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Quantitative determination of the anticancer agent tubeimoside I in rat plasma by liquid chromatography coupled with mass spectrometry

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Abstract

Tubeimoside I is an important component isolated from *Bolbostemma paniculatum*. Tubeimoside I has been demonstrated to possess many pharmacological activities, including anti-inflammatory, antitumor, and antitumor-promoting effects. The purpose of the present study was to examine *in vivo* pharmacokinetics and bioavailability of tubeimoside I in rats by using a liquid chromatography coupled with mass spectrometry quantitative detection method (LC/MS). The plasma samples were deproteinated, evaporated and reconstituted in 100 μl methanol prior to analysis. The separation was performed by Waters Symmetry® C18 reversed-phase column (3.5 μm, 150 mm × 2.1 mm, Waters Inc., USA) and a SB-C18 guard column (5 μm, 20 mm × 4.0 mm). The mobile phase was a mixture of acetonitrile and water containing 5 μM NaAc (60:40, v/v). The method was validated within the concentration range 20–5000 ng/ml, and the calibration curves were linear with correlation coefficients >0.999. The lowest limit of quantitation (LLOQ) for tubeimoside I was 20 ng/ml in 0.1 ml rat plasma. The intra-assay accuracy and precision ranged from 92.4 to 104.9% and from 5.8 to 10.5%, respectively, while inter-assay accuracy and precision ranged from 94.2 to 95.0% and from 5.1 to 8.8%, respectively. The method was further applied to assess pharmacokinetics and oral bioavailability of tubeimoside I after intravenous and oral administration to rats. The oral bioavailability of tubeimoside I is only 0.23%, which indicates that tubeimoside I has poor absorption or undergoes acid-induced degradation. Practical utility of this new LC/MS method was confirmed in pilot pharmacokinetic studies in rats following both intravenous and oral administration.

Keywords: Tubeimoside I; Bioavailability; Pharmacokinetics; LC/MS

1. Introduction

Rhizoma Bolbostematts, as one of widely used traditional Chinese medicine (TCM), is prepared from the bulb of *Bolbostemma paniculatum* (Maxim.) Franquet [1]. It possesses well-documented anticancer [2,3], antiviral [4], anti-inflammatory [5] and immunosuppressive activities [6]. As reported previously, the chemical components of *R. Bolbostematts* mainly involve saponins [7,8], sterols [9] and alkaloids [10], etc., among which saponins are considered as main bioactive constituents [2–6]. Up to now, 14 saponins were isolated and elucidated from this TCM [7,8]. Among them, tubeimoside I is the main component in the saponins of *R. Bolbostematts* [11].

Tubeimoside I (structure, see Fig. 1), a triterpenoid saponin, isolated from the tubers of *B. paniculatum* [8,12], showed potent antitumor and antitumor-promoting effects [13–15]. Previous results showed that tubeimoside I induced apoptosis in HeLa cells, decreased deltapsim and facilitated cytochrome c release [16]. Tubeimoside I can also induce the apoptosis of CNE-2Z cells, and the induction of apoptosis by tubeimoside I is closely associated with down-regulation and phosphorylation of bcl-2 and bax activation [17].

Other experimental results also demonstrated that exposure of HeLa cells to 40 μmol/l of tubeimoside I induced nuclear shrinkage, chromation condensation and margination against

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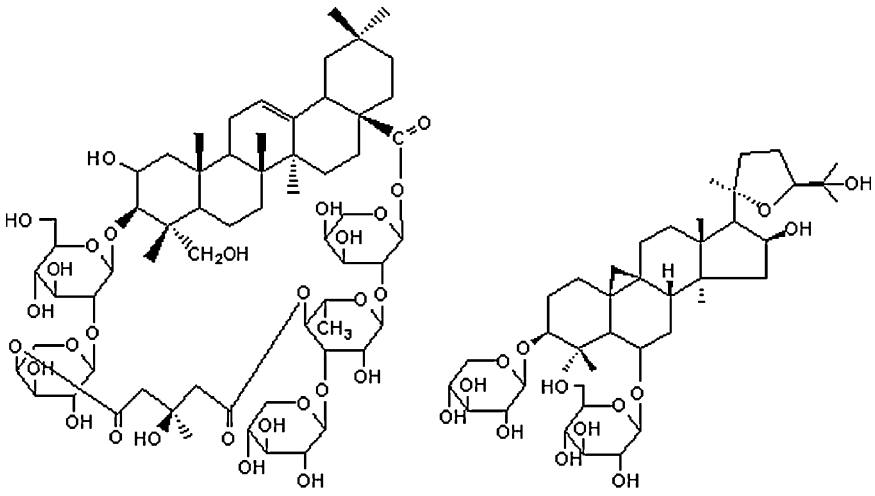

Fig. 1. Chemical structure of tubeimoside I and the internal standard (IS) astragaloside IV.

the nuclear envelope, subdiploid peak, and DNA fragmentation, characteristics seen in apoptotic cells [18]. The previous results also showed that tubeimoside I exerts potent anti-tumor activity with low toxicity [19–21]. The compound inhibited DNA synthesis and induced phenotypic reverse transformation of tumor cells. In addition, tubeimoside I had an inhibitory action on the infection of HIV-1 isolates and would be a promising candidate for treatment of AIDS [22,23]. These multiple pharmacological activities of tubeimoside I make it worth to further study its pharmacokinetic properties in animal bodies.

To our knowledge there was no analytical method for the quantitative determination of tubeimoside I, an active ingredient isolated from *B. paniculatum* in the biological samples, and there were no systematic pharmacokinetic studies for this active component in animal bodies. We describe a rapid, selective and sensitive LC/MS method for the determination of tubeimoside I in plasma. Using the described method in the paper, pilot pharmacokinetic studies of tubeimoside I were characterized after intravenous and oral administration.

2. Materials and methods

2.1. Chemicals and drug

Tubeimoside I was isolated and purified by the Department of Natural Medicinal Chemistry of Second Military Medical University (purity >99%). Internal standard (IS), astragaloside IV, was provided by Second Military Medical University (Shanghai, China). Chemical structures of the two compounds are shown in Fig. 1.

HPLC grade methanol and acetonitrile were obtained from TEDIA Company (Tedia Fairfield, OH, USA). Formic acid, purity >99%, was purchased from ACROS Organics (New Jersey, USA). All other reagents were of analytical grade. Distilled de-ionized water was produced by a Milli-Q Reagent Water System (Millipore, MA, USA).

2.2. Drug administration and plasma sample collection

Eight male Sprague-Dawley rats (180–220 g) were provided by Shanghai SLAC Lab Animal Co. Ltd. (Shanghai, China) and housed four to a cage with unlimited access to food and water except for 12 h before and during the experiment. The animals were maintained on a 12 h light–dark cycle (light on from 8:00 to 20:00) at ambient temperature (22–24 °C) and at 60% relative humidity. Animal studies were approved by the Second Military Medical University Animal Ethics Committee (Shanghai, China) and carried out in Shanghai Medicilon Inc. (Shanghai, China).

In order to determine the pharmacokinetics of tubeimoside I in a rat after intravenous or oral administration, the animals were deprived of food but had free access to water for 12 h before and during the experiment. Tubeimoside I was dissolved in 0.9% saline immediately before oral or intravenous administration and the injected volume was adjusted at 0.5 ml/100 g for rats. The dose is 5 mg/kg for intravenous administration and 50 mg/kg for oral administration. The blood samples (0.3 ml) were withdrawn in heparinized tubes from the carotid vein using carotid cannulation at 0, 5, 15, 30 min, 1, 1.5, 2, 4, 6, 8, 24 h after intravenous administration and at 0, 15, 30 min, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 24 h after oral administration. The plasma samples were placed in heparinized tubes and were separated following centrifugation at 3000 g for 5 min and stored at −20 °C until analysis.

2.3. Standard and sample preparation

A stock solution of tubeimoside I was prepared by dissolving the drug in methanol, obtaining a final concentration of 100 μg/ml. The appropriate volume of this solution was placed in a glass tube, and the solvent was evaporated under a compressed air stream. The dried analyte was reconstituted using blank plasma to yield a final concentration of 5000 ng/ml in plasma. From this solution, seven calibration standard solutions

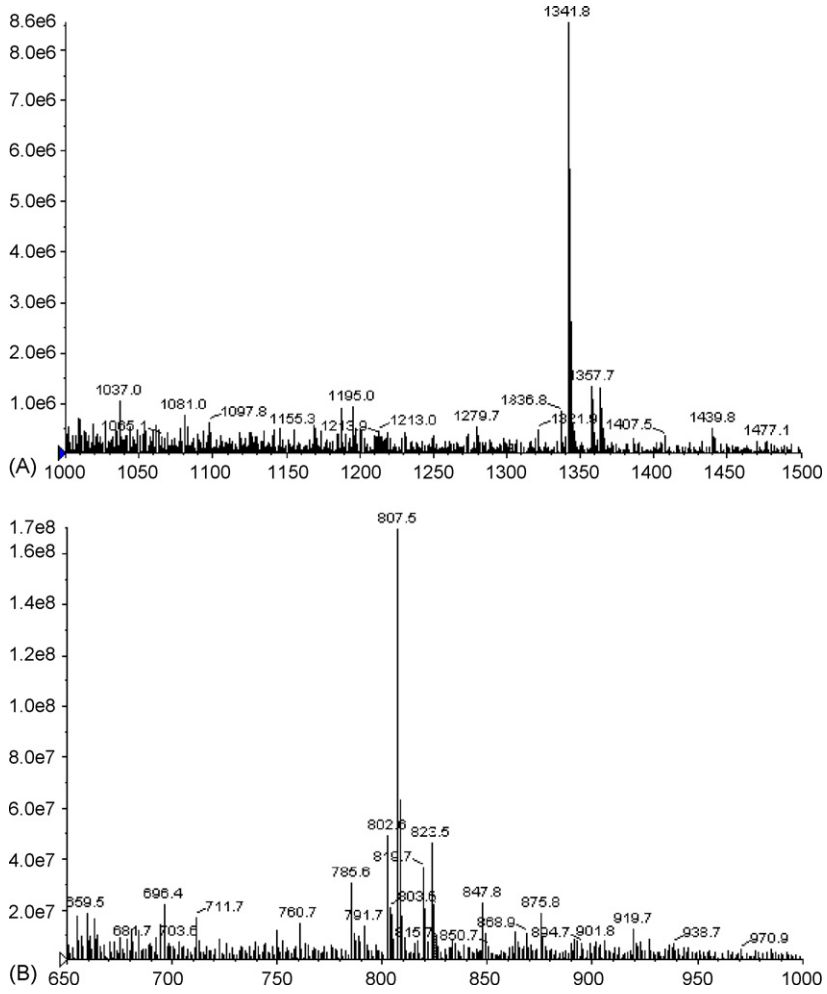

Fig. 2. Positive electrospray mass spectrums of tubeimoside I (A) and IS (B).

were prepared at concentrations of 20, 50, 100, 500, 1000, 2000 and 5000 ng/ml. Three quality control solutions at concentrations of 0.4, 4 and 40 ng/ml were also prepared in blank plasma. Aliquots (0.1 ml) of calibration standards and quality control plasma samples were dispensed into labeled Eppendorff tubes and stored at −20 °C until required for assay. A 100 μg/ml stock solution of astragaloside IV was prepared by dissolving the drug in methanol. This solution was diluted with methanol to a final concentration of 0.5 μg/ml.

The plasma sample (100 μl) was deproteined by adding 300 μl 0.5 μg/ml IS methanol solution. After stirring on a vortex mixer (XW-80A Vortex mixer, Shanghai) for 30 s, the sample was centrifuged at 16,000 rpm in a TGL-16B high speed centrifuger (Shanghai, China). Then the supernatant was transferred to a glass tube and evaporated using a Speed Vac (Savant, USA). The dried sample was reconstituted in 100 μl mobile phase for injection into the HPLC.

2.4. LC/MS condition

The HPLC system (Angilent 1100, Angilent Inc., MA, USA) consisted of a quaternary pump, an autosampler, a degasser, an automatic thermostatic column compartment and a computer with a Chemstation software (Analyst 1.4, Applied Biosystems Inc., USA). The analytical column used was a Waters Symmetry® C18 reversed-phase column (3.5 μm, 150 mm × 2.1 mm, Waters Inc., USA) and a SB-C18 guard column (5 μm, 20 mm × 4.0 mm). The mobile phase was a mixture of acetonitrile and water containing 5 μM NaAc (60:40, v/v). The mobile phase was degassed automatically using the electronic degasser system. The column was equilibrated and eluted under isocratic conditions utilizing a flow rate of 0.2 ml/min. Column temperature was maintained at 25 °C.

The mass spectrometer employed was a triple quadropole mass spectrometer with TurboIonSpray ion source (MDS Sciex Inc., Toronto, Candada), which was connected to the liquid chromatography system. High-purity nitrogen was provided by a liquid nitrogen tank. The conditions for mass spectrometry were set at positive selective ion mode (SIM) on the quasimolecular ion of two compounds (tubeimoside I at *m/z*: 1341.8 $[M + Na]^+$, and astragaloside IV at *m/z*: 807.5 $[M + Na]^+$, shown in Fig. 2A and B). Other parameters of the mass spectrometers were listed as following: Curtain Gas (CUR): 6, Ionspray Voltage (IS): 5000, Temperature (TEM): 500, Nebulized gas: 10, Heated gas: 7000, Declustering Potential (DP): 170, Focusing Potential (FP): 200, Entrance Potential (EP): 10, Dwell Time: 200 ms for each ion.

2.5. Method validation

The analytical curves were constructed using seven non-zero standards ranging from 20 to 5000 ng/ml and prepared in four samples. A blank sample (matrix sample processed without IS) was used to exclude contamination. The linear regression analysis was performed by plotting the peak area ratio (y) against the analyte concentration (x) in ng/ml. The linearity of the relationship between peak area ratio and concentration was demonstrated by the correlation coefficient (R) obtained for the linear regression. The relative standard deviation (R.S.D.) was calculated for all of the non-zero standards and they were compared to the nominal value.

The intra-day and inter-day assay of the method were evaluated by quintuplicate analyses of three quality control samples. The calibration standards and quality controls were analyzed on five different days in order to determine intra-day and inter-day precision and accuracy. The accepted criteria for each quality control were that the R.S.D. and accuracy should not exceed 15%.

Three QC samples were determined before and after one frozen-thawed cycle to evaluate the frozen-thawed stability. To evaluate the long-term stability, a long time period was defined as the time elapsed between the start of sampling and the end of sample analysis. Aliquots of each sample type were initially frozen at −20 °C and then thawed to be extracted and tested. The difference between the starting concentration and the concentration after 20 days will show whether the drug in plasma can be degraded under these conditions.

2.6. Pharmacokinetic and statistical analysis

Data fitting and pharmacokinetic parameter calculations were carried out using the DAS 2.0 pharmacokinetic program (Chinese Pharmacology Society). An appropriate pharmacokinetic model was chosen on the lowest Akaike's information criterion (AIC) value, lowest weighted squared residuals, lowest standard errors of the fitting parameters, and dispersion of the residual under equal weight scheme [24–27]. The area under the curve (AUC) was calculated by the trapezoidal rule between first (0 h) and last sampling time plus C_n/λ_n, where C_n is the concentration of last sampling, and λ_n is the elimination rate constant. $AUC_{0\to t} = \sum(C_i + C_{i-1}) \times (t_i - t_{i-1})/2$; $AUC_{0\to\infty} = AUC_{0\to t} + C_n/\lambda_n$. Bioavailability was calculated according to the equation:

$$\text{Bioavailability}\% = \frac{AUC_{0\to\infty}(\text{p.o.}) \times \text{Dose(i.v.)}}{AUC_{0\to\infty}(\text{i.v.}) \times \text{Dose(p.o.)}} \times 100\%$$

where AUC is for the area under the Conc. versus time curve; i.v. is for intravenous administration; p.o. is for oral administration.

3. Results and discussions

3.1. Method validation

The LC/MS method described in this report has high sensitivity and specificity that enables the determination of tubeimoside I in plasma with the lowest limit of quantitation (LLOQ) 20 ng/ml in 0.1 ml rat plasma (S/N > 10). Under the described chromatographic conditions with a mobile phase of acetonitrile and water containing 5 μM NaAc (60: 40, v/v), the retention time was about 3.0 min for tubeimoside I and 4.2 min for the IS. At the retention time, the test substance tubeimoside I and the IS were eluted without an interference peak from the blank rat plasma (Fig. 3A and C) and nor from urine (Fig. 3B and D).

The standard curve obtained from the detection of plasma containing known amounts of tubeimoside I was linear over the concentration ranges from 20 to 5000 ng/ml in 0.1 ml rat plasma. The calibration curves were found to be linear and could be described by the regression equations, $Y = 0.00163 \times X - 0.0109$, with correlation coefficient of over 0.999. This sensitivity has proven useful in the analysis of pharmacokinetic data of rats treated both intravenously and orally.

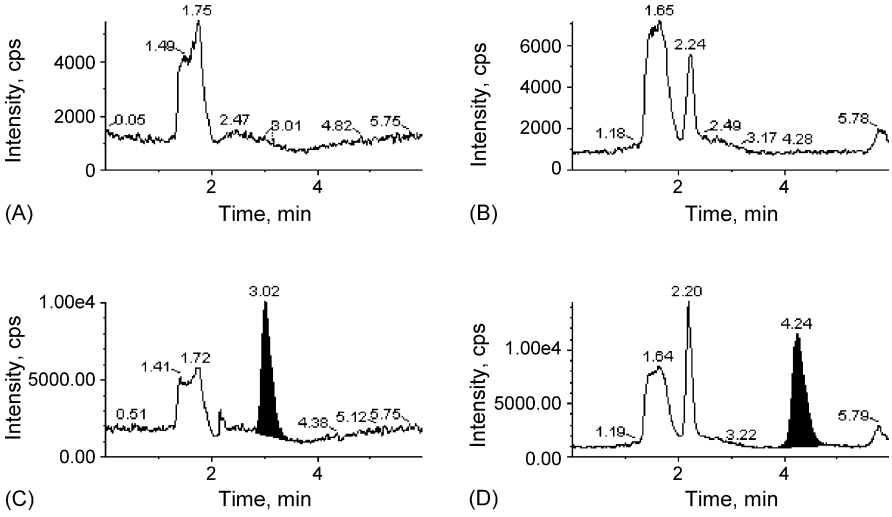

Fig. 3. Chromatograms of LC/MS: (A) SIM 1341.8, blank plasma; (B) SIM 807.5, blank plasma; (C) SIM 1341.8, 200 ng/ml tubeimoside I in plasma; and (D) SIM 807.5, 200 ng/ml astragalosie IV in plasma.

Table 1
Nominal and calculated concentration in the standard curve in rat plasma

Nominal concentration (ng/ml)	Calculated concentration ($n=4$)			
	Mean (ng/ml)	S.D.	R.S.D. (%)	Accuracy (%)
20	18.7	2.6	13.9	93.5
50	50.2	5.3	10.6	100.4
100	94.7	5.6	5.9	94.7
200	211.3	13.2	6.3	105.5
500	507.3	30.1	5.9	101.4
1000	1070.2	46.7	4.4	107.0
2000	1979.8	50.5	2.6	99.0
5000	4939.6	71.2	1.4	98.8

Table 2
Intra-day and inter-day assay variation in rat plasma

Nominal concentration (ng/ml)	Intra-assay precision ($n=5$)			Inter-assay precision ($n=5$)		
	Measured (ng/ml)	R.S.D. (%)	Accuracy (%)	Measured (ng/ml)	R.S.D. (%)	Accuracy (%)
40	42.0 ± 4.4	10.5	104.9	38.0 ± 3.3	8.8	94.9
800	742.8 ± 45.6	6.1	92.8	760.1 ± 39.0	5.1	95.0
4000	3646.2 ± 214.6	5.8	92.4	3768.3 ± 161.8	5.2	94.2

Table 3
Frozen-thawed stability and long-term stability in rat plasma at −20 °C

Nominal concentration (ng/ml)	Calculated concentration after one frozen-thawed cycle ($n=3$)	Calculated concentration after storing at −20 °C for 2 weeks
40	40.4 ± 2.8 (101.1 ± 7.0%)	37.5 ± 2.3 (93.8 ± 6.2%)
800	740.7 ± 30.5 (92.6 ± 3.8%)	805.7 ± 65.6 (100.7 ± 8.2%)
4000	3690.0 ± 173.5 (92.3 ± 4.7%)	3843.0 ± 137.1 (96.1 ± 3.3%)

The R.S.D. values of three concentrations ranged from 5.8 to 10.5% for intra-day assay and from 5.1 to 8.8% for inter-day assay. The accuracy at three concentrations ranged from 92.4 to 104.9% for intra-day assay and from 94.2 to 95.0% for inter-day assay, respectively (Tables 1 and 2). These results suggest that the procedures described above are satisfactory with respect to both accuracy and precision.

The frozen-thawed and long-term stability results are summarized in Table 3. The results show that there is no significant difference between before and after the storage at −20 °C for 2 weeks.

3.2. Pharmacokinetics in rats following intravenous and oral administration

Following intravenous and oral administration, plasma drug concentration–time profiles can be best described as a two compartmental model (Fig. 4). The pharmacokinetic parameter values are summarized in Tables 4 and 5. Tubeimoside I was eliminated with systemic clearance (CL) of 0.080 ± 0.027 l/(h kg) after intravenous administration, which is much lower than the hepatic blood flow rate (3.3 l/(h kg) in rats) [28], suggesting that this compound was very slowly cleared via hepatic clearance. The elimination half-life ($t1/2\beta$) of tubeimoside I was 6.70 ± 1.90 h following intravenous injection. The volume of distribution at terminal phase (V) was 0.106 ± 0.039 l/kg, which was lower than the total body water at 0.67 l/kg. The results suggested that tubeimoside I had limited distribution into extravascular systems.

The absolute oral bioavailability of tubeimoside I was only 0.23 ± 0.13%, which suggests that this tubeimoside I may be very difficult to be absorbed via the gastrointestinal or may have undergone acid-induced degradation. The time to reach the maximum plasma drug concentration (T_{max}) was 2.75 ± 0.96 h. The results revealed that tubeimoside I showed strong activities in anti-cancer, but *B. paniculatum* is not efficacious in oral administration. It is suggested that intravenous administration might be chosen as the therapeutic route in the clinic [16,17].

In summary, we have developed a simple and accurate analytical LC/MS method for determination of tubeimoside I, a

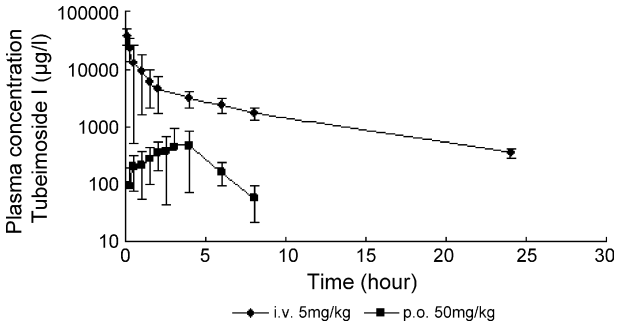

Fig. 4. Plasma tubeimoside I concentration vs. time curve after i.v. and p.o. administration.

Table 4
Pharmacokinetic parameters in rats after i.v. administration 5 mg/kg

Animal no.	1	2	3	4	Mean	S.D.
$t1/2\alpha$ (h)	0.586	0.155	0.109	0.131	0.245	0.228
$t1/2\beta$ (h)	5.34	9.43	5.47	6.56	6.70	1.90
V (l/kg)	0.101	0.162	0.092	0.070	0.106	0.039
CL (l/(h kg))	0.053	0.117	0.075	0.073	0.080	0.027
AUC_{0-24} (mg h/l)	89.06	36.24	60.76	59.46	61.38	21.62
$AUC_{0-\infty}$ (mg h/l)	95.09	42.62	66.44	68.05	68.05	21.45

Note: CL is for the clearance, no.1 is for the animal no.

Table 5
Pharmacokinetic parameters in rats after p.o. administration at 50 mg/kg

Animal no.	1	2	3	4	Mean	S.D.
$t1/2\alpha$ (h)	1.35	0.67	1.78	1.55	1.33	0.48
$t1/2\beta$ (h)	1.53	8.41	1.95	1.59	3.37	3.37
$t1/2$ka	0.046	0.491	1.225	1.310	0.768	0.606
AUC_{0-8} (mg h/l)	2.41	1.52	0.55	0.93	1.35	0.81
$AUC_{0-\infty}$ (mg h/l)	2.67	1.86	0.62	1.05	1.55	0.91
Bioavailability (%)	0.39	0.27	0.09	0.15	0.23	0.13
T_{max} (h)	3.00	2.00	2.00	4.00	2.75	0.96
C_{max} (μg/l)	1000.2	611.3	192.3	895.2	674.8	361.2

Note: where $t1/2$ka is for the half-life of the absorption.

compound extracted from *B. paniculatum*, in rat plasma. The method was further applied to evaluate the pharmacokinetics and oral bioavailability of tubeimoside I after intravenous and oral administration to rats. The results demonstrated tubeimoside I was limitedly absorbed into the system circulation and slowly eliminated. The availability of this assay will now permit detailed pharmacokinetic studies of tubeimoside I in rats and other animal species. To our knowledge, this is the first study demonstrating a bioanalytical method and pharmacokinetics of tubeimoside I.

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