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Journal of Chromatography B, 826 (2005) 108–113
JOURNAL OF CHROMATOGRAPHY B
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Chiral liquid chromatography resolution and stereoselective pharmacokinetic study of tetrahydropalmatine enantiomers in dogs

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Received 3 November 2004; accepted 16 August 2005
Available online 13 September 2005

Abstract

A selective chiral high performance liquid chromatographic (HPLC) method coupled with achiral column was developed and validated to separate and quantify tetrahydropalmatine (THP) enantiomers in dog plasma. Chromatography was accomplished by two steps: (1) racemic THP was separated from biological matrix and collected on a Kromasil C_{18} column (150 mm × 4.6 mm, 5 μm) with the mobile phase acetonitrile-0.1% phosphoric acid solution, adjusted with triethylamine to pH 6.15 (47:53); (2) enantiomeric separation was performed on a Chiralcel OJ-H column (250 mm × 4.6 mm, 5 μm) with the mobile phase anhydrous ethanol. The detection wavelength was set at 230 nm. (+)-THP and (−)-THP were separated with a resolution factor (Rs) of at least 1.6 and a separation factor (α) greater than 1.29. Linear calibration curves were obtained over the range of 0.025–4 μg/ml in plasma for each of (+)-THP and (−)-THP ($R^2 > 0.999$) with a limit of detection (LOD) of 0.005 μg/ml and the recovery was greater than 88% for each enantiomer. The relative standard deviation (R.S.D.) and relative error values were less than 10% at upper and lower concentrations. The method was used to determine the pharmacokinetics of THP enantiomers after oral administration of racemic THP. The results presented herein showed the stereoselective disposition kinetics of THP in dogs and were a further contribution to the understanding of the kinetic behavior of THP analogues.

Keywords: Enantiomer separation; Tetrahydropalmatine; Pharmacokinetics

1. Introduction

Rhizoma Corydalis (*yanhusuo*), the dried tuber of *Corydalis yanhusuo* W.T. Wang, has been traditionally used in China for the treatment of chest pain, epigastric pain, dysmenorrheal, traumatic swelling and pain for thousands of years [1]. Tetrahydropalmatine (THP) was one of the active ingredients isolated from Rhizoma Corydalis, which possessed the effects of anodyne and hypnosis without drug addiction [2]. It had also reported that THP had effects including hypotensive effect [3,4], anti-arrhythmia [5,6], inhibiting the aggregation of thrombocytes [7] and the secretion of gastric acid [8].

The chemical structure of THP was shown in Fig. 1. It had an asymmetric carbon on the structure. Pharmacological studies revealed that (−)-THP act as the blocker of dopamine receptor in nerve central system, accounting for most of antalgic activity, while (+)-THP acted as initiative substance of dopamine emptier [9]. THP was applied as the racemic mixture clinically. It was necessary to evaluate the pharmacokinetic behavior of each enantiomer rather than that of the racemate, to use a racemic drug effectively and safely. Several nonstereoselective analytical methods have been described for the determination of racemic THP in plasma [10–12]. To our knowledge, no enantiomeric separation of THP was reported until now and so did the pharmacokinetics of individual enantiomers.

Chiral separation by high-performance liquid chromatography using chiral stationary phases is now a well-established method for the enantioselective determination of chiral drugs. The commonly used chiral stationary phases are polysaccharides, such as cellulose, cyclodextrin and macrocyclic glycopeptides [13–15].

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doi:10.1016/j.jchromb.2005.08.012

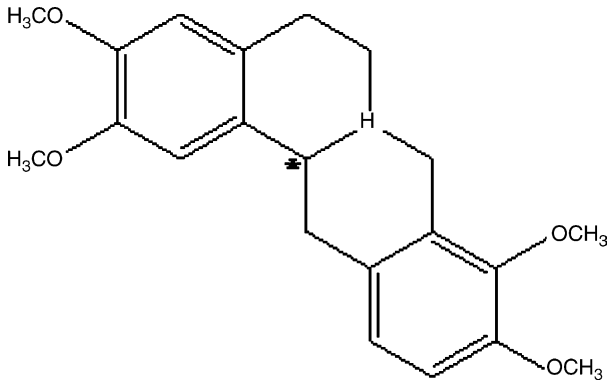

Fig. 1. Chemical structure of racemic tetrahydropalmatine (THP).

In the present study, the two enantiomers of THP were separated on Chiralcel OJ column, a cellulose tris(4-methylbenzoate)-based stationary phase on silica. A sequential achiral–chiral high performance liquid chromatographic (HPLC) method was thus developed and validated for the quantification of the two enantiomers in dog plasma. The pharmacokinetics of (+)-THP and (−)-THP in dogs was studied and compared using this method.

2. Experimental

2.1. Chemicals and reagents

Racemic THP (purity 99.5%) and one of its enantiomers, (−)-THP (optical purity 99.5%) were provided by Nanning Pharmaceuticals (Guangxi, China). HPLC-grade acetonitrile and ethanol were obtained from Merck Company (Darmstadt, Germany). Hexane, 2-propanol, triethylamine, sodium hydroxide and phosphoric acid, of analytical reagent grade, were purchased from Shanghai Reagents Company (Shanghai, China). Double-distilled water was used for the preparation of all solutions and 0.45 μm pore size filters (Millipore, MA) was used to filter the solutions.

2.2. Animals

Six healthy dogs weighing 10–15 kg were obtained from the Laboratory Animal Center of Chinese Academy of Sciences, Shanghai, China. Animals were housed under normal conditions and allowed to acclimatize for at least 1 week before initiation of studies. Water and standard laboratory food were given until 12 h before the experiments.

2.3. Drug administration and blood sampling

Six dogs received racemic THP (40 mg/kg) by gastric intubation followed by 5 ml flush with tap water. Blood samples (each 2.0 ml) were collected before drug administration and post-dose at 0.083, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12 and 24 h. Each collected blood sample was transferred to a heparinized microcentrifuge tube and plasma was separated out by centrifugation at 3000 rpm for 10 min. All plasma samples were stored at −20 °C until analysis.

2.4. Sample preparation

The plasma samples (0.5 ml) were alkalinized with 0.1 ml of 1 M NaOH and shaken for 20 s. The mixture was extracted with 3.0 ml of a mixture of hexane and 2-propanol (95:5), using a vortex mixer for 2 min and centrifugation at 3500 rpm for 10 min. An accurately measured 2.4 ml of the supernatant organic layer was evaporated to dryness in a stream of nitrogen on a 45 °C water bath. The residue was reconstituted in 100 μl mobile phase (I), followed by centrifugation at 15,000 rpm for 5 min. An 80 μl aliquot of the supernatant was directly injected into the HPLC system.

2.5. Sample analysis

A Shimadzu 10A HPLC system (Shimadzu Corporation, Kyoto, Japan), consisting of a standard vacuum degasser, LC-10ATvp pump, column oven and a multi-wavelength detector were used to conduct the analysis. Eighty microlitres was injected into the HPLC system fitted with a C_{18} reversed-phase column (150 mm × 4.6 mm, 5 μm), protected by a guard column (10 mm × 4.6 mm) packed with the same packing material (Kromasil, Sweden). The mobile phase (I) was acetonitrile–0.1% phosphoric acid solution, adjusted with triethylamine to pH 6.15 (47:53). The flow rate was 1.0 ml/min and the detection wavelength was set at 230 nm.

During the reverse-phase HPLC analysis, the THP fraction was collected into a glass tube at a retention time of 8.5 min. The collected fraction (∼1 ml) was evaporated under nitrogen stream for 15 min and the resulting solution was alkalinized with 1 M NaOH and prepared as the procedure in Section 2.4. The residue was reconstituted in 80 μl anhydrous ethanol mobile phase (II), followed by centrifugation at 15,000 rpm for 5 min. An 60 μl aliquot of the supernatant was directly injected into the same HPLC system fitted with a chiral stationary phase column (Chiralcel OJ-H, 250 mm × 4.6 mm, 5 μm), protected by a guard column (10 mm × 4 mm) packed with the same packing material (Daicel Chemicals, Japan). The mobile phase (II) was set at a flow rate of 0.5 ml/min. The detection wavelength was set at 230 nm and the temperature was maintained at 30 °C.

2.6. Calibration curves and assay validation

Stock solution of racemic THP was prepared in water (1.0 mg/ml) and working standard solutions were obtained by dilutions of the stock solution. Blank plasma samples were spiked with racemic THP at concentrations of 0.05–8 μg/ml. The calibration samples were prepared as described above. The calibration curves were generated by the peak area of racemic THP or each enantiomer versus the concentration of racemic THP or half of the concentration of racemic THP spiked in the samples. The peak area of the individual enantiomer was calculated by multiplying the peak area measured on the achiral column by the ratios of the enantiomers obtained from the chiral column. Linear regression analysis was performed using Microsoft Excel 97 (Microsoft, Redmond, WA). The precision (expressed as R.S.D.) and accuracy (expressed as relative error) of the

assay were obtained by comparing the predicted concentration (obtained from the calibration curve) to the actual concentration of racemic THP spiked in blank plasma. The limit of detection (LOD) was considered as the concentration resulting in a signal-to-noise ratio (S/N) of 3. The recovery was calculated from the ratio of the peak area of each enantiomer after extraction from plasma samples to the peak area of an equivalent amount of the working standard solution. The calibration curves and assay validation were all performed in duplicate on five separate occasions ($n = 5$).

2.7. Pharmacokinetic analysis

The pharmacokinetic parameters were determined based on the non-compartment model and calculated with an in-house validated computer program. The maximum plasma concentration (C_{max}) and the time to reach maximal plasma concentration (T_{max}) were obtained from the observed data of the concentration–time curves. The area under the plasma concentration–time curve (AUC) and the area under the first-moment time curve (AUMC) were calculated by the trapezoidal method, and were extrapolated to infinity using the last detectable plasma concentration and the terminal elimination rate constant. Mean residence time (MRT) was calculated using the equations MRT = AUMC/AUC. The terminal elimination half-life ($t_{1/2}$) was derived by linear regression analysis of the terminal phase of the plasma concentration–time curve.

All values were reported as mean ± standard deviation. Differences between pharmacokinetic parameters of the two enantiomers were evaluated by Pair *t*-test. A value of $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Sequential achiral–chiral HPLC method

Direct injection of the extracts was tested on the Chiralcel OJ column. Due to the interference by biological matrix components, the retention times of the enantiomers were increased, drastically diminishing the sensitivity of the method and the life of the column. Therefore, a sequential achiral–chiral system was preferable. The chromatographic conditions for the separation of THP enantiomers were optimized.

3.1.1. Mobile phase composition for chiral separation

Cellulose derivative-based phases, introduced by Ichida et al. [16] and Okamoto et al. [17] found application to the chiral separation of a broad range of compounds having different structures [18]. Chiralcel OJ is a cellulose tris(4-methylbenzoate)-based stationary phase on silica and this support is usually used in normal phase liquid chromatography. In this study, various preliminary trials were conducted in the normal phase mode with different combinations of hexane, ethanol and 2-propanol to select the mobile phase that would give an optimum separation and selectivity for THP enantiomers. There was no indication that separation was possible in normal phase mode. Separation was observed when the percentage of ethanol reached 100%. Therefore, the mobile phase was decided to be 100% ethanol.

Table 1
Effect of column temperature on the separation of THP enantiomers

Temperature (°C)	k'-(+)-THP	k'-(−)-THP	α	Rs
20	1.28	1.66	1.30	1.75
25	1.25	1.63	1.30	1.63
30	1.15	1.49	1.30	1.61
35	1.03	1.33	1.29	1.54

Column: Chiralcel OJ-H (250 mm × 4.6 mm i.d.). Mobile phase: anhydrous ethanol. Rs is the resolution factor; k', the capacity factor; and α, the separation factor.

3.1.2. Column temperature

The effect of raising the column temperature in 5 °C increments was investigated on separation of the THP enantiomers and the results were showed in Table 1. The separation factor remained constant at 1.29 while the resolution decreased from 1.75 to 1.54 across the tested temperature range. A raise in column temperature shortened the elution time and reduced the column pressure. Therefore, with regards to the right balance of resolution and elution time, the column temperature was set at 30 °C.

3.1.3. Effect of flow rate

The influence of flow rate upon resolution was examined under the optimum mobile phase and temperature conditions changing the flow rate from 0.3 to 0.7 ml/min. The results were listed in Table 2. It was found that the flow rate has little effect on separation factor but significant effect on the resolution. Although high flow rates would accelerate the speed of analysis, the column pressure increased. In order to maintain high resolution and avoid long analysis time, the flow rate was set at 0.5 ml/min.

3.2. Specificity and chromatography

Under the optimum conditions described, in achiral system, the retention time of racemic THP was about 8.5 min. Specificity of the method was demonstrated by the absence of any endogenous interference at retention time of peaks of interest as evaluated by chromatograms of blank dog plasma and plasma spiked with racemic THP. The chiral system was suitable for the separation of THP enantiomers. The order of elution was

Table 2
Effect of flow rate on the separation of THP enantiomers

Flow rate (ml/min)	k'-(+)-THP	k'-(−)-THP	α	Rs
0.3	1.16	1.50	1.29	2.48
0.4	1.13	1.47	1.30	1.66
0.5	1.14	1.48	1.30	1.61
0.6	1.13	1.47	1.30	1.61
0.7	1.19	1.54	1.29	1.57

Column: Chiralcel OJ-H (250 mm × 4.6 mm i.d.). Mobile phase: anhydrous ethanol. Rs is the resolution factor; k', the capacity factor; and α, the separation factor.

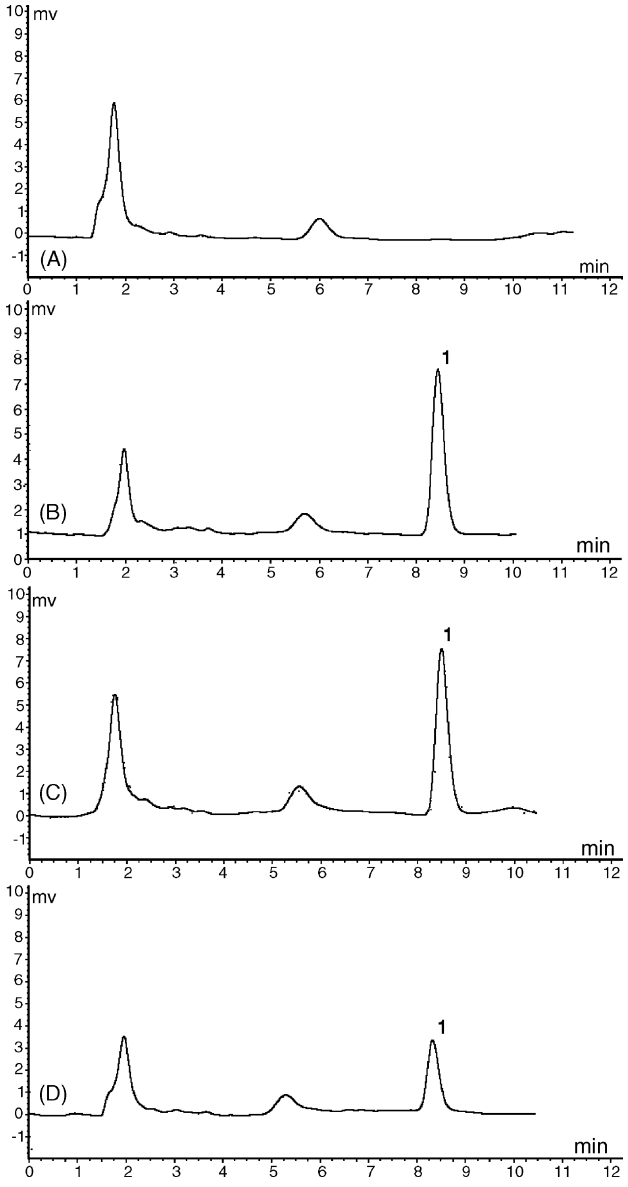

Fig. 2. Typical chromatograms of racemic THP on achiral system: (A) a blank plasma; (B) plasma spiked with racemic THP (2.0 μg/ml of racemic THP); (C) dog plasma drawn at 1 h (2.164 μg/ml of racemic THP) and (D) dog plasma drawn at 4 h (0.806 μg/ml of racemic THP) after oral administration of racemic THP (40 mg/kg), where (1) is racemic THP.

identified by injection of the isolated (−)-THP. The retention times for (+)-THP and (−)-THP were about 16.8 and 19.2 min. A resolution factor of at least 1.6 and a separation factor greater than 1.29 for THP enantiomers were obtained. Representative chromatograms of blank plasma and spiked plasma sample from the achiral system and the chiral system were shown in Figs. 2 and 3, respectively. The enantiomers were stable and no conversion or racemization was observed from the results obtained by chiral HPLC analysis of racemic THP solution and (−)-THP solution in this study.

3.3. Assay validation

Linear calibration curves were obtained over the concentration range of 0.05–8 μg/ml in plasma for racemic THP

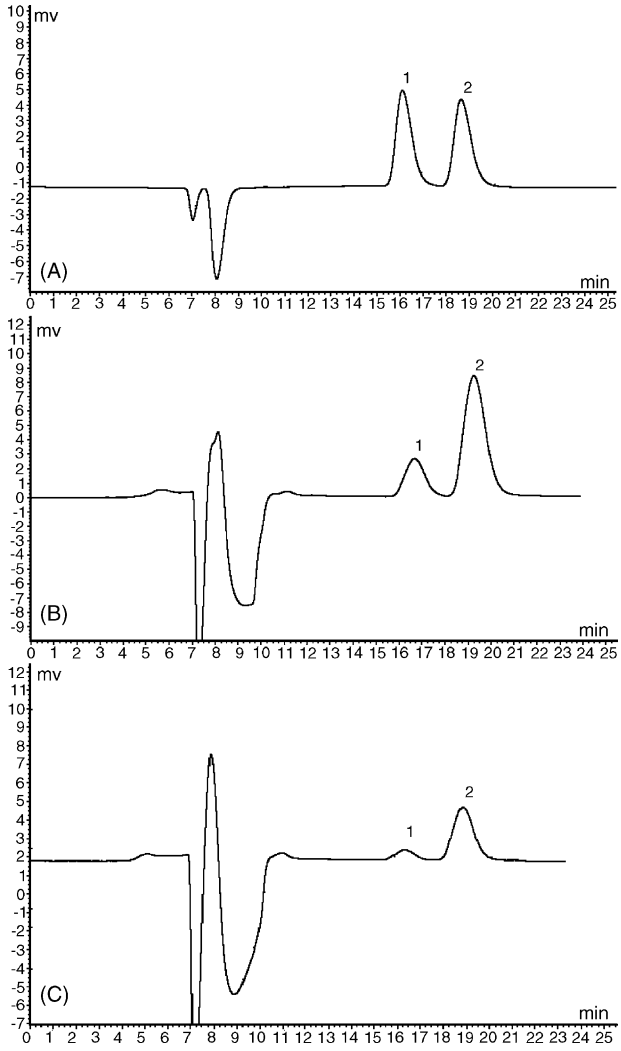

Fig. 3. Typical chromatograms of THP enantiomers obtained from chiral system: (A) standard solution of racemic THP; (B) dog plasma drawn at 1 h and (C) dog plasma drawn at 4 h after oral administration of racemic THP (40 mg/kg), where (1) is (+)-THP, (2) is (−)-THP.

[$y = (217208 \pm 4618)C - (699 \pm 1152)$, $R^2 = 0.9995$] and 0.025–4 μg/ml in plasma for both (+)-THP [$y = (217093 \pm 4626)C - (468 \pm 576)$, $R^2 = 0.9992$] and (−)-THP [$y = (218094 \pm 4638)C - (573 \pm 583)$, $R^2 = 0.9993$]. The accuracy and precision of the assay for racemic THP and individual enantiomer were presented in Table 3. R.S.D. and error values were less than 10%. The results of the comparison of plasma-extracted standards versus neat standards were estimated at 0.05, 0.125, 0.5, 1 and 4 μg/ml for individual enantiomer. The absolute recoveries for individual enantiomer ranged from 88.7% to 96.4%.The LODs was found to be 0.01 μg/ml for racemic THP and 0.005 μg/ml for individual enantiomer.

3.4. Pharmacokinetic analysis

Plasma concentration–time profiles of THP enantiomers following an, e.g. administration of 40 mg/kg THP were illustrated in Fig. 4. The plasma concentrations of (−)-THP reached a C_{max}

Table 3
Accuracy and precision for the determination of tetrahydropalmatine enantiomers in dog plasma ($n = 5$)

Nominal concentration (μg/ml)	Measured concentration (μg/ml)	Relative error (%)	R.S.D. (%)	
			Intra-day	Inter-day
rac-THP				
0.1	0.105 ± 0.006	5.3	5.9	6.4
0.25	0.243 ± 0.005	−2.9	1.9	1.7
1	0.988 ± 0.011	−1.2	1.2	3.3
2	1.986 ± 0.04	−0.7	2.0	2.7
8	8.24 ± 0.33	3.0	4.0	3.6
(+)-THP				
0.05	0.053 ± 0.003	5.1	5.8	6.3
0.125	0.121 ± 0.002	−3.1	2.0	1.6
0.5	0.493 ± 0.007	−1.4	1.4	3.5
1	0.991 ± 0.022	−0.9	2.2	2.6
4	4.11 ± 0.16	2.8	3.9	3.8
(−)-THP				
0.05	0.053 ± 0.003	5.5	5.9	6.6
0.125	0.122 ± 0.002	−2.7	1.8	1.9
0.5	0.495 ± 0.005	−1.0	1.1	3.2
1	0.995 ± 0.019	−0.5	1.9	2.8
4	4.13 ± 0.17	3.3	4.0	3.5

of 1.72 μg/ml at approximately 1.5 h (T_{max}) after dosing and then decreased with a terminal phase $t_{1/2}$ of about 6.77 h. The plasma concentrations of (+)-THP reached a C_{max} of 0.30 μg/ml at approximately 1.2 h (T_{max}) after dosing and then decreased with a terminal phase $t_{1/2}$ of about 15.44 h. The plasma concentration of (−)-THP was higher than that of (+)-THP at each time point in every dog, as shown in Fig. 4. Pharmacokinetic parameters determined by non-compartment analysis method were summarized in Table 4. The C_{max} and $AUC_{0\sim\infty}$ of (−)-THP were about five times and nine times as high as those of (+)-THP, respectively, indicating greater adsorption and distribution of (−)-THP. And the $t_{1/2}$ and MRT of (+)-THP were greater than those of (−)-THP, suggesting faster elimination of (−)-THP. Similarly, the other pharmacokinetic parameters of the enantiomers were also significantly different. The stereoselectivity in the pharmacokinetics of THP enantiomers was evident in both plasma concentrations versus time profiles and estimated pharmacokinetics for the individual enantiomers of

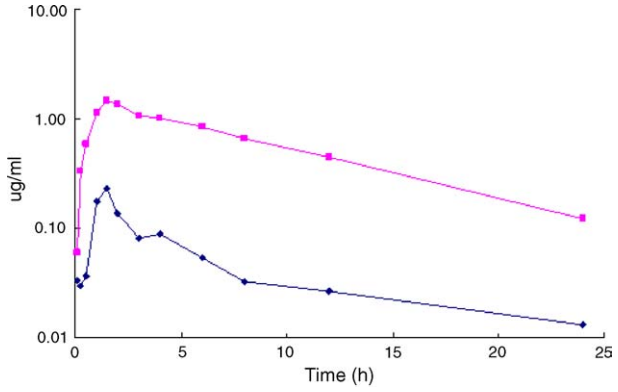

Fig. 4. Mean plasma concentration–time profiles for THP enantiomers in six dogs following oral administration of racemic THP (40 mg/kg): (♦) (+)-THP, (■) (−)-THP.

Table 4
Pharmacokinetic parameters of THP enantiomers following oral administration of racemic THP in six dogs (40 mg/kg)

Parameter	(+)-THP	(−)-THP
C_{max} (μg/ml)	0.30 ± 0.18	1.72 ± 0.75*
T_{max} (h)	1.17 ± 0.52	1.50 ± 0.45
$AUC_{0\sim\tau}$ (μg h/ml)	0.85 ± 0.54	8.63 ± 1.27*
$AUC_{0\sim\infty}$ (μg h/ml)	1.14 ± 0.64	10.02 ± 2.48*
$t_{1/2}$ (h)	15.44 ± 8.12	6.77 ± 2.70*
MRT (h)	24.10 ± 10.91	14.64 ± 3.64*

* Significantly different from (+)-THP at $P < 0.05$.

THP. It would appear that the pharmacokinetic behavior of (−)-THP is responsible for the greater pharmacological effects as anodyne and hypnosis.

4. Conclusion

The sequential achiral–chiral HPLC method described in this paper was suitable for the quantification of THP enantiomers and the study of the stereoselective pharmacokinetics of THP in dogs. The use of the sequential achiral–chiral system avoided direct injections of interfering components from the biological matrix and preserved the chiral stationary phase from rapid degradation. Pharmacokinetic analysis showed (−)-THP had higher C_{max}, $AUC_{0\sim\tau}$ and $AUC_{0\sim\infty}$ than that of the (+)-THP after the oral administration of racemic THP at 40 mg/kg, indicating the disposition of THP was stereoselective in dogs.

Acknowledgement

This work was financially supported by a grant (No. 30271603) from the National Natural Science Foundation of China.

References

[1] Pharmacopoeia commission of RPC, Chinese Pharmacopoeia (Part I, 2000 edition), Chemical Industry Publishing House, Beijing, 2000, p. 216.
[2] S. Sha, Assay Methods for Active Components in Chinese Herb Medicines [M], The People's Medical Publishing House, Beijing, 1985, p. 53.
[3] S.H. Xing, X.Q. Ge, M. Yan, Acta Pharmacol. Sin. 15 (1994) 92.
[4] S.H. Xing, J.L. Zheng, C.F. Bian, Chin. J. Mod. Appl. Pharm. 16 (1999) 8.
[5] J.H. Zhou, J.H. Wang, Advances in Pharmacological and Clinical Research of Traditional Chinese Medicines, Chinese Science and Technology Publishing House, Beijing, 1993, p. 201.
[6] Y.S. Wang, Y.M. Zheng, Y.H. Tan, Chin. Pharmacol. Bull. 9 (1993) 358.
[7] J. Xing, M. Wang, X. Ma, Chin. Pharmacol. Bull. 13 (1997) 258.
[8] Y. Li, J.H. Wang, S.X. Lao, Chin. Pharmacol. Bull. 9 (1993) 44.
[9] G.Z. Jin, Acta Pharm. Sin. 22 (1987) 472.
[10] B.M. Liu, P. Lei, G.F. Zhou, Chin. J. Pharm. Anal. 8 (1988) 299.
[11] Z.H. Han, X.L. Niu, J. Xi'an Med. Univ. 20 (1999) 454.
[12] Z.G. Pang, B.Q. Wang, C.Y. Wang, Chin. J. Anal. Chem. 22 (1994) 612.
[13] Y. Okamoto, Y. Kaida, J. Chromatogr. A 666 (1994) 403.
[14] F. Bressolle, M. Audran, T.N. Pham, J.J. Vallon, J. Chromatogr. B 687 (1996) 303.
[15] T.J. Ward, AB. Farris III, J. Chromatogr. A 906 (2001) 73.
[16] A. Ichida, T. Schibata, I. Okamoto, Y. Yuki, H. Namikoshi, Y. Toga, Chromatographia 19 (1984) 280.
[17] Y. Okamoto, K. Hatada, M. Kawashima, J. Am. Chem. Soc. 106 (1984) 5357.
[18] E. Yashima, J. Chromatogr. A 906 (2001) 105.