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Enantioselective analysis of unbound tramadol, O-desmethyltramadol and N-desmethyltramadol in plasma by ultrafiltration and LC–MS/MS: Application to clinical pharmacokinetics

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ABSTRACT

This study describes the enantioselective analysis of unbound and total concentrations of tramadol and its main metabolites O-desmethyltramadol (M1) and N-desmethyltramadol (M2) in human plasma. Sample preparation was preceded by an ultrafiltration step to separate the unbound drug. Both the ultrafiltrate and plasma samples were submitted to liquid/liquid extraction with methyl *t*-butyl ether. Separation was performed on a Chiralpak® AD column and tandem mass spectrometry consisting of an electrospray ionization source, positive ion mode and multiple reaction monitoring was used as the detection system. Linearity was observed in the following ranges: 0.2–600 and 0.5–250 ng/mL for analysis of total and unbound concentrations of the tramadol enantiomers, respectively, and 0.1–300 and 0.25–125 ng/mL for total and unbound concentrations of the M1 and M2 enantiomers, respectively. The lower limits of quantitation were 0.2 and 0.5 ng/mL for analysis of total and unbound concentration of each tramadol enantiomer, respectively, and 0.1 and 0.25 ng/mL for total and unbound concentrations of M1 and M2 enantiomers, respectively. Intra- and interassay reproducibility and inaccuracy did not exceed 15%. Clinical application of the method to patients with neuropathic pain showed plasma accumulation of (+)-tramadol and (+)-M2 after a single oral dose of racemic tramadol. Fractions unbound of tramadol, M1 or M2 were not enantioselective in the patients investigated.

1. Introduction

Tramadol, a drug with analgesic activity, is available in clinical practice as racemic mixtures of the (+)-tramadol [(1R, 2R)-tramadol] and (−)-tramadol [(1S, 2S)-tramadol] enantiomers [1,2]. (+)-Tramadol and its active metabolite (+)-O-desmethyltramadol (M1) act as μ opioid receptor agonists. (+)-Tramadol inhibits the reuptake of serotonin and (−)-tramadol inhibits the reuptake of norepinephrine [2]. The affinity of (+)-M1 for the μ opioid receptor is approximately 300 times higher than that observed for the unaltered drug [2,3]. Other tramadol metabolites include N-desmethyltramadol (M2) and conjugates with glucuronic acid or sulfonates [2,4].

The plasma protein binging of tramadol is approximately 20% [2,5,6]. However, no data are available in the literature regarding the plasma protein binding of the individual tramadol enantiomers and of the active (+)-M1 metabolite. The binding of drugs to plasma proteins is an important covariate in pharmacokinetics since only the fraction unbound of the drug in plasma is available for distribution, elimination, and pharmacodynamic effects. However, data regarding the influence of drug binding to plasma proteins on pharmacodynamic parameters are scarce, and the unbound instead of the total concentration should be used whenever possible in pharmacokinetic–pharmacodynamic (PK–PD) models for drugs that are highly or poorly bound to plasma proteins [7,8]. Therefore, the development of methods for analysis of the plasma concentration of the unbound drug instead of total concentration has been recommended. For analysis of unbound drug concentrations, preparation of the plasma samples should be preceded by equilibrium dialysis, ultracentrifugation or ultrafiltration techniques [7,9,10].

Since the binding of drugs to plasma proteins can be stereoselective [11,12], the present study describes, for the first time, the analysis of unbound concentrations of the tramadol, M1 and M2

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enantiomers in human plasma and the application of this method to pharmacokinetic studies on patients with neuropathic pain treated with a single dose of racemic tramadol. The method described is derived from a study recently published by our group in which total concentrations of the tramadol, M1 and M2 enantiomers were analyzed in rat plasma [13]. In the present investigation, the step of preparation of human plasma samples was preceded by ultrafiltration for analysis of the unbound drug. The method can be applied to studies investigating drug interactions and the influence of diseases on pharmacokinetics, as well as to PK–PD studies using the unbound plasma concentration of individual tramadol enantiomers and of the active (+)-M1 metabolite.

2. Experimental

2.1. Chemical agents and reagents

Racemic tramadol hydrochloride (96.9%) and the racemic M1 metabolite were kindly provided by Janssen-Cilag Farmacêutica (São José dos Campos, São Paulo, Brazil). N-desmethyltramadol (M2) was purchased from Toronto Research Chemicals Inc. (Ontario, Canada). The solvents methanol and ethanol (Merck, Darmstadt, Germany), methyl *t*-butyl ether (J.T. Baker, Xastoloc, Mexico), and 95% *n*-hexane (Tedia Company, Fairfield, OH, USA) were HPLC grade. Diethylamine (J.T. Baker, Phillipsburg, NJ, USA), sodium hydroxide (Synth, Diadema, Brazil) and ammonium acetate (J.T. Baker, Xastoloc, Mexico) were P.A. grade. Water purified with the Milli-Q® Plus system (Millipore Corp., Bedford, MA, USA) was used throughout the experiment.

The stock solution of racemic tramadol was prepared in methanol at a concentration of 1 mg/mL. The stock solutions of the racemic M1 and M2 metabolites were prepared in methanol at concentrations of 0.2 and 0.1 mg/mL methanol, respectively. For the analysis of unbound plasma concentrations, working solutions at concentrations of 4, 10, 20, 100, 400, 1000 and 2000 ng of each tramadol enantiomer/mL methanol, and at concentrations of 2, 5, 10, 50, 200, 500 and 1000 ng of each M1 and M2 enantiomer/mL methanol were prepared. The working solutions of tramadol for the analysis of total plasma concentration were prepared at concentrations of 4, 10, 20 and 400 ng and 1, 2, 4 and 12 μg of each enantiomer/mL methanol, respectively. For M1 and M2, working solutions of 2, 5, 10, 200 and 500 ng and 1, 2 and 6 μg of each enantiomer/mL methanol were used. The internal standard verapamil (Sigma, St. Louis, MO, USA) was prepared in methanol at a concentration of 100 ng/mL. All standard solutions were stored at −20 °C.

2.2. Chromatographic analysis

The tramadol, M1 and M2 enantiomers were analyzed by LC–MS/MS as described by Godoy et al. [13]. Briefly, the enantiomers were separated on a Chiralpak® AD chiral column (Chiral Technologies, Exton, PA, USA; 250 mm × 4.6 mm, particle size of 10 μm) maintained at 24 °C. The mobile phase consisted of a mixture of *n*-hexane:ethanol (95.5:4.5, v/v) supplemented with 0.1% diethylamine, with a flow rate of 1.2 mL/min. The enantiomers were eluted in the following sequence: (+)-tramadol at 5.2 min; (−)-tramadol at 5.9 min; (+)-M1 at 8.4 min; (−)-M1 at 9.2 min; (+)-M2 at 10.9 min; internal standard at 12.3 min, and (−)-M2 at 15.4 min. The effluent of the chromatographic column was mixed with a solution of ethanol:10 mM ammonium acetate (95:5, v/v) at a flow rate of 250 μL/min, with 200 μL/min of the mixture being directed towards the Quattro Micro LC Triple Quadrupole Mass Spectrometer (MS/MS) (Micromass, Manchester, UK). Tandem mass spectrometry was performed in positive mode using electrospray ionization.

The MS/MS conditions were optimized by direct infusion of the tramadol and metabolite standard solutions (10 μg/mL) prepared in the mobile phase with an infusion pump at a rate of 20 μL/min. The analyses were performed in the multiple reaction monitoring mode. Protonated ions $[M+H]^+$ and their respective ion products were monitored at transitions of 264>58 for the tramadol enantiomers, 250>58 for the M1 enantiomers, 250>44 for the M2 enantiomers, and 455>165 for the internal standard (verapamil). Data acquisition and sample quantification were performed using the MassLynx Program, version 4.1 (Micromass).

2.3. Sample preparation

For analysis of unbound plasma concentrations, 1-mL plasma aliquots were transferred to a Centrifree® Ultrafiltration Device (Millipore Corp., Carrigtwohill, Ireland). The samples were centrifuged at 1875 × *g* for 40 min in a centrifuge with a fixed-angle rotor (angle of 36°) (Model NT 825, Nova Técnica, Piracicaba, Brazil), refrigerated at 4 °C, to obtain the plasma ultrafiltrate. Aliquots of the ultrafiltrate (200 μL) were mixed with 25 μL of the internal standard solution (verapamil, 100 ng/mL), 50 μL 1 M sodium hydroxide, 50 mg sodium chloride, and 3 mL methyl *t*-butyl ether. The tubes were shaken for 30 min in a horizontal reciprocating shaker (Model MA 139, CTF da Marconi) and centrifuged at 1800 × *g* for 10 min at 4 °C. The organic phases (2.5 mL) were transferred to conic tubes and evaporated to dryness in a rotational vacuum concentrator (Christ, Osterode am Harz, Germany). The residues were resuspended in 100 μL of the mobile phase and shaken in a Phoenix AP-56 tube shaker for 10 s. An aliquot of 70 μL was injected into the HPLC system.

For analysis of total plasma concentration, 500-μL plasma aliquots were enriched with 25 μL of the internal standard solution (verapamil, 100 ng/mL), 100 μL 1 M sodium hydroxide, 0.1 g sodium chloride, and 6 mL methyl *t*-butyl ether. The tubes were shaken in a horizontal reciprocating shaker for 30 min and then centrifuged at 1800 × *g* for 10 min at 5 °C. The organic phases (5 mL) were transferred to conic tubes and evaporated to dryness under an air stream. The residues were resuspended in 140 μL of the mobile phase and shaken for 10 s, and 120 μL was injected into the chromatographic column.

2.4. Determination of the matrix effect

The matrix effect of human plasma was evaluated based on total plasma concentration by direct comparison of the peak areas obtained for the tramadol enantiomers, its metabolites and the internal standard verapamil, injected directly into the mobile phase, with the peak areas obtained for the standard solutions (0.6, 200 and 480 ng of each tramadol enantiomer/mL plasma, and 0.3, 100 and 240 ng of each M1 and M2 enantiomer/mL plasma) added to blank plasma extracts derived from six different healthy volunteers (according to the extraction procedure described in Section 2.3).

2.5. Racemization test

For analysis of the occurrence of racemization, the tramadol, M1 and M2 enantiomers were collected individually with the HPLC system equipped with a fluorescence detector and operating at 275 nm (excitation) and 300 nm (emission). The enantiomers were eluted on a Chiralpak® AD column (250 mm × 4.6 mm), with the mobile phase consisting of *n*-hexane:ethanol (95.5:4.5, v/v) supplemented with 0.1% diethylamine, at flow rate of 1.2 mL/min. The solvent was evaporated to dryness and the residues corresponding to the individual enantiomers were resuspended in the mobile phase. Part of the residues was analyzed directly with the LC–MS/MS system.

The other part was used to enrich blank plasma aliquots (500 μL), which were submitted to the extraction procedure for the analysis of total plasma concentration (Section 2.3) and subsequently analyzed with the LC–MS/MS system as described in Section 2.2.

2.6. Calibration/linearity curves

The blank plasma samples were obtained from the Blood Center of the University Hospital, Faculty of Medicine of Ribeirão Preto, University of São Paulo (HCFMRP-USP). For analysis of unbound plasma concentrations of tramadol, M1 and M2, aliquots of the ultrafiltrate (200 μL) derived from blank plasma were enriched with 25 μL of each working solution of tramadol, M1 and M2. The samples were prepared as described in Section 2.3 and the calibration curves were constructed at concentration ranges of 0.5–250 ng of each tramadol enantiomer/mL plasma and of 0.25–125 ng of each M1 and M2 enantiomer/mL plasma.

For analysis of total plasma concentrations of tramadol, M1 and M2, aliquots of blank plasma (500 μL) were enriched with 25 μL of each working solution of tramadol, M1 and M2. The samples were extracted and analyzed as described in Sections 2.2 and 2.3. The calibration curves were constructed at concentration ranges of 0.2–600 ng of each tramadol enantiomer/mL plasma and of 0.1–300 ng of each M1 and M2 enantiomer/mL plasma.

2.7. Validation

The method developed for analysis of the enantiomers of tramadol and its M1 and M2 metabolites in human plasma was validated according to the U.S. FDA Guidance for Industry Bioanalytical Method Validation [14].

Recovery evaluates the efficiency of the extraction procedure of the tramadol, M1 and M2 enantiomers and internal standard from human plasma. The recovery of tramadol, M1 and M2 was analyzed in quintuplicate at concentrations of 0.6, 200 and 480 ng of each tramadol enantiomer/mL human plasma and of 0.3, 100 and 240 ng of each M1 and M2 enantiomer/mL human plasma. Recovery was calculated by comparison of the results obtained for samples extracted according to the analytical procedure of total plasma concentration with those obtained for the standard solutions added to the blank plasma extracts, corresponding to 100% recovery.

Plasma samples spiked with tramadol, M1 and M2 at decreasing concentrations in relation to those used for construction of the calibration curves and prepared according to the analytical procedures of unbound and total plasma concentrations were analyzed in quintuplicate, and calculated through the calibration curve. The lower limit of quantitation (LOQ) was defined as the lowest concentration of each enantiomer quantified with a precision of up to 20% and inaccuracy of ±20%.

The precision and accuracy of the analytical methods for the measurement of unbound and total plasma concentrations were evaluated by intra- and interassay analysis at three levels of concentration: low quality control (LQC), medium quality control (MQC), and high quality control (HQC). For analysis of total plasma concentration, the LQC, MQC and HQC samples were prepared in blank plasma at concentrations of 0.6, 200 and 480 ng of each tramadol enantiomer/mL and of 0.3, 100 and 240 ng of each M1 and M2 enantiomer/mL. For analysis of unbound plasma concentration, the LQC, MQC and HQC samples were prepared at concentrations of 1, 80 and 200 ng of each tramadol enantiomer/mL and of 0.5, 40 and 100 ng of each M1 and M2 enantiomer/mL plasma. All solutions were divided into aliquots and stored at −20 °C. Intra-assay precision and accuracy was evaluated by the analysis of five aliquots of each sample in a single analytical run. Interassay precision and accuracy were evaluated by the analysis, in quintuplicate, of aliquots of samples in four consecutive assays. Intra- and interassay precision was evaluated by calculation of the coefficient of variation, and accuracy is expressed as the ratio between the mean concentration determined experimentally and the corresponding theoretical concentration using the following equation: % inaccuracy = (mean experimental concentration − nominal concentration)/nominal concentration × 100.

Short-term stability, post-processing stability, stability after three freeze–thaw cycles, and long-term stability were evaluated according to the analytical procedure of total plasma concentration. For this purpose, quality control samples were prepared at low (LQC: 0.6 ng of each tramadol enantiomer/mL plasma and 0.3 ng of each M1 and M2 enantiomer/mL plasma) and high concentrations (HQC: 480 ng of each tramadol enantiomer/mL plasma and 240 ng of each M1 and M2 enantiomer/mL plasma). The enriched samples were maintained at room temperature (23 °C) for 6 h for the analysis of short-term stability. Post-processing stability was evaluated by maintaining the samples in the autoinjector at 12 °C for 12 h, followed by injection into the chromatographic system. For the evaluation of stability after three freeze–thaw cycles, the quality control samples were frozen at −20 °C for at least 24 h. After this period, the samples were again thawed and frozen for 12 h and this process was repeated until the third thawing cycle when the samples were extracted and analyzed. For the evaluation of long-term stability, the quality control samples were maintained at −20 °C for a period of 11 months and then extracted and analyzed. The results were compared to those obtained for freshly prepared samples and are expressed as relative error (% inaccuracy).

2.8. Application

The study was approved by the Ethics Committee of HCFMRP-USP and all patients signed a free informed consent form. Twelve patients of both genders, ranging in age from 31 to 59 years (median: 44 years), with a BMI of 17.16–38.9 kg/m^2 (median: 26.0 kg/m^2), who had self-reported neuropathic pain above 4 on a numeric pain intensity scale (0–10), were investigated [15–17]. The cause of chronic neuropathic pain was non-surgical lumbar disc (L4 and L5) herniation (protrusion) in seven patients, cervical disc (C5–C7) herniation (protrusion) in three, and carpal tunnel syndrome in two. In all patients, lumbar or cervical disc herniation was confirmed by lumbar or cervical magnetic resonance imaging and carpal tunnel syndrome was confirmed by comparative electroneuromyography between the upper limbs. Neuropathic pain was classified by all patients as a “burning sensation” and “paresthesia” that carried out at least one specific nerve, i.e., sciatic for lumbar pain and radial/median nerves for both cervical and carpal tunnel syndrome. Patients with associated somatic and/or visceral nociceptive pain and sympathetic neurovegetative component of pain were excluded. All patients investigated were phenotyped as extensive metabolizers of CYP2D6 using metoprolol (100 mg) as probe drug (urinary metoprolol/α-hydroxymetoprolol ratio < 12.6) [18].

On the day of investigation, after an 8-h fast, the patients received a single dose of 100 mg racemic tramadol hydrochloride in capsule form (Tramal®, Pfizer, Guarulhos, Brazil) with 200 mL water. Breakfast was served 2 h after the administration of the drug. Blood samples were collected with heparin syringes (5000 IU Liquemine, Roche, São Paulo, Brazil) through an intravenous catheter at times 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 16 and 24 h after drug administration. Plasma was separated by centrifugation and the samples were stored at −70 °C until the time for analysis.

Kinetic disposition was evaluated using a two-compartment model for the tramadol enantiomers and a one-compartment model for the enantiomers of the M1 and M2 metabolites, first-order kinetics, and inclusion of lag time. The WinNonlin Software

(Pharsight Corp., Mountain View, CA, USA) was used for analysis. Unbound plasma concentrations of the tramadol, M1 and M2 enantiomers were evaluated at the time of observation of peak total plasma concentration of tramadol for each patient. The fraction unbound in plasma (fu) was determined as follows: fu = unbound plasma concentration/total plasma concentration.

Statistical analysis was performed using the GraphPad Instat® (GraphPad Software, San Diego, CA, USA) and Origin 8.0 Softwares (OriginLab Corporation, Northampton, MA, USA). Data are reported as medians (25th and 75th percentiles). The Wilcoxon test was used to evaluate (+)-tramadol/(−)-tramadol, (+)-M1/(−)-M1 and (+)-M2/(−)-M2 enantiomer ratios that differed from one. A p value ≤ 0.05 was considered to indicate statistical significance.

3. Results

Fig. 1 shows the chromatograms obtained for the analysis of plasma blank (Fig. 1A) and of total (Fig. 1B) and unbound (Fig. 1C) plasma concentrations of tramadol, M1 and M2 in a sample collected 3 h after administration of single oral dose of 100 mg racemic tramadol to a patient with neuropathic pain. The enantiomers were resolved on a Chiralpak® AD column, with the mobile phase consisting of a mixture of n-hexane and ethanol (95.5:4.5, v/v) supplemented with 0.1% diethylamine.

The analytic method for the measurement of total plasma concentration of the tramadol, M1 and M2 enantiomers showed the absence of racemization and a nonsignificant matrix effect in six different batches of human plasma.

Tables 1–3 show the confidence limits of the analytical methods for the measurement of unbound and total plasma concentrations of the tramadol, M1 and M2 enantiomers. The total plasma concentration versus time curves are shown in Fig. 2. Fig. 3 illustrates the median (+)/(−) enantiomer ratios of plasma tramadol, M1 and M2 concentrations in patients with neuropathic pain ($n = 12$). Table 4 shows the pharmacokinetic parameters, including fu, of each tramadol, M1 and M2 enantiomer obtained for patients with neuropathic pain ($n = 12$) treated with a single oral dose of 100 mg racemic tramadol.

4. Discussion

The present study describes an analytical method for the measurement of unbound and total plasma concentrations of the enantiomers of tramadol and of its M1 and M2 metabolites, and application of the method to the pharmacokinetic study of administration of a single oral dose of 100 mg racemic tramadol to 12 patients with neuropathic pain. To our knowledge, there are no studies in the literature analyzing the unbound plasma concentration of the enantiomers of tramadol and its M1 and M2 metabolites.

An analytical method for the measurement of the fraction unbound (fu) of tramadol, M1 and M2 enantiomers in plasma was developed and validated. The method is similar to that used for the analysis of total plasma concentration, except for the requirement of a procedure that is able to separate the free drug from the drug bound to plasma proteins. Ultrafiltration using the Centrifree® device was used in the present study. This system results in the formation of an ultrafiltrate in which the unbound drug is present at the same concentration as in the sample. Protein-bound drug does not cross the membrane and, consequently, does not reach the ultrafiltrate [19]. The ultrafiltration procedure has been used for the determination of unbound plasma concentrations of other drugs, such as moxifloxacin, paclitaxel and mycophenolic acid [20–22].

The tramadol, M1 and M2 enantiomers were resolved on a Chiralpak® AD column with a run time of approximately 19 min, using a mixture of n-hexane:ethanol:diethylamine (95.5:4.5:0.1, v/v/v) as the mobile phase (Fig. 1). Fig. 1 illustrates the LC–MS/MS chromatograms obtained for the analysis of total and unbound plasma concentrations in a blood sample collected 3 h after the administration of a single oral dose of 100 mg racemic tramadol to a patient with neuropathic pain.

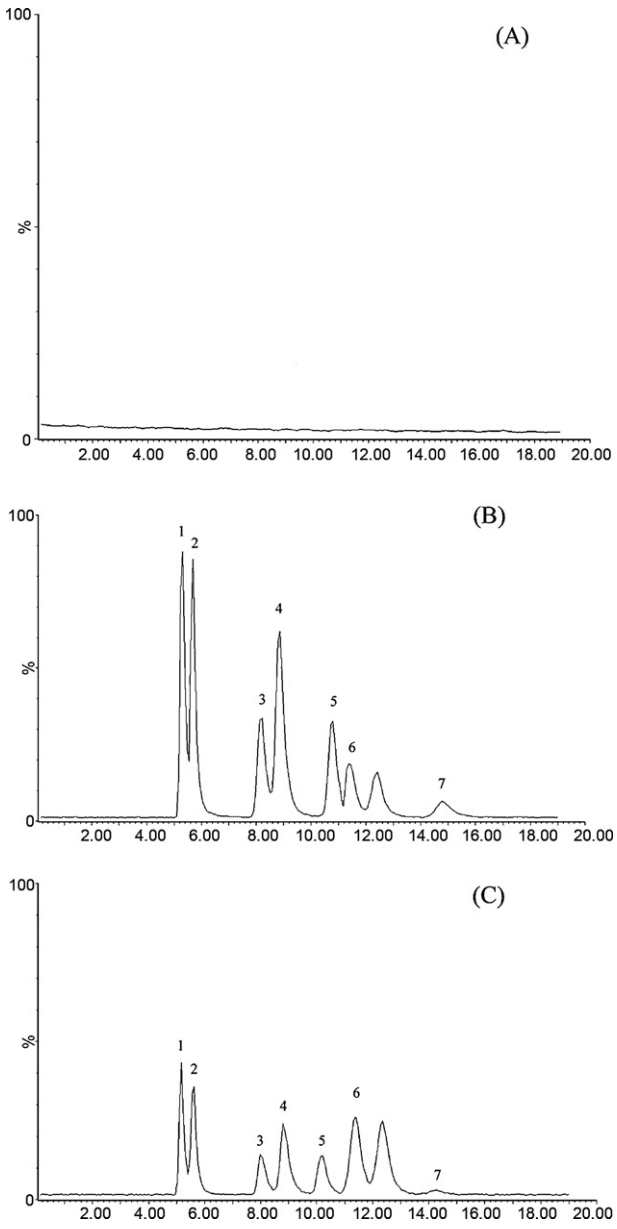

Fig. 1. Total ion chromatograms (TIC) obtained for the analysis of plasma blank (A), total (B) and unbound (C) plasma concentrations of the tramadol, M1 and M2 enantiomers in human plasma collected 3 h after oral administration of 100 mg racemic tramadol to a patient with neuropathic pain. Chiralpak® AD column; mobile phase: n-hexane:ethanol (95.5:4.5, v/v) supplemented with 0.1% diethylamine. Peaks: (1) (+)-tramadol; (2) (−)-tramadol; (3) (+)-M1; (4) (−)-M1; (5) (+)-M2; (6) internal standard; (7) (−)-M2.

No matrix effect was observed in the analysis of the tramadol, M1 and M2 enantiomers in human plasma. Values of approximately 100% were obtained when the peak areas resulting from the direct injection of standard solutions in the mobile phase were compared with the areas obtained for standard solutions added to blank plasma extracts obtained from six different volunteers. Godoy et al. [13], using rat plasma for the analysis of tramadol, M1 and M2 enantiomers, also reported the absence of a matrix effect. The racemization test was performed before the validation

Table 1
Validation parameters of the analytical method for the measurement of total and unbound plasma concentrations of tramadol enantiomers in human plasma.

	Total (+)-tramadol	Total (−)-tramadol	Unbound (+)-tramadol	Unbound (−)-tramadol
Recovery (%, $n = 5$)				
0.6 ng/mL	91.95	91.12	–	–
200 ng/mL	92.34	102.35	–	–
480 ng/mL	98.18	85.90	–	–
Linearity (ng/mL)	0.2–600	0.2–600	0.5–250	0.5–250
r	0.9991	0.9993	0.9996	0.9995
Lower limit of quantitation (ng/mL)	0.2	0.2	0.5	0.5
Precision (CV%, $n = 5$)	6.04	7.59	11.21	8.16
Accuracy (% inaccuracy)	0.40	2.00	1.4	−8.13
Interassay precision (CV%, $n = 20$)				
LQC	6.94	6.70	7.69	8.59
MQC	8.18	7.14	7.97	10.27
HQC	8.80	8.13	10.00	10.16
Intra-assay precision (CV%, $n = 5$)				
LQC	6.76	5.34	8.68	11.53
MQC	10.06	3.99	5.31	7.67
HQC	12.94	10.66	9.16	7.94
Interassay accuracy (% inaccuracy, $n = 20$)				
LQC	1.83	−0.08	−3.91	0.59
MQC	−6.22	−5.32	−5.47	−7.45
HQC	−0.24	3.43	1.70	−2.77
Intra-assay accuracy (% inaccuracy, $n = 5$)				
LQC	4.00	2.00	−4.96	−1.71
MQC	−9.46	−10.51	5.20	5.17
HQC	−5.14	−0.81	9.02	3.34
Stability (% inaccuracy, $n = 5$)				
Short-term (6 h at 23 °C)				
LQC	−11.86	−13.40	–	–
HQC	7.23	3.88	–	–
Post-processing (12 h at 12 °C)				
LQC	−2.88	−7.52	–	–
HQC	13.73	10.86	–	–
Freezing/thawing				
LQC	−9.96	−7.84	–	–
HQC	8.90	5.74	–	–
Long-term (11 months at −20 °C)				
LQC	0.01	−4.15	–	–
HQC	7.11	2.04	–	–

CV: coefficient of variation [(standard deviation/mean) × 100]; r: linear correlation coefficient; % inaccuracy = [(experimentally obtained concentration − nominal concentration)/nominal concentration] × 100; LQC, MQC and HQC: low, medium and high quality control. For total tramadol: LQC: 0.6 ng/mL, MQC: 200 ng/mL, and HQC: 480 ng/mL; for unbound tramadol: LQC: 1 ng/mL, MQC: 80 ng/mL, and HQC: 200 ng/mL.

procedure and showed no chiral inversion of the tramadol, M1, or M2 enantiomers.

The enantiomers were extracted from plasma in alkaline medium using the salting-out technique with sodium chloride and methyl *t*-butyl ether as extracting solvent. Recovery values (Tables 1–3) of approximately 90% were obtained for the tramadol, M1 and M2 enantiomers at the different concentrations analyzed. In addition to liquid–liquid extraction using methyl *t*-butyl ether [23], the use of ethyl acetate as an extracting solvent with recovery

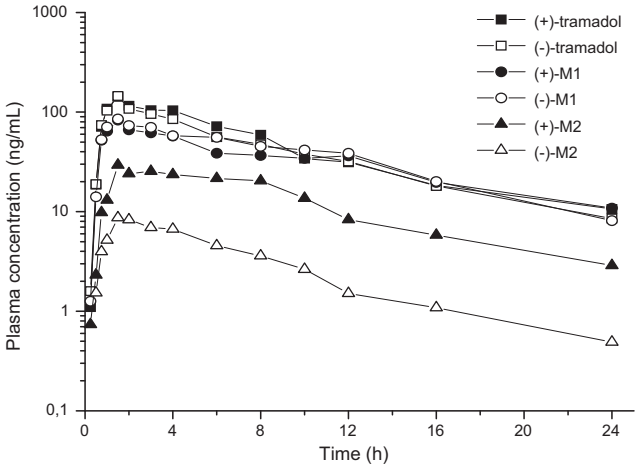

Fig. 2. Plasma concentration versus time curves for total concentration of tramadol, M1 and M2 enantiomers after administration of oral racemic tramadol (100 mg) to patients with neuropathic pain. Data are reported as medians ($n = 12$).

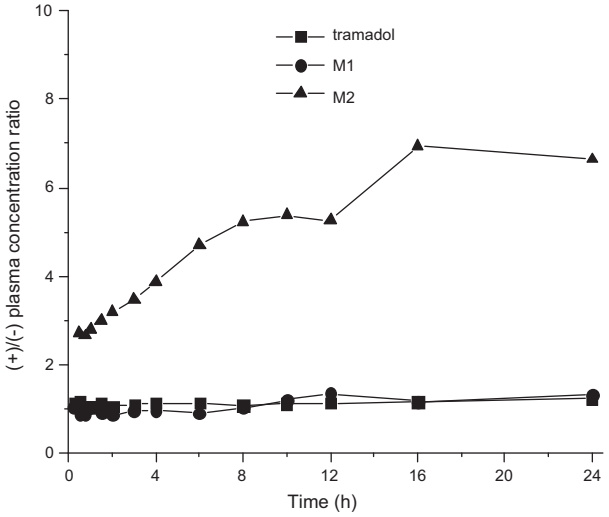

Fig. 3. Plasma (+)/(−) enantiomer ratios of tramadol, M1 and M2 after administration of a single oral dose of 100 mg racemic tramadol to patients with neuropathic pain. Data are reported as medians ($n = 12$).

Table 2
Validation parameters of the analytical method for the measurement of total and unbound plasma concentrations of M1 enantiomers in human plasma.

	Total (+)-M1	Total (−)-M1	Unbound (+)-M1	Unbound (−)-M1
Recovery (%, $n = 5$)				
0.3 ng/mL	94.06	93.72	–	–
100 ng/mL	100.20	96.71	–	–
240 ng/mL	98.42	97.73	–	–
Linearity (ng/mL)	0.1–300	0.1–300	0.25–125	0.25–125
r	0.9997	0.9966	0.9997	0.99905
Lower limit of quantitation (ng/mL)	0.1	0.1	0.25	0.25
Precision (CV%, $n = 5$)	2.95	4.63	11.82	11.47
Accuracy (% inaccuracy)	−4.00	−1.60	−5.33	−3.00
Interassay precision (CV%, $n = 20$)				
LQC	8.11	9.49	8.62	5.67
MQC	9.83	5.29	9.70	5.11
HQC	10.04	7.16	9.78	11.08
Intra-assay precision (CV%, $n = 5$)				
LQC	7.78	6.68	2.86	7.50
MQC	14.70	6.42	7.97	5.53
HQC	8.29	2.92	12.84	8.48
Interassay accuracy (% inaccuracy, $n = 20$)				
LQC	−5.33	−3.33	−6.02	−6.98
MQC	−1.11	4.86	−6.67	−9.14
HQC	−4.54	2.53	0.70	−1.90
Intra-assay accuracy (% inaccuracy, $n = 5$)				
LQC	−1.33	−6.67	−7.67	2.00
MQC	−1.90	0.41	−6.48	−7.80
HQC	6.32	7.11	−8.10	−9.10
Stability (% inaccuracy, $n = 5$)				
Short-term (6 h at 23 °C)				
LQC	−0.65	5.71	–	–
HQC	−12.67	−5.03	–	–
Post-processing (12 h at 12 °C)				
LQC	−1.30	0.71	–	–
HQC	−13.90	−2.68	–	–
Freezing/thawing				
LQC	−1.95	14.29	–	–
HQC	−11.21	−13.64	–	–
Long-term (11 months at −20 °C)				
LQC	1.82	13.79	–	–
HQC	−13.87	−14.93	–	–

CV: coefficient of variation [(standard deviation/mean) × 100]; r: linear correlation coefficient; % inaccuracy = [(experimentally obtained concentration − nominal concentration)/nominal concentration] × 100; LQC, MQC and HQC: low, medium and high quality control. For total M1: LQC: 0.3 ng/mL, MQC: 100 ng/mL, and HQC: 240 ng/mL; for unbound M1: LQC: 0.5 ng/mL, MQC: 40 ng/mL, and HQC: 100 ng/mL.

rates of 75–90% [24,25], and solid-phase extraction with recovery rates of 90% [26] have been reported.

The analytical method for the measurement of total plasma concentrations resulted in LOQ values of 0.2, 0.1 and 0.1 ng for each tramadol, M1 and M2 enantiomer/mL plasma, respectively. For the analysis of unbound plasma concentrations, the LOQ was 0.5, 0.25 and 0.25 ng for each tramadol, M1 and M2 enantiomer/mL plasma (Tables 1-3). These values were obtained after extraction of only 200 μL of the ultrafiltrate derived from 1 mL plasma for the analysis of unbound plasma concentration and from 500 μL plasma for the analysis of total plasma concentration. These results permit the determination of the total concentration of these analytes in plasma up to 24 h after the administration of a single oral dose of 100 mg racemic tramadol. The unbound concentration of the drug was evaluated at the time when the maximum total plasma concentration of tramadol (C_{max}) was reached, which ranged from 1 to 4 h after administration of racemic tramadol (Table 4). Other investigators employing LC–MS/MS reported LOQ of 0.15, 3 and 1 ng/mL for each tramadol enantiomer using plasma volumes of 1 mL [26], 500 μL [27] and 200 μL [28], respectively. For the active M1 metabolite, these investigators found LOQ of 0.3, 4 and 0.5 ng/mL for each enantiomer, respectively. Therefore, the method developed in the present study is the most sensitive among those using LC–MS/MS reported in the literature [26–28]. The kinetic disposition of (+)-M1 after oral administration of racemic tramadol in CYP2D6 poor metabolizers has not been described, since plasma concentrations of (+)-M1 were below the quantification limit [29,30]. Therefore, sensitive enantioselective methods are needed for the analysis of plasma concentrations of tramadol metabolites, including in CYP2D6 poor metabolizers.

The calibration curves for the analysis of total plasma concentration were linear within the range of 0.2–600 ng of each tramadol enantiomer/mL plasma and of 0.1–300 ng of each M1 and M2 enantiomer/mL plasma, with a linear coefficient of correlation higher than 0.99 (Tables 1–3). The calibration curves for the analysis of unbound plasma concentration were linear within the range of 0.5–250 ng of each tramadol enantiomer/mL plasma and within the range of 0.25–125 ng of each M1 and M2 enantiomer/mL plasma (Tables 1–3).

The coefficients of variation indicating precision and the percentage of inter- and intra-assay inaccuracy < 15% obtained for the tramadol, M1 and M2 enantiomers evaluated at three levels of concentration (LQC, MQC and HQC) guaranteed the reproducibility and accuracy of the analytical methods for the measurement of total and unbound plasma concentrations (Tables 1–3).

The stability of the tramadol, M1 and M2 enantiomers in human plasma was evaluated by the determination of short-term stability (6 h at room temperature), post-processing stability for 12 h at 12 °C, after three freeze–thaw cycles, and long-term stability over a period of 11 months at −20 °C. The analytes were considered to be stable under the conditions studied, with the observation of relative error of less than 15% in relation to freshly prepared samples

Table 3
Validation parameters of the analytical method for the measurement of total and unbound plasma concentrations of M2 enantiomers in human plasma.

	Total (+)-M2	Total (−)-M2	Unbound (+)-M2	Unbound (−)-M2
Recovery (%, $n = 5$)				
0.3 ng/mL	93.85	90.69	–	–
100 ng/mL	95.06	89.05	–	–
240 ng/mL	88.39	99.44	–	–
Linearity (ng/mL)	0.1–300	0.1–300	0.25–125	0.25–125
r	0.9989	0.9991	0.9952	0.9952
Lower limit of quantitation (ng/mL)	0.1	0.1	0.25	0.25
Precision (CV%, $n = 5$)	8.50	7.99	10.54	7.10
Accuracy (% inaccuracy)	−2.40	−2.4	0.87	−5.53
Interassay precision (CV%, $n = 20$)				
LQC	7.16	7.24	10.68	8.12
MQC	4.55	5.83	11.08	11.99
HQC	9.06	6.70	12.58	11.13
Intra-assay precision (CV%, $n = 5$)				
LQC	10.66	6.68	11.20	3.95
MQC	1.75	3.91	13.79	11.78
HQC	6.95	9.26	9.61	4.04
Interassay accuracy (% inaccuracy, $n = 20$)				
LQC	−1.50	−1.50	−1.22	4.3
MQC	5.68	2.72	0.53	3.33
HQC	2.24	4.17	−9.69	−10.46
Intra-assay accuracy (% inaccuracy, $n = 5$)				
LQC	−2.67	−6.67	−0.17	−6.58
MQC	8.84	7.51	3.11	0.51
HQC	−6.86	1.92	−6.47	−9.04
Stability (% inaccuracy, $n = 5$)				
Short-term (6 h at 23 °C)				
LQC	4.79	−5.00	–	–
HQC	9.95	11.63	–	–
Post-processing (12 h at 12 °C)				
LQC	3.42	4.28	–	–
HQC	12.19	1.72	–	–
Freezing/thawing				
LQC	2.74	−7.14	–	–
HQC	12.43	0.44	–	–
Long-term (11 months at −20 °C)				
LQC	4.93	5.42	–	–
HQC	9.58	5.15	–	–

CV: coefficient of variation [(standard deviation/mean) × 100]; r: linear correlation coefficient; % inaccuracy = [(experimentally obtained concentration − nominal concentration)/nominal concentration] × 100; LQC, MQC and HQC: low, medium and high quality control. For total M2: LQC: 0.3 ng/mL, MQC: 100 ng/mL, and HQC: 240 ng/mL; for unbound M2: LQC: 0.5 ng/mL, MQC: 40 ng/mL, and HQC: 100 ng/mL.

(Tables 1–3). Previous studies have reported the stability of tramadol, M1 and M2 enantiomers in plasma samples stored for 3 months at −20 °C [25].

The confidence limits obtained for the analytical method of total plasma concentration are compatible with the application in studies of kinetic disposition and metabolism after administration of a single oral dose of racemic tramadol. Considering only the chromatographic methods for the sequential analysis of tramadol and its metabolites, the method developed is the most sensitive and fastest technique among those published in the literature.

The kinetic disposition of the tramadol, M1 and M2 enantiomers was evaluated in patients with neuropathic pain after administration of a single oral dose of 100 mg racemic tramadol. The plasma concentrations of (+)-tramadol were significantly higher than those of (−)-tramadol (AUC: 1311.8 versus 1264.2 ng h/mL), with $AUC_{(+)/(-)}$ ratios of 1.13 (Table 4 and Fig. 3). Plasma accumulation of (+)-tramadol has been reported previously, with $AUC_{(+)/(-)}$ ratios ranging from 1.22 to 1.28 [31–33]. Patients with neuropathic pain presented a non-enantioselective unbound fraction of approximately 0.65 for (+)-tramadol, 0.54 for (−)-tramadol, 0.16 for (+)-M1, 0.18 for (−)-M1, 0.24 for (+)-M2, and 0.21 for (−)-M2 (Table 4). Therefore, plasma accumulation of (+)-tramadol cannot be explained by plasma protein binding.

Table 4
Kinetic disposition of the tramadol, M1 and M2 enantiomers based on total plasma concentration in patients with neuropathic pain ($n = 12$) treated with 100 mg racemic tramadol.

	(+)-Tramadol	(−)-Tramadol	(+)-M1	(−)-M1	(+)-M2	(−)-M2
$AUC^{0-\infty}$ (ng h/mL)	1311.8 (1042.2–2166.6)	1264.2[a] (851.5–1496.4)	1246.6 (621.2–1414.0)	998.9 (689.4–1293.8)	402.3 (198.8–728.4)	80.9[a] (47.1–104.5)
C_{max} (ng/mL)	167.5 (137.5–253.1)	159.5 (119.7–223.6)	83.0 (62.5–110.5)	85.6 (69.1–103.7)	36.1 (16.1–52.8)	9.2[a] (4.6–13.4)
t_{max} (h)	1.53 (1.29–2.00)	1.46 (1.20–1.97)	1.54 (1.28–2.06)	1.66 (1.31–2.17)	2.12 (1.57–3.03)	1.53[a] (1.25–2.14)
$t_{1/2}\beta$ (h)	7.61 (6.55–9.11)	7.09[a] (6.25–8.88)	–	–	–	–
$t_{1/2}$ (h)	–	–	7.53 (6.18–9.29)	6.47 (5.42–8.83)	6.50 (5.66–7.96)	5.51[a] (4.26–6.37)
fu	0.65 (0.37–0.68)	0.54 (0.43–0.70)	0.16 (0.12–0.22)	0.18 (0.14–0.21)	0.24 (0.11–0.33)	0.21 (0.10–0.33)
$AUC^{0-\infty}{}_{(+)/(-)}$	1.13 (1.00–1.27)		1.18 (0.90–1.36)		4.80 (3.75–6.20)	

Data are reported as the median (25th–75th percentile). $AUC^{0-\infty}$: area under plasma concentration versus time curve; C_{max}: maximum plasma concentration; t_{max}: time to reach C_{max}; $t_{1/2}\beta$: elimination half-life; fu: fraction unbound.

[a] Wilcoxon test ($p \leq 0.05$).

M1 is an intermediary metabolite produced from tramadol and eliminated by N-demethylation reactions, with the formation of M5 and M4 metabolites, and by conjugation reactions with glucuronic acid or sulfonate. The pharmacokinetic parameters shown in Table 4 and Figs. 2 and 3 revealed no significant differences between M1 enantiomers. In healthy volunteers, the glucuronidation reaction is favored in the case of (−)-M1, with the observation of a 4-fold higher urinary excretion of (−)-M1 compared to (+)-M1 [34,35]. The preferential elimination of (−)-M1 glucuronide is therefore a determining factor to explain the plasma accumulation of (+)-tramadol observed in the patients with neuropathic pain studied here.

The pharmacokinetics of M2 was enantioselective, with the observation of significantly higher plasma concentrations of (+)-M2 compared to (−)-M2 (AUC: 402.3 versus 80.9 ng h/mL) and an $AUC_{(+)/(-)}$ ratio of 4.64 (Fig. 2 and Table 4). Similar results have been reported by García-Quetglas et al. [32], who obtained an $AUC_{(+)/(-)}$ ratio of 4.5 for M2 after oral administration of racemic tramadol. However, the present results showed an increase in the (+)/(−) enantiomer ratio of plasma M2 concentrations over time, with the observation of higher values approximately 16 h after the administration of tramadol, suggesting the preferential formation of (+)-M2 and/or preferential elimination of (−)-M2 (Fig. 3).

5. Conclusion

The analytical methods for the measurement of total and unbound plasma concentrations of tramadol, M1 and M2 enantiomers in human plasma using LC–MS/MS were found to be sensitive, precise and accurate. The kinetic disposition of tramadol and its M2 metabolite was enantioselective, with the plasma accumulation of (+)-tramadol and (+)-M2, whereas the pharmacokinetics of M1 was not enantioselective in patients with neuropathic pain phenotyped as extensive metabolizers of CYP2D6. Plasma protein binding was not enantioselective, although the fraction unbound of the two tramadol enantiomers was approximately 4 times higher than that of the fraction unbound of the active (+)-M1 metabolite. The developed method can be used in the future for the evaluation of the influence of drug interactions and of diseases on the fraction unbound of the tramadol, M1 and M2 enantiomers, as well as in PK–PD studies using the unbound concentration of the tramadol and (+)-M1 enantiomers involved in the analgesic activity of the drug.

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