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Journal of Chromatography B, 864 (2008) 109–115
JOURNAL OF CHROMATOGRAPHY B
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# Enantioselective determination of tramadol and its main phase I metabolites in human plasma by high-performance liquid chromatography

Yalda H. Ardakani [a], Reza Mehvar [b], Alireza Foroumadi [c], Mohammad-Reza Rouini [a,*]

[a] *Biopharmaceutics and Pharmacokinetics Division, Department of Pharmaceutics, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran 14155-6451, Iran*
[b] *School of Pharmacy, Texas Tech University Health Sciences Center, 1300 Coulter, Amarillo, TX 79106, USA*
[c] *Pharmaceutical Sciences Research Center, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran 14155-6451, Iran*

Received 27 October 2007; accepted 28 January 2008
Available online 3 February 2008

**Abstract**

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


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

## 1. Introduction

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

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

* Corresponding author. Tel.: +98 21 66959056; fax: +98 21 66461178.
*E-mail address:* rouini@sina.tums.ac.ir (M.-R. Rouini).

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doi:10.1016/j.jchromb.2008.01.038

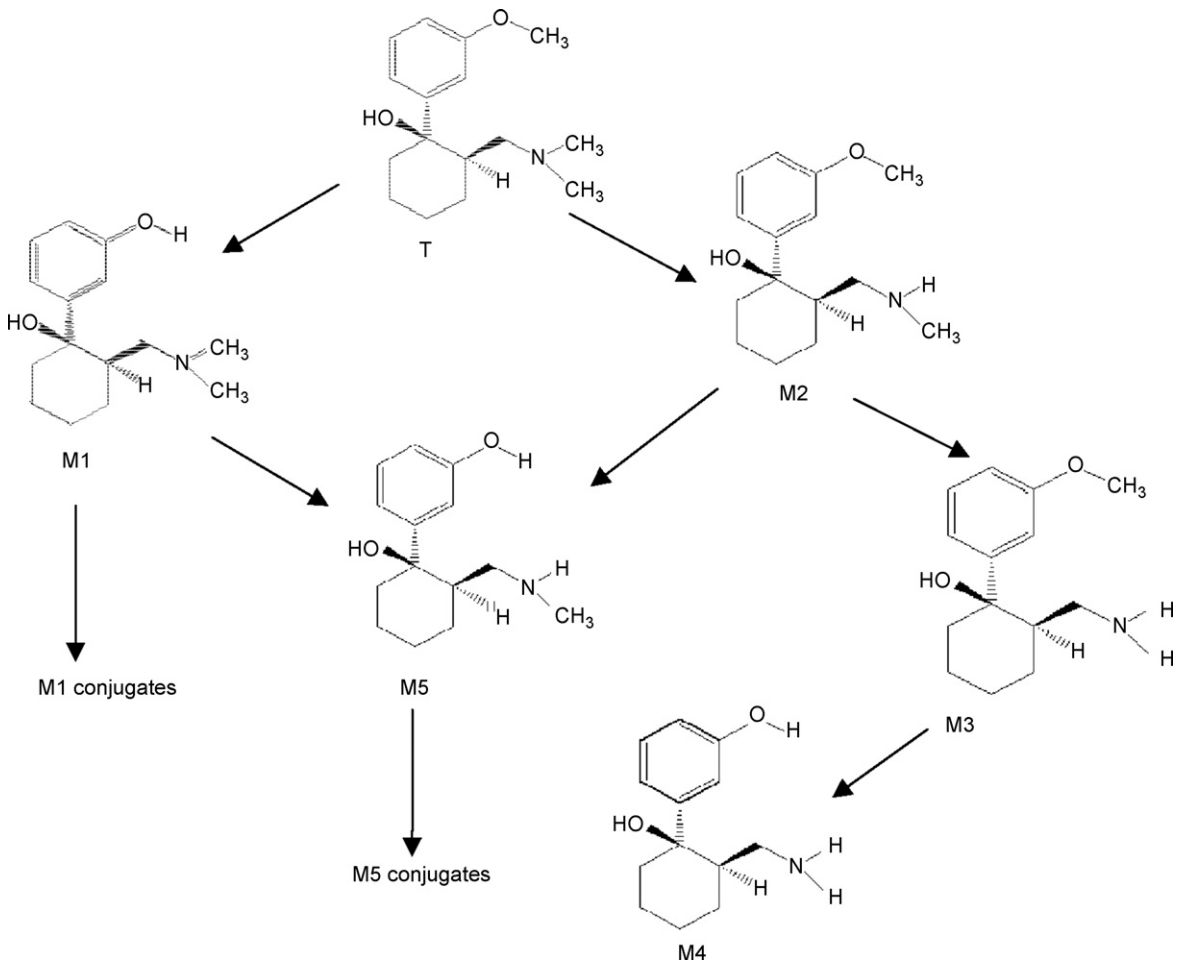


Fig. 1. Metabolic pathway of tramadol.

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

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

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

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

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

In this study, we developed and validated a new analytical method based on fluorescence detection coupled to CHIRAL-AGP column for selective and sensitive determination of the enantiomers of T and its main metabolites (M1 and M2) in