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Full Length Research Paper

Determination of levo-tetrahydropalmatine in rat plasma by HPLC and its application to pharmacokinetics studies

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The objective of this study was to establish a high performance liquid chromatography (HPLC) method for the determination of levo-tetrahydropalmatine (*l*-THP) in rat plasma, and to investigate the pharmacokinetics after oral administration of *l*-THP in rats. The plasma samples were extracted by ethyl acetate. The mobile phase consisted of a mixture of phosphoric acid (0.1%) and methanol (60: 40 v/v). The flow rate was 1.0 ml/min and the ultraviolet detection wavelength was at 280 nm. Plasma concentrations at different time were determined after oral administration at the dose of 20, 40 and 80 mg/kg. The data of concentration-time were fitted and the pharmacokinetics parameters were calculated with 3p97 program (Chinese Pharmacology Society). The limit of quantitation was 0.02 µg/ml, the linear range was 0.02-20.0 µg/ml (R^2= 0.9989). The mean absolute recoveries of *l*-THP at three different concentrations (0.04, 5.00 and 20.00 µg/ml) were 97.5 ± 4.9, 98.2 ± 3.6 and 99.2 ± 3.2%, respectively. The relative standard deviations (RSD) of intra-day and inter-day were both less than 10%. The parameters of low, middle and high doses were as follows: $t_{1/2}\alpha$ were (0.79 ± 0.04), (0.66 ± 0.02), (4.42 ± 0.07) h, $t_{1/2}\beta$ were (20.26 ± 1.21), (19.28 ± 1.04), (31.96 ± 0.85) h, while AUC were (6.95 ± 0.98), (9.91 ± 1.11), (19.19 ± 3.35) mg·h/L, respectively. The proposed method was found to be convenient, accurate and reliable, and it can be used for determination of *l*-THP in rat plasma. The pharmacokinetics studies also provided the theoretical foundation and reference for the safe and reasonable clinic exploitation of *l*-THP.

Key words: High performance liquid chromatography (HPLC), levo-tetrahydropalmatine, pharmacokinetics, rat.

INTRODUCTION

Rhizoma Corydalis (yanhusuo), the dried tuber of Corydalis yanhusuo W.T. Wang, has been traditionally used in China for the treatments of chest pain, epigastric pain, dysmenorrheal, and traumatic swelling and pain for thousands of years (Pharmacopoeia commission of RPC, Chinese Pharmacopoeia, 2000). Tetrahydropalmatine (THP) is one of the active ingredients isolated from *R. Corydalis*, which possesses the functions of anodyne and hypnosis without drug addiction.

It has also been reported that THP has other functions, such as hypotensive effect, anti-arrhythmia, inhibiting the aggregation of thrombocytes and the secretion of gastric acid (Hu et al., 1999a, b; Hong et al., 2005; Huang et al., 1999). THP, including both *d*- and *l*-conformations, belongs to the isoquinoline alkaloid family. The two enantiomers act on different targets in the CNS; *d*-THP acts as a dopamine (DA) depletory, while *l*-THP acts as a brain DA antagonist (Hu et al., 1999a, b). The pharma-

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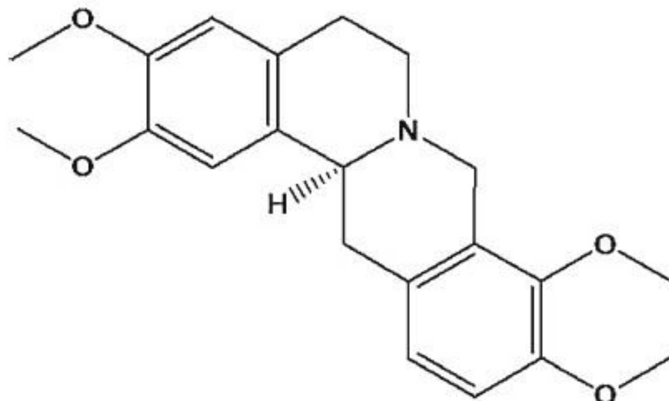

Figure 1. Chemical structure of levo-tetrahydropalmatine (*l*-THP).

ceutical industry has synthetically produced plenty of potent enantiomers: levo-tetrahydropalmatine (*l*-THP), which has been marketed worldwide under different brand names as an alternative to anxiolytic and sedative drugs of the benzodiazepine group and analgesics such as opiates (Li et al., 2011). The chemical structure of *l*-THP is shown in Figure 1. Methods for the determination of THP in rat plasma by e high-performance liquid chromatography electrospray ionization (-) tandem mass spectrometry (NP-(HPLC–ESI–MS) or HPLC–MS/MS have been described (Lu et al., 2006; Deng et al., 2008), because THP exists as the racemic mixture in Chinese herb. Hong et al. (2005) established a chiral HPLC method using an achiral column to separate and quantify THP enantiomers in dog plasma. As far as we know, there has been no report published for the determination of *l*-THP in rat plasma or its pharmacokinetics.

In this study, a robust, sensitive, simple and accurate HPLC method for the determination of *l*-THP in rat plasma was developed and the pharmacokinetics of *l*-THP in rats was also investigated at three different doses of 20, 40 and 80 mg/kg after oral administration, which provides the theoretical foundation and reference for the safe and reasonable clinic exploitation of *l*-THP.

MATERIALS AND METHODS

Drugs and Chemicals

l-THP (purity 99.72%) was provided by Shanghai Winherb Medical S&T Development Co. Ltd. (Shanghai, China). HPLC-grade methanol was obtained from Merck Company (Darmstadt, Germany). All other reagents were of analytical graded. Phosphoric acid was 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) were used to filter the solutions. Blank rat blood was collected from healthy, drug-free rats. Plasma was obtained by centrifugation of blood treated with anticoagulant. Plasma was prepared and stored at approximately -20°C.

Instruments and chromatographic conditions

The HPLC analysis was carried out according to a Waters 2695 HPLC system (Waters Associates, Milford, MA), which consisted of a photodiode array detector and an autosampler. The apparatus was interfaced to a DELL PC compatible computer with Empower Pro software. A Diamonsil C_{18} column (250 × 4.6 mm i.d; pore size 5 μM) was used. The mobile phase consisted of a mixture of phosphoric acid (0.1%) and methanol (60: 40 v/v). The flow rate was set at 1 $ml \cdot min^{-1}$ and a 20 μL aliquot was injected into the HPLC column. Column temperature was maintained at 30°C by a column heater controller and the peaks were monitored at 280 nm.

Standard solutions

The *l*-THP was weighed and dissolved in HPLC-grade methanol at room temperature to obtain a stock solution of 1.0 mg/ml. Serial dilutions of the stock solutions were made for spiking the calibration standards. The calibration curve for *l*-THP was prepared in rat plasma at eight concentrations: 0.02, 0.04, 0.08, 1.25, 2.50, 5.00, 10.00 and 20.00 μg/ml. The simulated samples were prepared by adding appropriate volumes of the diluted solutions to drug-free rat plasma. Stock and working standard solutions were protected from light and stored at -20°C until being used.

Sample preparation

To determine *l*-THP in rat plasma, blood samples were taken from the tail vein and placed in the tubes containing sodium heparin. The tubes were centrifuged (6000 rpm for 5 min) at 27°C in order to separate the plasma elements and then stored at -20C for the posterior analysis. The plasma samples (0.2 ml) were alkalinized with 50 μL of 1 M NaOH and shaken for 20 s. The mixture was extracted with 2.0 ml ethyl acetate by a vortex mixer for 3 min and then centrifugated at 4000 rpm for 10 min. An accurately measured 1.2 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, then centrifugated at 15,000 rpm for 5 min. A 20 μL aliquot of the supernatant was directly injected into the HPLC system.

Assay validation

To validate the assay in rat plasma, the following parameters were investigated: selectivity, sensitivity, recovery, precision and accuracy, linearity and Stability.

Selectivity and sensitivity

Selectivity was defined as the lack of interfering peaks at the retention times of the assayed drug. The specificity of the method was determined by comparing the chromatograms obtained from the samples containing *l*-THP with those obtained from blank plasma samples. The limit of quantification (LOQ) for *l*-THP was defined as the lowest concentration of spiked plasma that can be determined with sufficient precision and accuracy (RSD < 20% and -20% < RE < 20%) for both intra-day and inter-day runs.

Recovery and linearity

In the analysis of *l*-THP in rat plasma, the analytical recovery of *l*-THP was determined at concentrations of 0.04, 5.00 and 20.00 μg/ml (n=6). The samples of the plasma without drug were spiked

with known amounts of the drug to achieve the specified concentration. These samples were processed with the analytical method described above and peak areas were compared to that obtained by direct injection of the drug in the mobile phase. To calculate linearity, calibrations curves were constructed by linear regression within the range of 0.02-20.00μg/ml of *l*-THP, with eight standard solutions.

Precision and accuracy

Precision was determined as the coefficient of variation (CV) and accuracy as the percent relative error (RE). Intraday precision and accuracy data were obtained by analyzing aliquots of plasma samples at low (0.04 μg/ml), medium (5.00 μg/ml) and high (20.00 μg/ml) levels of the *l*-THP concentration (n=6). Inter-day reproducibility was determined over three days.

Stability

The ambient stability and freeze-thaw stability of *l*-THP in rat plasma were assessed with the samples of three different concentrations (0.04, 5.00 and 20.00μg/ml). The ambient stability was assessed by leaving the samples at room temperature for 24 h. The freeze-thaw stability was assessed over three cycles, thawed at room temperature and refrozen at -20°C.

Pharmacokinetics study

The analytical method was applied to evaluate the pharmacokinetics parameters of *l*-THP after oral administration in rats. The plasma pharmacokinetics of *l*-THP was studied in 30 Wistar rats with body weight 180 – 220 g (fifteen males and fifteen females, respectively). Wistar rats were from the Laboratory Animal Center of Third Military Medical University, Chongqing, China. All the rats were treated in accordance with the guidelines approved by Chongqing Science and Technology Commission (SYXK/2009-002).

The animals were randomly divided into three groups of ten, which were orally administered *l*-THP at the doses of 20, 40, and 80 mg/kg, respectively. The rats were fasted for 12 h prior to and during the experiments, and were allowed free access to water. *l*-THP solution was then orally administered to rats at different doses of *l*-THP (20, 40 and 80 mg/kg). Heparinized blood samples (0.5 ml) were collected before drug administration and 15 min, 30 min, 1, 1.5, 2, 3, 5, 7, 9, 12, 24, 36 and 48 h after oral administration. Thirty rats were used for each time point. After each sampling, the removed volume of blood was supplemented with an equal volume of sodium chloride. Plasma samples were obtained after centrifugation (6000rpm for 5 min) and were stored at -20°C until being analyzed.

Pharmacokinetics and statistical analysis

The pharmacokinetics parameters, including the area under the plasma concentration-time curve (AUC), elimination rate constant (Ke), elimination half-life ($t_{1/2}\beta$), apparent volume of distribution (Vd), and clearance (CL) were calculated with 3p97 program (Chinese Pharmacology Society). An appropriate pharmacokinetics model was chosen based on the lowest Akaike's information criterion (AIC) value, lowest weighed squared residuals, lowest standard errors of the fitting parameters, and dispersion of the residual under equal weight scheme. All data were expressed as mean ± standard deviation (S.D).

RESULTS

Method validation

Figure 2 shows the chromatograms of the blank rat plasma, blank rat plasma spiked with *l*-THP and rat plasma obtained 9 h after oral *l*-THP administration. The retention time for *l*-THP was 8 min, at a flow rate of 1 ml/min. The method described was shown to be selective for the analyte. The limit of quantification (LOQ) was defined as the lowest concentration of spiked plasma samples that can be determined with sufficient precision and accuracy, relative standard deviation (RSD) less than 20% and relative error (RE) of -20 to 20%. The LOQ was estimated at 0.02 μg/ml. The calibration curve for *l*-THP was constructed by plotting the area under peak versus drug concentration.

It was found to be linear over a concentration range from 0.02 to 20.00 μg/ml (y=23422x - 5061.1, R^2= 0.9989, y is the peak area of *l*-THP, and x is the plasma concentration of *l*-THP). The mean absolute recovery of *l*-THP at three concentrations was 97.5 ± 4.9, 98.2 ± 3.6 and 99.2 ± 3.2%, respectively. The results are shown in Table 1, which indicate a lack of interference from the sample preparation procedure. The analytical precision and accuracy for intraday (n=6) and inter-day (n=6) assays of three quality controls (0.04, 5.00 and 20.00 μg/ml) are presented in Table 2.

Stability investigation (Table 3) demonstrates that the concentrations of *l*-THP in processed samples had no significant difference to nominal values within 24 h at 25°C and between the three freeze-thaw cycles (room temperature to -20°C). These results indicates that the analyte in rat plasma was stable for up to 24 h at 25°C with the maximal loss of 2.5% and was stable over at least three freeze-thaw cycles with no significant loss(≤10.3%).

Pharmacokinetics study

The developed HPLC analytical method has been successfully used for the pharmacokinetics study after oral administration of *l*-THP in rats. The mean plasma concentration-time curves of *l*-THP after oral administration at doses of 20, 40 and 80 mg/kg in rats are shown in Figure 3, and the mean pharmacokinetics parameters (mean ± S.D) are summarized in Table 4. Pharmacokinetics analysis of *l*-THP concentrations in plasma was performed with two-compartment model methods via the 3p97 software package (Chinese Pharmacology Society).

DISCUSSION

There is an asymmetric carbon on the chemical structure of *l*-THP, as shown Figure 1. It reveals that the two enantiomers acted in different pharmacological activities.

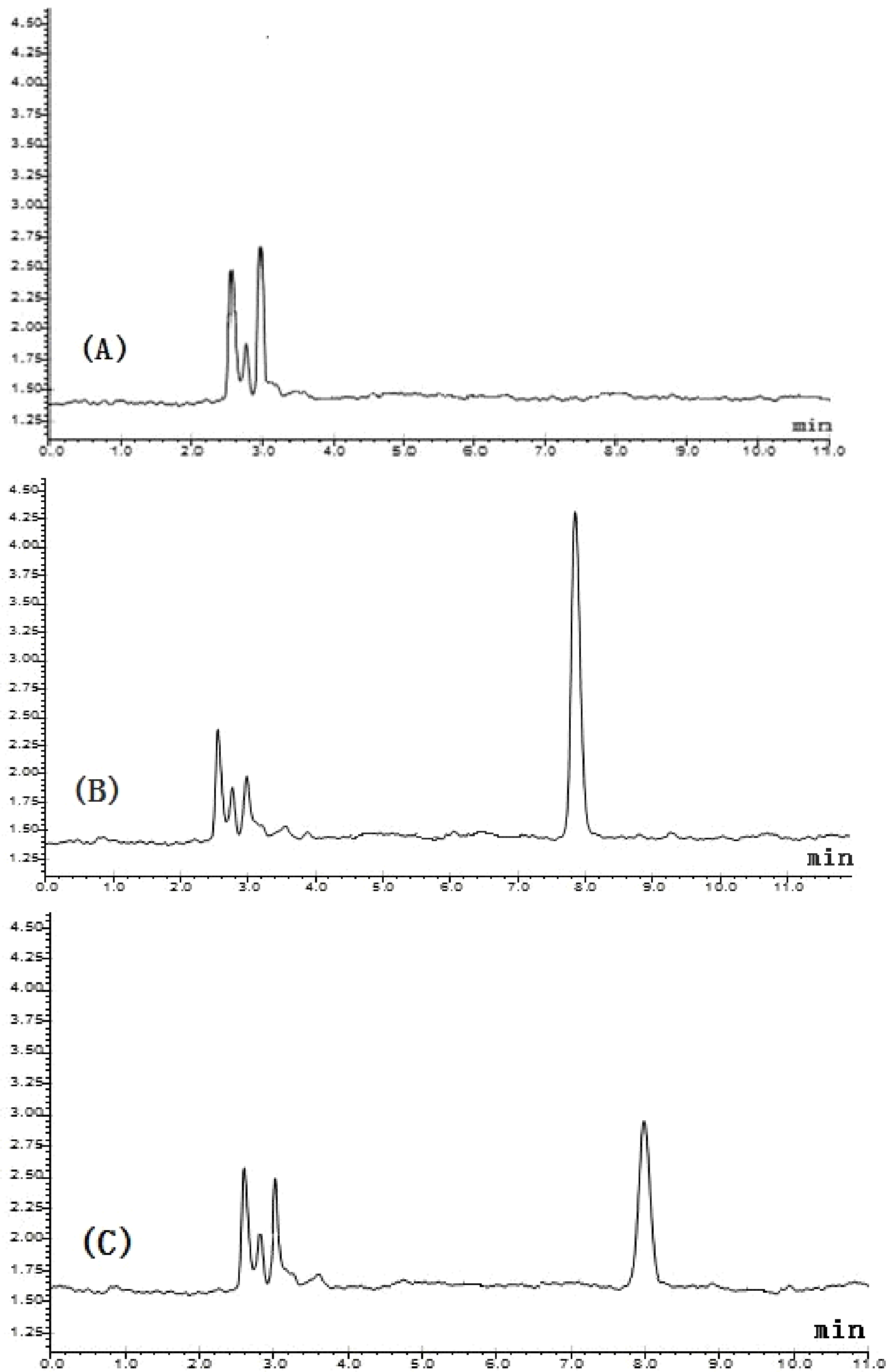

Figure 2. Chromatograms demonstrating selectivity: (A) blank rat plasma; (B) blank rat plasma spiked with *l*-THP; (C) plasma from rat 9 h after oral administration of *l*-THP.

Table 1. Recovery of l-THP from rat plasma (n=6).

Nominal concentration (μg·mL^{-1})	Measured concentration (μg·mL^{-1})	Recovery (%)	RSD
0.04	0.039 ± 0.002	97.5 ± 4.9	3.25
5.00	4.91 ± 0.008	98.2 ± 3.6	2.19
20.00	19.84 ± 0.348	99.2 ± 3.2	3.46

Table 2. Intra- and inter-day precision and accuracy of l-THP in rat plasma (n=6).

Parameter	Nominal concentration (μg·mL^{-1})	Measured concentration (μg·mL^{-1})	Accuracy (RE%)	Precision (RSD%)
	0.04	0.038 ± 0.003	95	4.67
Inter-day	5.00	4.96 ± 0.012	99.2	3.56
	20.00	20.12 ± 0.416	100.6	3.39
	0.04	0.039 ± 0.006	97.5	2.73
Intra-day	5.00	4.93 ± 0.006	98.6	3.29
	20.00	20.20 ± 0.371	101	2.42

Table 3. Assessment of stability in rat plasma.

Condition	Concentration (μg·mL^{-1})		
	0.04	5.00	20.00
Freeze-thaw stability(-20°C)*			
Cycle 1	97.6	96.9	98.2
Cycle 2	93.5	90.8	94.7
Cycle 3	89.9	89.7	92.6
Short-term stability(25°C)*			
Time=2.0h	98.2	99.4	99.9
Time=4.0h	99.4	99.0	100
Time=8.0h	97.8	102	99.7
Time=24.0h	97.5	101	99.6

*Expressed as the mean percentage change from time zero (nominal concentration).

Though THP exists as a racemic mixture in Chinese herbs, it is necessary to evaluate the pharmacokinetic behavior of each enantiomer rather than that of the racemate to use the racemic drug effectively and safely (Li et al., 2011). Early publications have described methods for the determination of racemic THP concentration in rat plasma by HPLC-ESI-MS (Ma et al., 2009) or HPLC-MS/MS (Deng et al., 2008; Lin et al., 2008). As it is well known, HPLC-MS or HPLC-MS/MS method is more expensive than HPLC-UV and the matrix effect is also insuperable. A sequential achiral-chiral HPLC method has been established to determine the enantiomer pharmacokinetics of THP in dogs. 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 (Hong et al., 2005). In this paper, a simple and effective HPLC method was established and used for *l*-THP pharmacokinetic studies.

The chromatograms showed a good baseline separation and the mobile phase used resulted in optimal separation. The method was selective for *l*-THP since it showed that no interfering peaks appeared near the retention time (8 min) of the compound of interest. The LOQ values were low (0.02 μg/ml), indicating the good sensitivity of this HPLC method. Moreover, the precision met the expected range. Accuracy and recovery were also in good agreement with acceptable values for the validation of an analytical procedure (100 ± 20%). The sample preparation used in this study involved only a

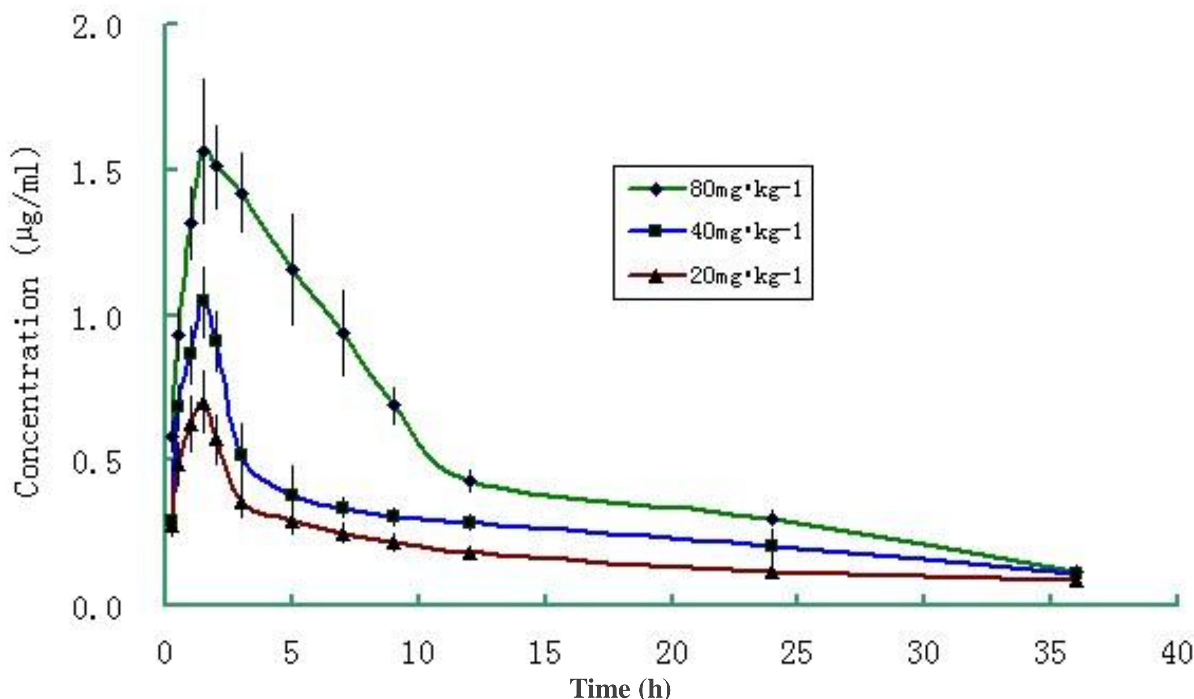

Figure 3. Mean Plasma concentration-time profile of *l*-THP after oral administration of three dosages.

Table 4. The pharmacokinetics parameters of *l*-THP after single oral administration of three dosages in rats (n=10).

Parameter	Dosage ($mg \cdot kg^{-1}$)		
	20	40	80
$t_{1/2}\alpha$/h	0.79 ± 0.04	0.66 ± 0.02	4.42 ± 0.07
$t_{1/2}\beta$/h	20.26 ± 1.21	19.28 ± 1.04	31.96 ± 0.85
$t_{1/2}k\alpha$/h	0.44 ± 0.05	0.47 ± 0.08	0.60 ± 0.06
K_{12}/ h^{-1}	0.55 ± 0.02	0.69 ± 0.03	0.04 ± 0.03
K_{21}/ h^{-1}	0.22 ± 0.01	0.23 ± 0.01	0.04 ± 0.02
K_{10}/h^{-1}	0.13 ± 0.01	0.17 ± 0.02	0.10 ± 0.01
$K\alpha$/ h^{-1}	1.57 ± 0.21	1.48 ± 0.32	1.16 ± 0.16
V_d/ $L \cdot kg^{-1}$	15.82 ± 1.22	18.22 ± 1.33	39.32 ± 2.09
CL/ $L \cdot kg^{-1} \cdot h^{-1}$	2.12 ± 0.32	3.10 ± 0.45	3.74 ± 0.28
AUC/ $mg \cdot h \cdot L^{-1}$	6.95 ± 0.98	9.91 ± 1.11	19.19 ± 3.35
Tmax/h	1.5 ± 0.08	1.5 ± 0.07	1.5 ± 0.06
Cmax/$mg \cdot L^{-1}$	0.70 ± 0.03	1.04 ± 0.05	1.56 ± 0.09

single step- that is extraction with ethyl acetate. This condition was optimal for sample preparation as it resulted in clean chromatograms. The validated method was employed in pharmacokinetic analysis of *l*-THP after oral administration in rats. The result (Table 4) shows that there was a significant difference in the elimination half-life $t_{1/2}\beta$ (20.26, 19.28, 31.96 h, respectively) when the oral dose of *l*-THP was increased from 20 to 80 mg/kg. These results suggested that the pharmacokinetics of *l*-THP is a nonlinear process. The nonlinear increasing of $t_{1/2}\beta$ with the increased dosage suggested the saturated elimination of *l*-THP and the nonlinear pharmacokinetics in rats from 20 to 80 mg/kg. The AUC were calculated to be 6.95, 9.91 and 19.19 mg·h/L at doses of 20, 40 and 80 mg/kg, respectively. The $t_{1/2}a$ were approximately 0.79, 0.66, 4.42 h after three dosages, indicating that *l*-THP was distributed quickly in rats. The value of V_d after three dosages were 15.82, 18.22 and 39.32 L/kg, respectively. Li et al. (2011) reported that the Tmax was 1.25 h and the $t_{1/2}\beta$ for *l*-THP was about 11.42 h in healthy Chinese after

oral administration. A similar Tmax of 1.5 h and a longer $t_{1/2}\beta$ was obtained in this study, which may be explained by species variation. In conclusion, a sensitive, specific, accurate and reproducible HPLC method for the determination of *l*-THP in rat plasma was developed, which had been successfully applied in the study of pharmacokinetics of *l*-THP in rat. This method can be applied efficiently to large number of biological samples. Pharmacokinetics studies provide the theoretical foundation and reference for the safe and reasonable clinic exploitation of *l*-THP.

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