Stereoselectivity in *trans*-tramadol metabolism and *trans-O*-demethyltramadol formation in rat liver microsomes

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ABSTRACT

AIM: To study the stereoselectivity in *trans*-tramadol [(±)-*trans*-T] metabolism and *trans-O*-demethyltramadol (M1) formation. **METHODS:** (+)-, (-)-, Or (±)-*trans*-T was separately incubated with rat liver microsomes *in vitro*. The concentrations of (±)-*trans*-T and M1 enantiomers were determined by high performance capillary electrophoresis (HPCE). **RESULTS:** When each enantiomer of (±)-*trans*-T was incubated with rat liver microsomers, the metabolic rate of (+)-*trans*-T was lower than that of (-)-*trans*-T. The kinetics of (+)-, (-)-M1 formation was found to fit the single-enzyme Michaelis-Menten model. The V_{max} and CL_{int} of (+)-M1 formation were lower than those of (-)-M1 formation. When (±)-*trans*-T was used as the substrate, the metabolic rates of (+)-, (-)-*trans*-T, and the formation rates of (+)-M1, (-)-M1 decreased to different extents. Dextromethorphan (Dex), propafenone (Pro), and fluoxetine (Flu) could inhibit both the metabolism of (±)-*trans*-T enantiomers and the formation of M1 enantiomers. Pro and Flu were shown to enhance the stereoselectivity in both (±)-*trans*-T metabolism and M1 formation, and Dex could only enhance that in M1 formation. **CONCLUSION:** (±)-*Trans*-T metabolism and M1 formation were stereoselective, (-)-*trans*-T being preferentially metabolized and (-)-M1 being preferentially formed. There was interaction in metabolism between (±)-*trans*-T enantiomers. Dex, Pro, and Flu had different effects on the stereoselectivity.

INTRODUCTION

Trans-tramadol [(±)-*trans*-T] hydrochloride, a chiral compound, is used as a centrally acting analgesic. (+)-, (-)-*Trans*-T take as the action mainly through inhibiting the reuptake of serotonin and norepinephrine, respectively[1]. In human and rat, the drug is mainly metabolized in the liver to form five phase I metabolites, with the main pathways being *O*-demethylation to *O*-demethyltramadol (M1) and *N*-demethylation to *N*-demethyltramadol[2]. Among the metabolites, M1 is an only active metabolite, and (+)-M1 has a high affinity to the opioid receptor[1]. Paar *et al* reported that (±)-*trans*-T was stereoselectively metabolized through *O*- and *N*-demethylation *in vitro*, (-)-*trans*-T and (+)-*trans*-T being preferentially metabolized, respectively[3]. It was proved that M1 formation was carried out by CYP2D6 in human[4].

Recently, we found that the pharmacokinetics of (±)-*trans*-T and M1 was stereoselective *in vivo*[5,6]. In order to explore the mechanism of the stereoselectivity in pharmacokinetics, we had studied the distribution in central nervous system and renal clearance of the enantiomers of (±)-*trans*-T and M1 in rats[6,7]. The aim of

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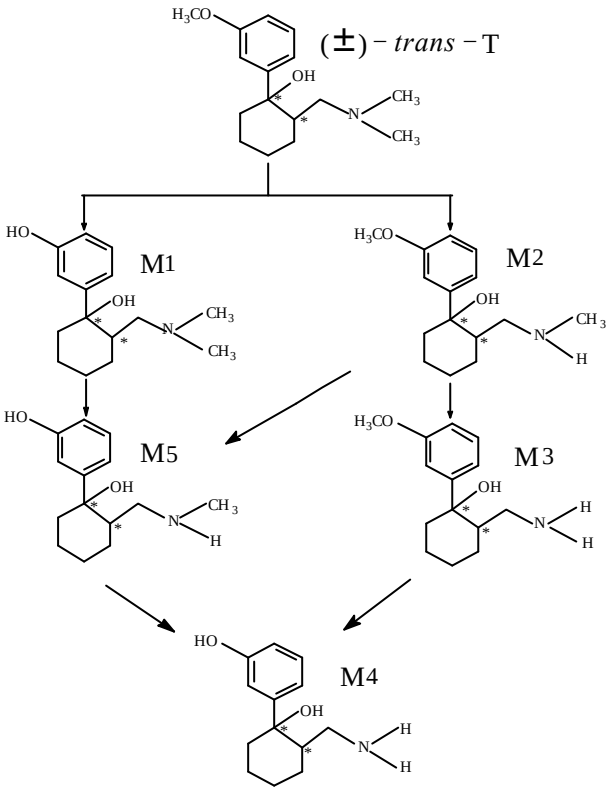

Structures of (±)-*trans*-T and its phase I metabolites, M1, *N*-demethyltramadol (M2), *N,N*-didemethyltramadol (M3), *N,N,O*-tridemethyltramadol (M4), and *N,O*-didemethyltramadol (M5). The chiral centers are marked with asterisks.

this paper was to investigate the stereoselectivity in (±)-*trans*-T metabolism and M1 formation, and also the effects of some inhibitors on their stereoselectivity.

MATERIALS AND METHODS

Chemicals and reagents (±)-*Trans*-T hydrochloride (purity 99.8 %) were obtained from Shijiazhuang First Pharmaceutical Factory (China). (+)-, (-)-*Trans*-T hydrochloride and M1 were kindly provided by Grünenthal Gmbh (Stolberg, Germany). *Cis*-T hydrochloride (internal standard) was a gift from Chemical Department of Jinzhou Medicine College (China). Sulfobutylether-β-cyclodextrin was a kind gift from Lanzhou Institute of Chemical Physics, the Chinese Academy of Sciences. Nicotinamide-adenine dinucleotide phosphate (NADPH, reduced form), dextromethorphan (Dex) hydrobromide monohydrate, propafenone (Pro) hydrochloride, and fluoxetine (Flu) hydrochloride were purchased from Sigma (USA).

Preparation of liver microsomes and condition of incubation Six Sprague-Dawley (SD) rats (♂, 244 g±15 g) were supplied by the Animal Center of Hebei Medical University. Microsomes were prepared by using differential ultracentrifugation[8]. Protein concentrations were measured by the method of Lowry *et al*, using bovine serum albumin as standard[9].

The incubation mixtures contained microsomal protein 0.6 g/L, $MgCl_2$ 6 mmol/L, Tris buffer (pH 7.5) 29 mmol/L, different concentrations of (+)-, (-)-, or (±)-*trans*-T with or without Dex, Pro, or Flu as the inhibitor. After 3 min pre-incubation at 37 °C, the reaction was started by adding 25 μL of NADPH 4 mmol/L. The final volume was 250 μL. The reaction was allowed to proceed at 37 °C for 90 min with constant shaking, then stopped by adding 10 μL ammonia solution (25 %) and cooling on ice. Preliminary experiments showed that the formation of M1 enantiomers was linear with respect to the incubation time and the microsomal protein concentration.

Determination of the enantiomers of (±)-*trans*-T and M1[5] After addition of *cis*-T, the enantiomers of (±)-*trans*-T and M1 in the incubation products were extracted with ethyl acetate. After centrifugation at 2000×*g* for 10 min, the organic layer was removed into another tube and evaporated to dryness under a gentle stream of nitrogen gas. The residue was redissolved in 200 μL of water, then an aliquot (30 μL) was removed out for high performance capillary electrophoresis (HPCE) analysis. Electrophoretic experiments were performed in a P/ACE 5000 automatic electrophoresis apparatus equipped with an UV detector (Beckman, California, USA). Data were collected with Gold software. The capillary was a fused silica one with a total length of 37 cm, an effective length of 30 cm, and an inner diameter of 75 μm. The background electrolyte (BGE) contained Tris buffer (pH 2.5) 40 mmol/L and sulfobutylether-β-cyclodextrin (chiral selector) 0.8 mmol/L. The samples were injected into the capillary by electrophoretic injection at the anode. The separation was performed at 25 °C with a positive voltage of 15 kV. The UV detector was set at 214 nm.

Data analysis The metabolic rates of (±)-*trans*-T enantiomers were obtained by dividing their depleted concentrations with the incubation time. The formation rates of M1 enantiomers were obtained by dividing their concentrations in the incubation products with the incubation time. For M1 formation, the maximum velocity (V_{max}) and apparent Michaelis-Menten constant (K_m) were obtained by fitting the data to the Michaelis-

Menten model. The intrinsic clearance (CL_{int}) was defined as the ratio of V_{max}/K_m. All data were expressed as mean±SD, and statistical analysis was carried out by unpaired *t* test or paired *t* test (for the comparison between the enantiomers), with $P<0.05$ as the minimal level of significance.

RESULTS

Electropherogram Under the analytical condition selected, the enantiomers of (±)-*trans*-T, M1, and *cis*-T could be well-separated. There was no interference from the incubation products or caused by the inhibitors used. Using the internal standard method, chromatograms were analyzed by measuring peak area. For the determination of the enantiomers of (±)-*trans*-T and M1, the within-day and between-day RSDs were less than 15 % and 20 %, and relative recoveries were 94 %– 106 % and 90 %– 107 %, respectively. The limit of quantitation was 0.04 μmol/L. When each enantiomer of (±)-*trans*-T was used as the substrate, (+)-M1 was formed from (+)-*trans*-T, whereas (-)-M1 was produced from (-)-*trans*-T. Thus, there was no chiral inversion between the enantiomers of (±)-*trans*-T (Fig 1).

Stereoselectivity in (±)-*trans*-T metabolism and M1 formation In the kinetic experiments, seven different concentrations of (+)- and (-)-*trans*-T (2.5- 100 μmol/L) were separately incubated with four rat liver microsomes. The metabolic rate of (+)-*trans*-T was lower than that of (-)-*trans*-T (Fig 2). The kinetics of (+)- and (-)-M1 formation was found to fit the single-enzyme Michaelis-Menten model. The V_{max} and CL_{int} of (+)-M1 formation were lower than those of (-)-M1 formation (Tab 1). After M1 (50 μmol/L) being incubated with six rat liver microsomes under the same experimental condition as for (±)-*trans*-T, the concentrations of (+)- and (-)-M1 decreased by 42 %±12 % and 38 %±14 %, respectively. Further study showed that M1 enantiomers had similar rates in phase I metabolism (Fig 3).

Interaction between (±)-*trans*-T enantiomers Separately using (+)-*trans*-T (12.5 μmol/L), (-)-*trans*-T (12.5 μmol/L), or (±)-*trans*-T (25 μmol/L) as the substrate, the interaction between (±)-*trans*-T enantiomers was investigated in six rat liver microsomes. Compared with those when (+)- or (-)-*trans*-T was used as the substrate, the metabolic rates of (±)-*trans*-T enantiomers and the formation rates of M1 enantiomers were lower when the racemate was used as the substrate.

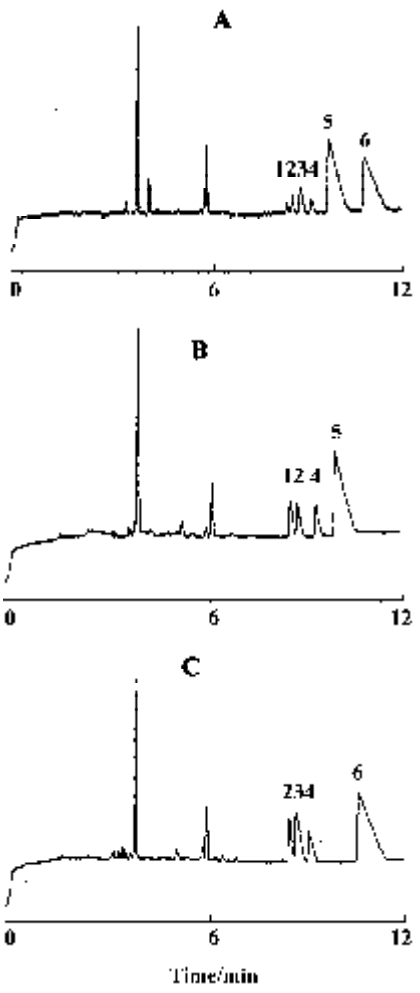

Fig 1. Typical electropherograms of rat liver microsomes incubation using (±)-*trans*-T (A), (+)-*trans*-T (B), or (-)-*trans*-T (C) as the substrate. 1: (+)-M1; 2: one enantiomer of *cis*-tramadol; 3: (-)-M1; 4: another enantiomer of *cis*-tramadol; 5: (+)-*trans*-T; 6: (-)-*trans*-T.

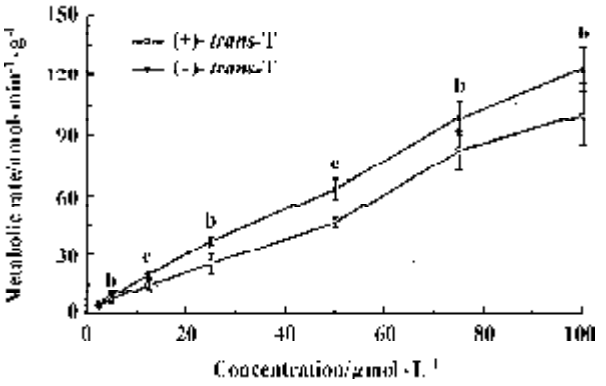

Fig 2. Metabolic rate *vs* substrate concentration plot for the metabolism of (±)-*trans*-T enantiomers in rat liver microsomes. $n=4$. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* (+)-*trans*-T.

Tab 1. Kinetic parameters for the formation of M1 enantiomers in rat liver microsomes. $^cP<0.01$ *vs* (+)-M1.

Specimen	K_m/μmol·L^{-1}		V_{max}/nmol·min^{-1}·g^{-1}		CL_{int}/mL·min^{-1}·g^{-1}	
	(+)-M_1	(-)-M_1	(+)-M_1	(-)-M_1	(+)-M_1	(-)-M_1
1	33	35	29	51	0.88	1.44
2	32	42	26	55	0.78	1.28
3	38	38	31	53	0.82	1.40
4	39	35	25	51	0.62	1.42
Mean±SD	36±4	38±3	28±3	52±2^c	0.77±0.11	1.39±0.07^c

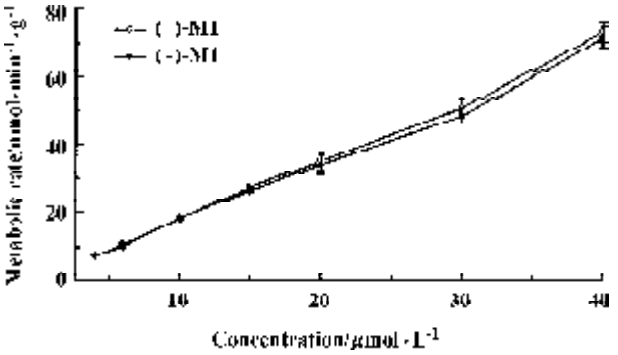

Fig 3. Metabolic rate *vs* substrate concentration plot for the metabolism of M1 enantiomers in rat liver microsomes. *n*=3. Mean±SD.

The metabolic rates of (+)- and (-)-*trans*-T decreased by 20 % and 5 %, respectively, and the formation rates of (+)- and (-)-M1 decreased by 60 % and 10 %, respectively. The ratio of (-)/(+)-*trans*-T metabolic rates increased from 1.2±0.1 to 1.5±0.2, and the ratio of (-)/(+)-M1 formation rates increased from 2.0±0.2 to 4.2±0.4 (Tab 2).

Inhibition studies Using each enantiomer (12.5 μmol/L) of (±)-*trans*-T as the substrate, the effects of Dex, Pro, and Flu on the metabolism of (±)-*trans*-T enantiomers and the formatiom of M1 enantiomers were investigated in six rat liver microsomes. In the incubation mixtures, the concentrations of Dex, Pro, and Flu were 20, 20, and 5 μmol/L, respectively. Dex, Pro, and Flu could reduce the rates of both the metabolism of (±)-*trans*-T enantiomers and the formation of M1 enantiomers. Pro and Flu could increase the ratios of (-)/(+)-*trans*-T metabolic rates and (-)/(+)-M1 formation rates, but Dex could only increase the ratio of (-)/(+)-M1 formation rates (Tab 3).

Tab 2. Metabolic rates of (±) -*trans*-T enantiomers and formation rates of M1 enantiomers in rat liver microsomes when (+)-, (-)-, or (±)-*trans*-T was used as the substrate. *n*=6. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* (+)- or (-)-*trans*-T as the substrate.

Substrate	Metabolic rate/ nmol· min^{-1}· g^{-1}		Formation rate/ nmol· min^{-1}· g^{-1}	
	(+)-*trans*-T	(-)-*trans*-T	(+)-M_1	(-)-M_1
(+)-*trans*-T	20±3		6±1	
(−)-*trans*-T		24±2		13±1
(±)-*trans*-T	16±3^b	23±2	3±1^c	12±2

DISCUSSION

In rat liver microsomes, the metabolic rate of (+)-*trans*-T was lower than that of (-)-*trans*-T, which indicated that (±)-*trans*-T metabolism was stereoselective. Meanwhile, the V_{max} and CL_{int} of (+)-M1 formation were lower than those of (-)-M1 formation, which indicated that M1 formation was also stereoselective. These findings were in agreement with the results found in human microsomes by Paar *et al*[3].

Since the ratio of (-)/(+)-*trans*-T metabolic rates was much lower than that of (-)/(+)-M1 formation rates,

Tab 3. Effects of dextromethorphan (Dex), propafenone (Pro), and fluoxetine (Flu) on the metabolism of (±)-*trans*-T enantiomers and the formation of M1 enantiomers in rat liver microsomes. *n*=6. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* control.

Inhibitor	Parent metabolism			Metabolite formation		
	Rate/nmol· min^{-1}· g^{-1}		Ratio	Rate/nmol· min^{-1}· g^{-1}		Ratio
	(+)-*trans*-T	(-)-*trans*-T	(-)/(+)-*trans*-T	(+)-M1	(-)-M1	(-)/(+)-M1
Control	20±3	24±2	1.24±0.10	6±1	13±1	2.02±0.24
Dex	13±3^c	17±3^c	1.39±0.19	1±0^c	6±1^c	4.6±1.8^b
Pro	8±2^c	13±3^c	1.68±0.25^c	2±0 ^{c}	5±1^c	3.6±0.7^c
Flu	4±2^c	10±3^c	3.3±1.4^b	2±1^c	8±2^c	4.3±1.4^b

the stereoselectivity in M1 formation was much higher than that in (±)-*trans*-T metabolism. There might be other pathway that preferentially metabolized (+)-*trans*-T. But, the preferential *O*-demethylation of (-)-*trans*-T could play an important role in the stereoselectivity in (±)-*trans*-T metabolism, which might be the main reason that (-)-*trans*-T was preferentially eliminated from blood *in vivo*[5]. In phase I metabolism, M1 could be further demethylated to form two other metabolites. Our results indicated that the further metabolism of M1 was not stereoselective.

It is common that the enantiomers of chiral drugs may be metabolized by the same enzyme at different rates. The enantiomers of (±)-*trans*-T might compete the *O*-demethylation and give rise to the possibility of an enantiomer/enantiomer interaction. Consequently, the *O*-demethylation of (+)-*trans*-T was inhibited more intensely than that of (-)-*trans*-T, and the stereoselectivity in (±)-*trans*-T metabolism and M1 formation was enhanced.

The *O*-demethylation of Dex is catalyzed by CYP2D1 in SD rat and CYP2D6 in human, and is used as a measurement of CYP2D6 and CYP2D1 activity[10]. In rat liver, Pro is metabolized to omega-OH-Pro, not 5-OH-Pro which is the major metabolite in human plasma[11]. Pro is a special rat CYP2D1 inhibitor[12]. As the substrates of CYP2D1, Dex and Pro might compete for the *O*-demethylation with the enantiomers of (±)-*trans*-T, then inhibited (±)-*trans*-T metabolism and M1 formation. Their effects on the stereoselectivity in (±)-*trans*-T metabolism and M1 formation were much different. Pro could preferentially inhibit (+)-*trans*-T metabolism and (+)-M1 formation, and enhance the stereoselectivity in (±)-*trans*-T metabolism and M1 formation. Dex could induce greater inhibition on (+)-M1 formation than on (-)-M1 formation, and only enhanced the stereoselectivity in M1 formation.

Flu is metabolized in the liver by *N*-demethylation to an active metabolite- norfluoxetine (*N*-Flu). Both Flu and *N*-Flu are potent inhibitors of CYP2D6 *in vivo* and *in vitro*[13]. In this study, the concentration of Flu was only one-fourth of that of Pro or Dex, but its inhibitory effect on the metabolism of (±)-*trans*-T enantiomers was greater than that of Pro or Dex, and its inhibitory effect on the formatiom of M1 enantiomers was lower than that of Pro or Dex. It might be due to the fact that Flu and/or *N*-Flu could inhibit other metabolic pathways of (±)-*trans*-T. It had been reported that Flu and *N*-Flu were also the potent inhibitors of CYP3A4 and CYP2C19[13].

The inhibition studies might provide convincing evidence for the involvement of CYP2D1 in the metabolism of (±)-*trans*-T enantiomers and the formation of M1 enantiomers. CYP2D1 is known as the rat counterpart of human CYP2D6[14,15]. Because *in vitro* and *in vivo* studies had shown that (±)-*trans*-T and its enantiomers were metabolized in a similar way in rat and human[2,3], these results would call the attention to the possible clinical interaction when (±)-*trans*-T is administrated simultaneously with these inhibitors.

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