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# Stereospecific high-performance liquid chromatographic analysis of tramadol and its *O*-demethylated (M1) and *N*,*O*-demethylated (M5) metabolites in human plasma

Reza Mehvar[a,*], Katherine Elliott[a], Ridhi Parasrampuria[a], Okponanabofa Eradiri[b]

[a] *School of Pharmacy, Texas Tech University Health Sciences Center, 1300 Coulter, Amarillo, TX 79106, USA*
[b] *Biovail Technologies, Ltd., 3701 Concorde Parkway, Chantilly, VA 2015, USA*

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**Abstract**

A stereospecific method for simultaneous quantitation of the enantiomers of tramadol (T) and its active metabolites *O*-demethyl tramadol (M1) and *O*-demethyl-*N*-demethyl tramadol (M5) in human plasma is reported. After the addition of penbutolol (IS), plasma (0.5 ml) samples were extracted into methyl *tert*-butyl ether, followed by back extraction into an acidic solution. The separation was achieved using a Chiralpak AD column with a mobile phase of hexanes:ethanol:diethylamine (94:6:0.2) and a flow rate of 1 ml/min. The fluorescence of analytes was then detected at excitation and emission wavelengths of 275 and 300 nm, respectively. All the six enantiomeric peaks of interest plus three unknown metabolite peaks and IS peak (a total of 10 peaks) eluted within 23 min, free from endogenous interference. The assay was validated in the plasma concentration range of 2.5–250 ng/ml, with a lower limit of quantitation of 2.5 ng/ml, for all the six analytes. The extraction efficiency ($n = 5$) was close to 100% for both T and M1 enantiomers and 85% for M5 and IS enantiomers. The application of the assay was demonstrated by simultaneous measurement of plasma concentrations of T, M1, and M5 enantiomers in a healthy volunteer after the administration of 50 mg oral doses of racemic T.


*Keywords:* Stereospecific assay; Active metabolite; Enantioselective assay; Stereoselective pharmacokinetics; Stereoselective metabolism; Tramadol

## 1. Introduction

Tramadol (T), *trans*-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)cyclohexanol, is a centrally acting analgesic [1], which contains two chiral carbon atoms (Fig. 1). The commercially available product is a racemate consisting of equal portions of (+)-1*R*,2*R* and (−)-1*S*,2*S* enantiomers. In humans, the drug undergoes significant metabolism [2], resulting in at least 23 metabolites [3], with major metabolites being *O*-demethyl (M1), *N*-demethyl (M2), *N*,*N*,-didemethyl (M3), *O*-demethyl-*N*-demethyl (M5), and *O*-demethyl-*N*,*N*-didemethyl (M4) tramadol (Fig. 1). The *O*-demethylated metabolites are then further metabolized by glucuronide or sulfate conjugation.

The pharmacodynamics of T are complex in that in addition to stereoselectivity in the effects of T itself, metabolism of the drug results in the formation of active metabolites with stereoselective effects [1,4–7]. Furthermore, the pharmacokinetics, including the metabolism, of T are stereoselective in humans [8–12]. The analgesic activity of T administration has been attributed to inhibition of reuptake of serotonin and norepinephrine in addition to activation of μ-opioid receptors [1,4]. Whereas the serotonin uptake inhibitory effect resides mostly in the (+)-enantiomer, the inhibition of norepinephrine reuptake is due to the (−)-enantiomer. As for metabolites, it is generally assumed that M1 is the only active metabolite, which is responsible for the opioid-like activity of the drug. However, studies by Gillen et al. [4] using cloned human μ-opioid receptors showed that the affinity of (±)-M5 for the μ receptor, although 30-fold less than that of (+)-M1, was 2.4-fold higher than that of (−)-M1 and 24-fold higher than that of (+)-T. On the other hand, the affinities of the other major metabolites (M2, M3, and M4) were very weak (>3000-fold less than that of (+)-M1 or >100-fold less than (±)-M5). Indeed, M2, M3, and M4 metabolites had no stimulatory effects on GTPγS binding, indicating lack of activation of μ-receptor by these

* Corresponding author. Tel.: +1 806 356 4015x337; fax: +1 806 356 4034.
*E-mail address:* reza.mehvar@ttuhsc.edu (R. Mehvar).

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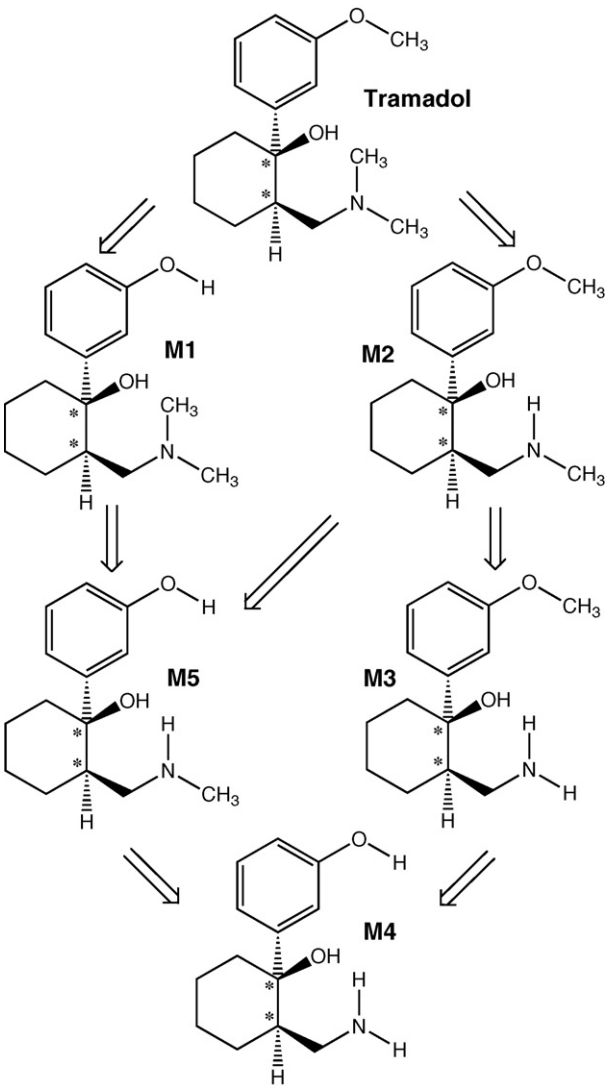


Fig. 1. Chemical structures of *trans*-tramadol and its major phase I metabolites. Asterisks denote chiral carbons.

metabolites [4]. These data suggest that M5 is the only other metabolite, in addition to M1, with the potential to contribute to the μ-opioid receptor-related effects of the drug in vivo. The in vivo contribution of M5 to the analgesic effects of T, if any, would be dependent on its plasma concentrations relative to those of the M1 enantiomers, in particular those of (+)-M1. However, to date, there are no data available with regard to the relative plasma concentrations of the individual enantiomers of T, M1, and M5 after administration of racemic T in humans. Such studies require an analytical method capable of simultaneous measurement of T, M1, and M5 enantiomers in biological samples. Despite the availability of a few validated stereospecific assays for measurement of the enantiomers of T and M1 metabolite [9,10,13–15], currently there is no validated assay for simultaneous quantitation of the enantiomers of T, M1, and M5 in human plasma. Therefore, the aim of the present study was to develop such an assay and test its application after administration of the recommended doses of T in humans.

## 2. Experimental

### 2.1. Chemicals

Racemic *trans*-tramadol (T) HCl was purchased from Heumann Pharma GmbH & Co. (Nürnberg, Germany). The *O*-demethyl (M1) and *O*-demethyl-*N*-demethyl (M5) tramadol powders were synthesized by Total Synthesis, Inc. (Toronto, Canada) with purities of >95 and 97%, respectively. Racemic penbutolol sulfate was generously provided by Dr. F.M. Pasutto of the University of Alberta, Faculty of Pharmacy (Edmonton, Canada). Methyl *tert*-butyl ether (Omnisolv Grade) and hexanes (HPLC Grade) were from EM Science/Merck (Darmstadt, Germany), and absolute ethanol (Reagent Grade) was from Mallinckrodt (Phillipsburg, NJ, USA). Diethylamine (Ultra Grade) and Trizma base were purchased from Sigma Chemicals (St. Louis, MO, USA). All other reagents were analytical grade and available from commercial sources.

### 2.2. Standard solutions

Stock solutions (500 μg/ml) of racemic T, M1, and M5 were prepared in distilled water. Calibration standards were prepared daily by spiking blank human plasma with stock solutions of T, M1, and M5 to produce final enantiomeric concentrations of 0 (blank), 2.5, 5, 10, 25, 50, 100, and 250 ng/ml of the drug and its two metabolites.

Internal standard (penbutolol, IS) was prepared by dissolving the powder in distilled water to produce a final concentration of 500 ng/ml of the racemate.

### 2.3. Sample preparation

To 0.5 ml of plasma in a glass tube were added 50 μl of IS solution and 0.5 ml of 0.05 M Trizma base. The analytes and IS were then extracted into 6 ml of methyl *tert*-butyl ether by mixing the samples (Rotamix; ATR, Laurel, MD) at a rate of 45 rpm for 30 min. After centrifugation at 2000 × *g* for 5 min, the organic layer was transferred to silanized glass test tubes, and 0.3 ml of 5 mM HCl was added. The analytes were then back extracted into the aqueous phase by vortex-mixing the samples for 30 s. Following a 5-min centrifugation, the organic layer was discarded and the aqueous layer was evaporated under a nitrogen stream at 45–50 °C. The residue was then reconstituted in 250 μl of mobile phase, and 100 μl of the injection solution was injected into the system.

### 2.4. Chromatography

Samples were analyzed using a 250 mm × 4.6 mm analytical, chiral column (Chiralpak AD, Chiral Technologies; Exton, PA, USA), preceded by a LichroCART 4-4 LiChrospher 100 Diol (5 μm) precolumn (EM Science/Merck, Darmstadt, Germany). The mobile phase consisted of hexanes:ethanol:diethylamine (94:6:0.2) and was pumped at a flow rate of 1.0 ml/min.

The HPLC instrument (Waters; Milford, MA) consisted of a 515 pump, a 717 plus autosampler, and a 2474 fluorescence

detector set at a excitation and emission wavelengths of 275 and 300 nm, respectively. Calibration curves were constructed using the Empower 2 software (Waters) by plotting the peak area ratio of each analyte to IS versus the analyte concentrations in plasma. The data was weighted by 1/concentration.

### 2.5. Extraction recovery

The efficiency of the extraction method to recover T, M1, M5, and IS from plasma was tested using samples containing 25 ng/ml of each enantiomer of T, M1, and M5 and appropriate concentration of IS. These samples were then subjected to the sample preparation procedure explained above. The peak areas of the analytes and IS in these extracted samples ($n = 5$) were then compared with those of unextracted samples ($n = 5$) containing equivalent concentrations of analytes and IS in the injection solution.

### 2.6. Assay validation

The intra- and inter-run precision and accuracy of the assay ($n = 5$) were determined by percent C.V. and percent error values, respectively, based on reported guidelines [16]. Briefly, each set of quality control samples containing the lowest, midpoint, and highest concentrations in the calibration curves was run along with a calibration curve. The concentrations of the quality control samples were then determined against the calibration curve and used for the calculation of percent C.V. and percent error values. The percent error values were calculated by the following equation:

$$\text{percent error} = \frac{\text{calculated conc.} - \text{added conc.}}{\text{added conc.}} \times 100$$

The quality control samples were run at enantiomeric concentrations of 2.5, 50, and 250 ng/ml for T, M1, and M5.

### 2.7. Application of the assay

After signing an informed consent, a healthy, 28-year-old Caucasian male volunteer, 1.88 m tall and weighing 81.8 kg, received a 50-mg tablet of T (Ultram, Ortho-McNeil Pharmaceutical, Inc., Raritan, NJ, USA) every 6 h on days 1 and 3–10. Blood samples (7 ml) were collected in pre-cooled potassium EDTA tubes at 0 (before drug administration), 0.5, 1.0, 1.5, 2.0, 4.0, 6.0, 6.5, 7.0, 7.5, 8.0, 10, 12, 12.5, 13, 13.5, 14, 16, 18, 18.5, 19, 19.5, 20, 22, and 24 h on days 1 and 10. Additional blood samples were also collected at 36 and 48 h after the start of the study. To confirm the achievement and maintenance of steady state, additional samples were also taken immediately before drug administration (trough samples) on days 7–10. The samples were immediately centrifuged and the resultant plasma samples were stored at −20 °C until analysis.

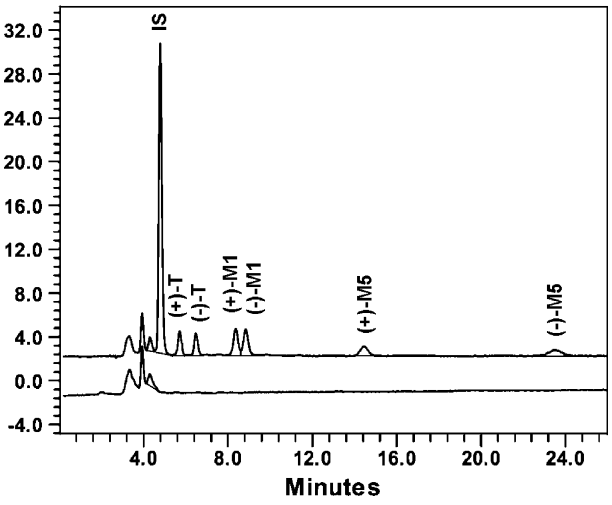


Fig. 2. Chromatograms of a blank human plasma sample (bottom) and a plasma sample spiked with 5 ng/ml of T, M1, and M5 enantiomers (top).

## 3. Results

Chromatograms of a blank plasma and a plasma sample spiked with T, M1, and M5 to yield a final concentration of 5 ng/ml for each enantiomer are illustrated in Fig. 2. Under the stated chromatographic conditions, IS, (+)-T, (−)-T, (+)-M1, (−)-M1, (+)-M5, and (−)-M5 eluted at approximately 4.8, 5.7, 6.5, 8.3, 8.9, 14.4, and 23.6 min, respectively, free from endogenous peaks (Fig. 2). Fig. 3 illustrates the chromatograms of 24-h samples on day 1 (top) and day 10 (bottom), taken from a healthy volunteer who received 50 mg racemic T orally every 6 h. The chromatograms of volunteer samples (Fig. 3) showed three extra peaks in addition to the seven peaks attributed to IS and the enantiomers of T and its M1 and M5 metabolites. These unknown peaks, which are most likely other metabolites of T, were well separated from the seven peaks of interest (Fig. 3).

The extraction recoveries of IS, and enantiomers of T, M1, and M5 are presented in Table 1. The recoveries were quantitative for the enantiomers of T and M1. The recoveries of the M5 enantiomers and IS, although lower than those for T and M1, were still high (Table 1). The recovery data indicate that the extraction method, despite having a back extraction step, is very efficient for all the analytes of interest.

The statistical characteristics of five calibration curves obtained during the inter-run validation of the assay are listed in Table 2. Linear relationships ($r^2 > 0.98$, Table 1) were found between peak area ratios of analyte:IS and plasma concentrations in the range of 2.5–250 ng/ml for all the six analytes of

Table 1
The extraction efficiencies of IS and the enantiomers of T, M1, and M5 ($n = 5$)

| | IS | (+)-T | (−)-T | (+)-M1 | (−)-M1 | (+)-M5 | (−)-M5 |
|---|---|---|---|---|---|---|---|
| Mean | 87.5 | 96.2 | 102 | 99.3 | 99.6 | 83.1 | 86.4 |
| S.D. | 5.4 | 5.6 | 5.1 | 4.7 | 5.7 | 8.3 | 4.4 |

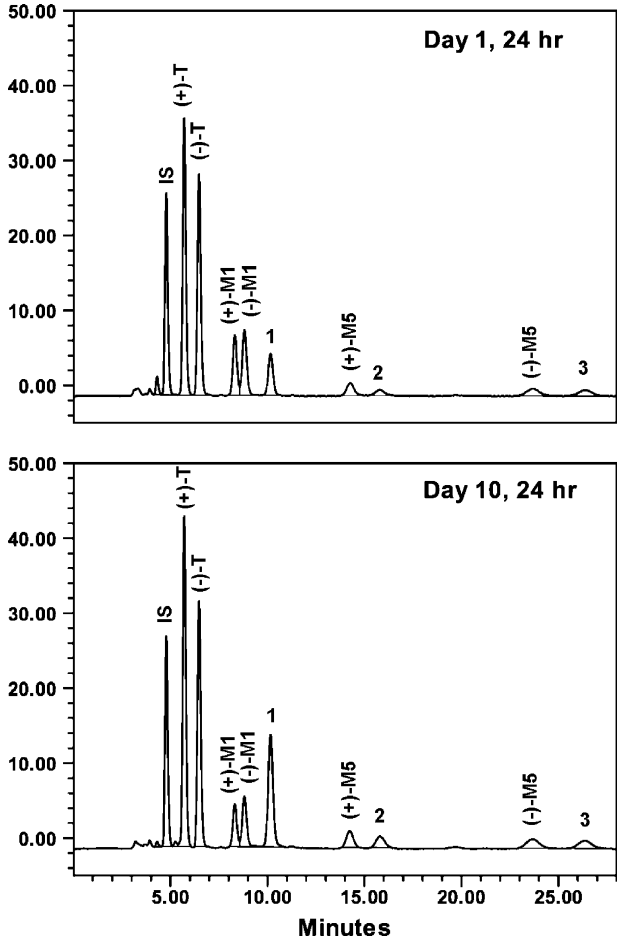


Fig. 3. Chromatograms of 24-h plasma samples on day 1 (top) and 10 (bottom), taken from a healthy volunteer who received 50 mg racemic T orally every 6 h. The day 1 sample (top) contained 95.6, 85.8, 17.4, 19.6, 8.62, and 9.14 ng/ml of (+)-T, (−)-T, (+)-M1, (−)-M1, (+)-M5, and (−)-M5, respectively. The day 10 sample (bottom) contained 111, 93.3, 13.2, 16.4, 12.9, and 13.0 ng/ml of (+)-T, (−)-T, (+)-M1, (−)-M1, (+)-M5, and (−)-M5, respectively.

interest. Furthermore, the slopes of the five inter-run calibration curves were very similar for each analyte (Table 1). Indeed, the C.V.s of the inter-run slopes were 4.99, 4.28, 9.77, 4.95, 7.58, and 7.67% for (+)-T, (−)-T, (+)-M1, (−)-M1, (+)-M5, and (−)-M5, respectively.

The intra- and inter-run assay validation data are reported in Table 3. The error values were <10% for the 50- and 250-ng/ml quality control samples and <15% for the 2.5-ng/ml quality control samples for all the six analytes (Table 3), indicating the accuracy of the assay within the 2.5–250 ng/ml concentration range. Additionally, the precision of the assay is shown by C.V. values of <10% for the 50 and 250 ng/ml concentrations and <17% for the 2.5 ng/ml concentration for both intra- and inter-run data (Table 3). Based on these validation data and reported guidelines [16], the lower limit of quantitation for all analytes is 2.5 ng/ml.

The plasma concentration–time courses of the individual enantiomers of T, M1, and M5 after the oral administration of the four daily doses of 50 mg racemic T on days 1 and 10 are depicted in Figs. 4 and 5, respectively. Additionally, the trough concentrations of the drug and its metabolites during days 7–10 are presented in Fig. 6. Multiple dosing of T resulted in substantial accumulation of the drug and both of its metabolites (Figs. 4–6). In terms of stereoselectivity, whereas the plasma concentrations of (+)-T were higher than those of its antipode, the opposite was true in most samples for the M1 and M5 metabolites. Comparing the data for the day 1 (Fig. 4) with those on day 10 (Fig. 5), it appears that the extent of stereoselectivity is higher on day 10 for both T and its metabolites. The relatively constant trough values measured during days 7–10 (Fig. 6) and reproducible plasma concentration–time profiles during the four dosage intervals on day 10 (Fig. 5) for all the measured analytes indicate the achievement and maintenance of the steady state condition with the stated dosage regimen. Although substantially less than those of the parent drug, the steady-state concentrations of the M5 enantiomers were close to those of the M1 enantiomers (Fig. 5).

Table 2
The relationship between the analyte:internal standard peak area ratio and the added concentration for inter-run data [peak area ratio = intercept + (slope × conc.)]

| Analyte | Calibration # | Slope | Intercept | $r^2$ | Standard error |
|---|---|---|---|---|---|
| (+)-T | 1 | 0.0186 | −0.00620 | 0.998 | 0.00670 |
| | 2 | 0.0176 | −0.00642 | 0.998 | 0.00680 |
| | 3 | 0.0174 | 0.00225 | 0.999 | 0.00234 |
| | 4 | 0.0171 | −0.00108 | 0.997 | 0.00877 |
| | 5 | 0.0162 | −0.00290 | 0.997 | 0.00808 |
| (−)-T | 1 | 0.0177 | 0.000111 | 0.998 | 0.00702 |
| | 2 | 0.0169 | −0.00135 | 0.998 | 0.00631 |
| | 3 | 0.0163 | 0.00471 | 0.999 | 0.00261 |
| | 4 | 0.0165 | 0.000147 | 0.997 | 0.00802 |
| | 5 | 0.0158 | 0.00499 | 0.997 | 0.00716 |
| (+)-M1 | 1 | 0.0240 | 0.00662 | 0.998 | 0.0083 |
| | 2 | 0.0210 | 0.000325 | 0.998 | 0.00913 |
| | 3 | 0.0200 | 0.00630 | 0.999 | 0.00272 |
| | 4 | 0.0203 | −0.00941 | 0.995 | 0.0125 |
| | 5 | 0.00185 | 0.00802 | 0.994 | 0.0127 |
| (−)-M1 | 1 | 0.0222 | −0.00283 | 0.998 | 0.00784 |
| | 2 | 0.0229 | −0.00896 | 0.999 | 0.00763 |
| | 3 | 0.0219 | 0.000862 | 0.999 | 0.00424 |
| | 4 | 0.0220 | 0.00204 | 0.997 | 0.0147 |
| | 5 | 0.0200 | 0.00111 | 0.997 | 0.0145 |
| (+)-M5 | 1 | 0.0163 | −0.0151 | 0.995 | 0.00992 |
| | 2 | 0.0158 | −0.0073 | 0.997 | 0.00706 |
| | 3 | 0.0145 | −0.0138 | 0.994 | 0.00893 |
| | 4 | 0.0153 | 0.0145 | 0.995 | 0.0105 |
| | 5 | 0.0134 | −0.00188 | 0.986 | 0.0142 |
| (−)-M5 | 1 | 0.0161 | −0.0217 | 0.994 | 0.0109 |
| | 2 | 0.0155 | −0.0227 | 0.998 | 0.00621 |
| | 3 | 0.0145 | −0.00568 | 0.996 | 0.00779 |
| | 4 | 0.0149 | 0.0106 | 0.995 | 0.0106 |
| | 5 | 0.0131 | 0.00428 | 0.989 | 0.0137 |

## 4. Discussion

There are several non-chiral assays reported in the literature that deal with the simultaneous measurement of T and one or more of its metabolites in human plasma [17–20]. As for individual enantiomers, the first stereospecific assay for T and

Table 3
Intra- and inter-run accuracy and precision of the assay ($n = 5$)

| Added conc. (ng/ml) | Analyte | Calculated conc. (μg/ml) | | % C.V. | | % error | |
|---|---|---|---|---|---|---|---|
| | | Intra-run | Inter-run | Intra-run | Inter-run | Intra-run | Inter-run |
| 2.5 | (+)-T | 2.37 | 2.66 | 8.07 | 16.5 | −5.04 | 6.56 |
| | (−)-T | 2.17 | 2.58 | 6.62 | 11.2 | −13.0 | 3.04 |
| | (+)-M1 | 2.43 | 2.61 | 9.81 | 10.4 | −2.8 | 4.24 |
| | (−)-M1 | 2.20 | 2.72 | 7.12 | 9.76 | −12 | 8.96 |
| | (+)-M5 | 2.54 | 2.62 | 8.45 | 12.4 | 1.52 | 4.72 |
| | (−)-M5 | 2.35 | 2.73 | 6.55 | 13.1 | −6.16 | 9.28 |
| 50 | (+)-T | 53.1 | 51.5 | 3.64 | 6.43 | 6.20 | 3.08 |
| | (−)-T | 51.7 | 51.0 | 4.66 | 5.58 | 3.40 | 1.92 |
| | (+)-M1 | 51.8 | 51.2 | 4.66 | 6.36 | 3.56 | 2.33 |
| | (−)-M1 | 52.2 | 51.3 | 5.55 | 6.10 | 4.44 | 2.52 |
| | (+)-M5 | 50.3 | 48.9 | 4.21 | 8.84 | 0.64 | −2.28 |
| | (−)-M5 | 49.4 | 50.0 | 6.26 | 9.55 | −1.12 | 0.092 |
| 250 | (+)-T | 254 | 254 | 2.51 | 5.83 | 1.68 | 1.52 |
| | (−)-T | 260 | 258 | 1.75 | 7.09 | 4.16 | 3.36 |
| | (+)-M1 | 247 | 252 | 2.70 | 5.99 | −1.20 | 0.72 |
| | (−)-M1 | 251 | 250 | 2.72 | 6.00 | 0.32 | 0.08 |
| | (+)-M5 | 230 | 250 | 9.06 | 5.76 | −8.16 | 0.00 |
| | (−)-M5 | 234 | 249 | 9.22 | 5.41 | −6.4 | −0.24 |

its metabolites was reported in 1993 by Elsing and Blaschke [21], who used two different chiral columns, Chiralpak AD and Chiralcel OD, along with non-aqueous mobile phases (normal phase) for quantitation of the enantiomers of T, M1, and M2 in human urine. Whereas the enantioseparation of T and M2 was achieved using Chiralpak AD, M1 enantiomers were separated using Chiralcel OD column. Since then, a few validated assays have been published describing the quantitation of the enantiomers of T in plasma along with simultaneous measurement of its main active metabolite M1 [9,10,14]. Additionally, Campanero et al. [15] have reported a validated HPLC assay for simultaneous quantitation of the enantiomers of T, M1, and M2 metabolites in human plasma. These authors also showed the applicability of the assay to the studies of the pharmacokinetic parameters of the drug and its two metabolites after oral administration of 100 mg racemic T. As for the quantitation of M5 enantiomers, a validated study [22] using capillary electrophoresis reported the quantitation of T, M1, M2, and M5 enantiomers in urine of a healthy volunteer. However, a subsequent report [13] from the same laboratories using capillary electrophoresis associated with electrospray ionization mass spectrometry, although capable of separating relatively high concentrations (100 ng/ml) of T, M1, M2, M3, M4, and M5 in non-biological samples, could detect only T and M1 in a plasma sample taken 2 h after a single 100-mg dose of T administered to a healthy subject. Unfortunately, no information was made available regarding the validity or sensitivity of this assay [13]. Therefore, its usefulness for measurement of the plasma concentrations of metabolites of T, other than M1, after therapeutic doses of T was not shown. As demonstrated in representative chromatograms (Figs. 2 and 3), validation statistics (Table 3), and application data (Figs. 4–6), our assay is capable of simultaneous and accurate measurement of the enantiomers of T and M1 and M5 metabolites in human plasma.

Most of the available chiral assays for separation of the enantiomers of T and/or its metabolites use liquid chromatography with Chiralcel OD-R or Chiralpak AD as a chiral column operating in a reversed- or normal-phase mode, respectively. The reversed-phase chromatography with Chiralcel OD-R was first used by Ceccato et al. [23] for quantitation of T enantiomers in human plasma. However, the enantiomers of the metabolites were not quantitated using this assay. Campanero et al. [9] modified this reversed-phase method by attaching an achiral column before the Chiralcel OD-R to simultaneously measure T and M1 in human plasma. They later [15] extended their work to the simultaneous measurement of T, M1, and M2 enantiomers by using a longer achiral column operated at a low temperature (5 °C). In both studies [9,15], the use of an achiral column before the chiral column was necessary to avoid co-elution of T and metabolite enantiomers. Although successful in separating T, M1, and/or M2 enantiomers, the combined use of achiral–chiral column in these studies resulted in relatively long run times of up to 70 min [15]. Our method for simultaneous quantitation of T, M1, and M5, which constitute all the potential active moieties after T administration, requires a much shorter run time of <30 min (Figs 2 and 3).

The column used in our studies, based on normal-phase operation, has been used before for simultaneous quantitation of T and M1 enantiomers in human plasma [10,14]. Ceccato et al. [10] reported a validated chromatographic assay for quantitation of T and M1 enantiomers based on solid phase extraction and tandem mass spectrometric detection of the analytes. Although using the same column as ours, their mobile phase contained only 3% ethanol, as opposed to 6% ethanol used in our assay. Consequently, the retention times of T and M1 enantiomers in our study (Figs 2 and 3) are significantly shorter than those reported by these authors [10]. Additionally, these authors showed that their method is capable of separat-

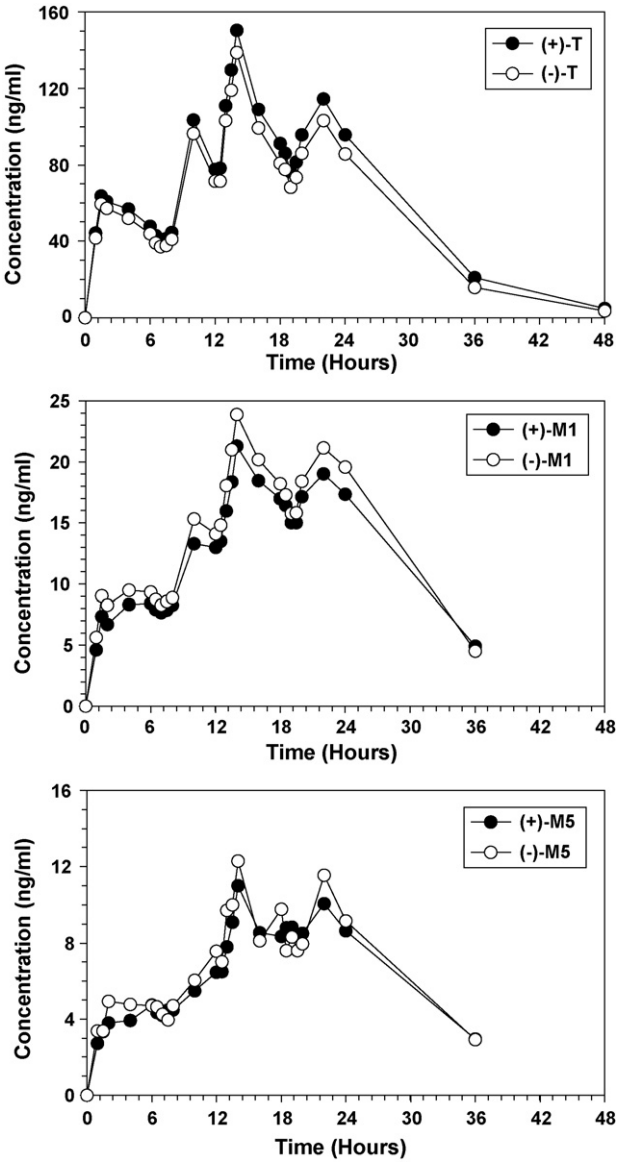


Fig. 4. Plasma concentration–time courses of T (top), M1 (middle), and M5 (bottom) enantiomers in a healthy volunteer after the oral administration of four 50-mg doses of racemic T at 0, 6, 12, and 18 h.

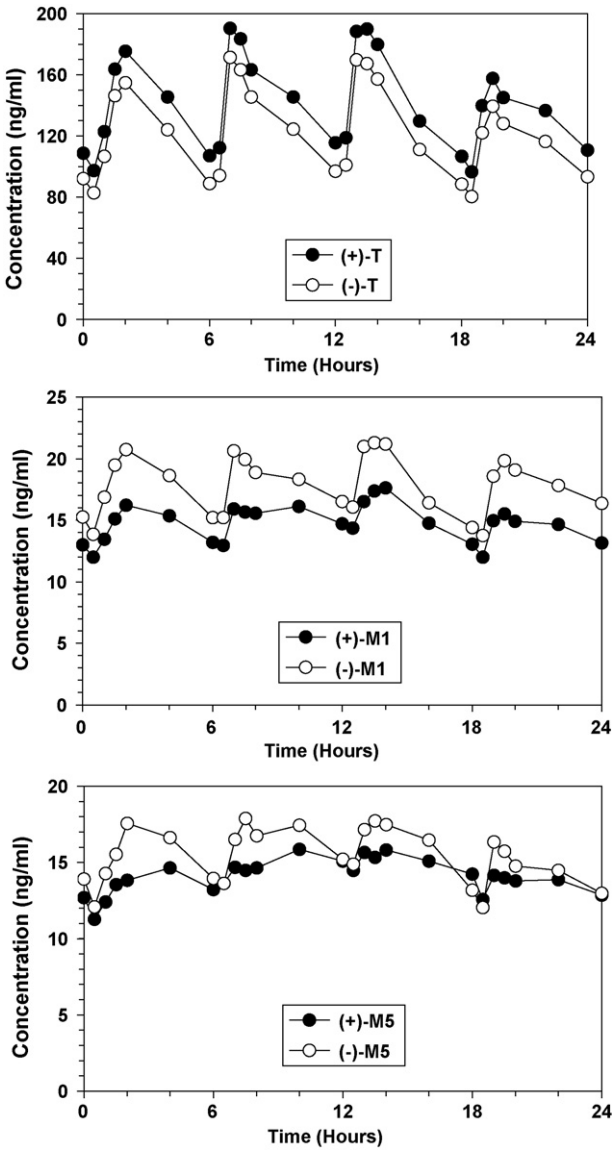


Fig. 5. Plasma concentration–time courses of T (top), M1 (middle), and M5 (bottom) enantiomers in a healthy volunteer after the oral administration of 50-mg doses of racemic T every 6 h during day 10.

ing the enantiomers of M2 and M5 (Fig. 3, ref. [10]), although the method was not validated for these metabolites nor was it applied to their quantitation in plasma samples after T dosing. Our attempt to use a similar mobile phase, although successful in separating the enantiomers of T, M1, and M5 in spiked plasma samples, resulted in very long retention times (∼60 min for the (−)-enantiomer) and broad peaks for the M5 metabolite. More importantly, when the actual samples from the healthy volunteers dosed with T were analyzed using a mobile phase with 3% ethanol, additional peaks (unknown peaks 1–3 in Fig. 3) interfered with the measurement of M1 and M5 enantiomers. Therefore, the concentrations of ethanol in the mobile phase was changed until a complete resolution of the unknown peaks, present in the plasma of volunteers, from the M1 and M5 enantiomers was achieved using our mobile phase with 6% ethanol.

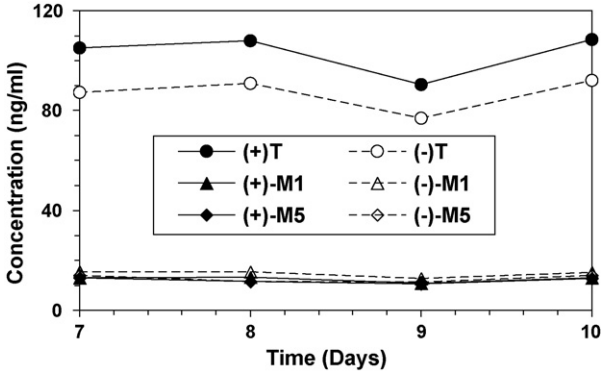


Fig. 6. Trough plasma concentrations of T, M1, and M5 enantiomers on days 7–10 after the oral administration of 50-mg doses of racemic T every 6 h.

The unknown peaks 1–3, present in the samples of the volunteer after T administration (Fig. 3), are most likely due to other metabolites of T, such as M2, M3, and/or M4 because they were not present in the predose plasma samples. Because the standards of M2, M3, and M4 metabolites were not available to us, we could not identify and quantitate these peaks. However, based on the comparison of the chromatograms on days 1 and 10 (Fig. 3), it seems that peak 1 is substantially more accumulated than the other analytes. Nevertheless, because M2, M3, and M4 metabolites are inactive [4], this accumulation may not have a clinical significance.

Different methods have been used in the literature for the extraction of T and its metabolites from plasma. Whereas methyl *tert*-butyl ether has been used to efficiently extract T, M1, and/or M2 metabolites in reversed-phase chiral chromatography [9,15], solid-phase extraction has been reported [10,14,23] for normal-phase chiral chromatography of T and M1. We tested the use of methyl *tert*-butyl ether for the extraction of T, M1, and M5 metabolites from plasma for further normal-phase chiral chromatography. Although the analytes, including M5 enantiomers, were efficiently extracted by this solvent, normal-phase chromatography of the samples revealed several interfering peaks in the blank plasma. This observation suggests that although one-step extraction with methyl *tert*-butyl ether is appropriate for reversed-phase chiral chromatography, it cannot be applied to normal phase chiral chromatography of T utilized in our assay. A simple back extraction of the analytes into HCl solution, however, resulted in clean chromatograms, which were free of any interfering peaks (Fig. 2), along with very high extraction efficiencies for all the analytes (Table 1).

It has been reported that the formation of (+)-M1, and to a lesser extent that of (−)-M1, is affected by the CYP2D6 phenotype of the subject [12,24]. Poulsen et al. [24] reported that within 10 h after a single 2 mg/kg dose of racemic T, the plasma concentrations of (+)-M1 were at or below the detection limit of 3 ng/ml in all the studied poor metabolizers and substantially higher than the detection limit in all the studied extensive metabolizers. We did not determine the phenotype of the volunteer with regard to CYP2D6 status. However, given that the plasma concentrations of (+)- and (−)-M1 were quantifiable in all the samples (Figs. 4–6), including after the first dose of 50 mg racemic T (Fig. 4), it is likely that our volunteer is an extensive metabolizer with regard to CYP2D6.

The plasma concentration–time courses of T enantiomers at steady-state (Fig. 5) are very similar to those reported before using an identical dosage regimen of immediate release racemic T [25]. Our maximum and minimum concentrations of 190 and 96.7 ng/ml for (+)-T are very close to the reported average of 190 and 111 ng/ml, respectively. Additionally, our maximum and minimum plasma concentrations of 171 and 80.6 ng/ml for (−)-T are also in complete agreement with the reported average values of 157 and 86 ng/ml, respectively. However, the concentrations of M1 enantiomers in our study (Fig. 5) are almost half of the average values reported before [25].

Reports, stereoselective or otherwise, of the concentrations of M5 metabolite in biological samples obtained from humans after T dosing are rare [2,3,22]. Using non-stereospecific assays, it has been suggested [2,3] that M1, M2, and M5 are the major metabolites of T in urine after administration of tramadol to humans, whereas M3 and M4 metabolites are formed in much lesser quantities. Rudaz et al. [22] used a stereospecific capillary electrophoresis method to determine the concentrations of the enantiomers of T and its major metabolites (M1, M2, and M5) in the urine of healthy subjects after oral administration of a single 100-mg dose of racemic T. Whereas the cumulative amount of (±)-M1 and (±)-M5 excreted in urine were relatively high (∼15%) and similar, that of (±)-M2 metabolite was only ∼2%. As for the individual enantiomers, the excretion of the metabolites into the urine was stereoselective, with the enantiomeric ratio of the second eluting peak:first eluting peak being ∼2 for the M5 metabolite [22]. However, the exact optical rotation of the enantiomers could not be detected in this study because of the lack of availability of the individual enantiomers of metabolites to the authors. Nevertheless, this study suggested stereoselective disposition for the M5 metabolites after T administration [22].

We are not aware of any study reporting the concentrations of M5 metabolite (total or individual enantiomers) in human plasma after T dosing. However, our plasma data (Figs. 4–6) in one healthy volunteer is in agreement with the urine data reported by Rudaz et al. [22]. Our data show that M5 achieves plasma concentrations comparable to those of M1 after multiple dosing of T (Fig. 5), confirming that M5 is a major metabolite of T, in addition to M1. Because M5 enantiomers are 2.4-fold more potent that (−)-M1 [4], similar plasma concentrations of M5 and M1 enantiomers (Fig. 5) suggests that the contribution of M5 enantiomers to the analgesic activity of T, if any, may be more than that of (−)-M1. Further studies are needed to determine the pharmacokinetics and pharmacodynamics of M5 generated from T in humans.

In conclusion, a sensitive, accurate, and precise method for simultaneous quantitation of the enantiomers of T and its active metabolites M1 and M5 in human plasma is reported. The assay is based on liquid–liquid extraction, chiral chromatography, and fluorescence detection and has successfully been applied to quantitation of the enantiomers of the drug and its active metabolites after oral administration of normal doses of racemic T.

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