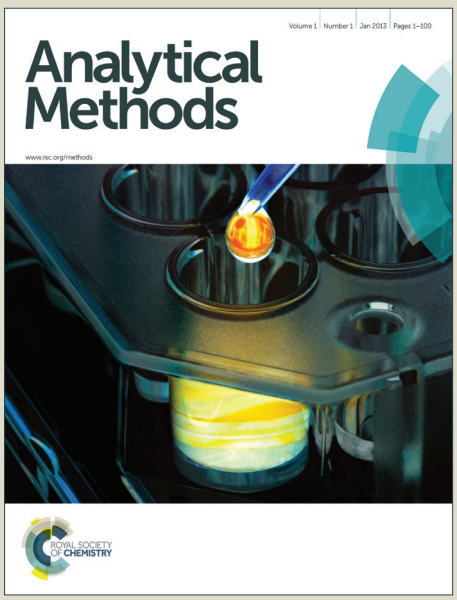
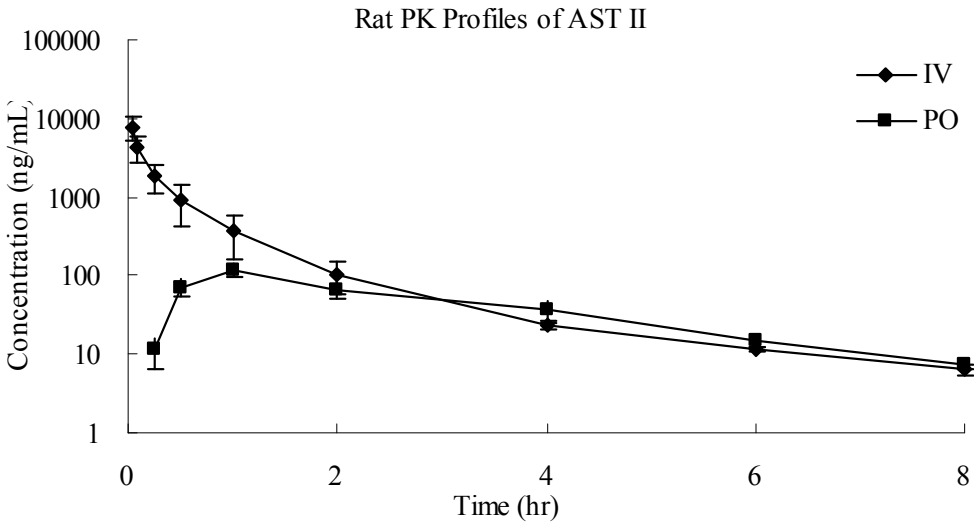
Analytical Methods

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods



Mean plasma concentration-time profiles of AST II determined by LC-MS/MS method after intravenous and oral administration of AST II to rats. The oral absolute bioavailability (F) of AST II in rats was calculated to be 0.79 ± 0.16%, suggesting its poor absorption and/or strong metabolism *in vivo*.

Quantification and Pharmacokinetics of Astragaloside II in Rats by rapid Liquid Chromatography–Tandem Mass Spectrometry

Haijun Qu[1], Meijuan Sun[1], Yu Cao[1], Longyuan Wang[1], Zhiwu Han[1*]

[*]Correspondence to: Zhiwu Han, Department of Pharmacy, the Affiliated Hospital of Medical College Qingdao University, Qingdao 266003, China. E-mail: navy828@126.com

[1] The Affiliated Hospital of Medical College Qingdao University, Qingdao 266003, China.

ABSTRACT

This study firstly describes the development of a rapid and accurate high-performance liquid chromatography–tandem mass spectrometry (LC-MS/MS) assay for the quantification of astragaloside II (AST II) in rat plasma. The assay involved a simple protein precipitation (PPT) step with methanol:acetonitrile (50:50, v/v) and a gradient elution using a mobile phase consisting of water containing 0.1% formic acid and acetonitrile containing 0.1% formic acid. Chromatographic separation was successfully achieved on an Agilent Zorbax XDB C_{18} column (2.1 mm × 50 mm, 3.5 μm) with a flow rate of 0.50 mL/min. The multiple reaction monitoring (MRM) was based on the transitions of *m/z* = 827.3→143.2 for AST II and 386.3→122.3 for buspirone (IS). The assay was validated to demonstrate the specificity, linearity, recovery, accuracy, precision and stability. The lower limit of quantification (LLOQ) was 5.0 ng/mL in 50 μL of rat plasma. The developed and validated method has been successfully applied to the quantification and pharmacokinetic study of AST II in rats after intravenous and oral administration of AST II. The oral absolute bioavailability (F) of AST II was calculated to be 0.79 ± 0.16% with an elimination half-life ($t_{1/2}$) value of 1.92 ± 0.30 hr, suggesting its poor absorption and/or strong metabolism *in vivo*.

Key Words: *Radix astragali*; Astragaloside II; Pharmacokinetics; LC-MS/MS; Rat

1. Introduction

Radix *Astragali* (Huangqi in Chinese) is one of the most popular traditional Chinese medicines (TCM) in China. It is derived from the dried roots of *Astragalus membranaceus* (Fisch.) Bge. var. *mongholicus* (Bge.) Hsiao or *A. membranaceus* (Fisch.) Bge. [1, 2]. It has been widely used for centuries in the prevention and treatment of various diseases such as nephritis, diabetes, cancer, etc [3-5].

Astragaloside II (AST II, (3b,6a,16b,20R,24S)-3-((2-O-Acetyl-beta-D-xylopyranosyl)oxy)-20,24-epoxy-16,25-dihydroxy-9,19-cyclolanostan-6-yl beta-D-glucopyranoside, chemical structure shown in Fig.1) was one kind of saponins isolated and identified from *Radix Astragali* and other natural plants[6-9]. In recent years, extensive phytochemical and pharmacological studies have confirmed that AST II exhibits a significant induction of proliferation, differentiation and mineralization in primary osteoblasts, and stimulates osteoblast differentiation at various stages, from early to late stage of differentiated osteoblasts [10]. AST II also shows protective effects against intermittent hypoxia-induced hippocampal neurons impairment in rats [11]. Furthermore, AST II could trigger T cell activation through regulation of CD45 protein tyrosine phosphatase activity [12]. AST II also shows some inhibition effects on human tumor cells [13-15]. As a result, as one phyto-saponin of Radix Astragalus, AST II may become a novel drug candidate that is beneficial for human health. However, based on its relative large molecular, the bioavailability of AST II might be of great concern in the period of drug discovery. Therefore, it is necessary to develop a rapid and accurate bioanalytical method to quantify AST II in biological fluids and then apply to the bioavailability study of AST II in animals.

A quantitative analytical method of several saponins was developed by high-performance liquid chromatography coupled with diode array and evaporative light scattering detectors [16]. But this method

only aimed at the quality control of Radix Astragali, and it is not suitable for analysis of AST II in biological samples, including rat plasma. In addition, a qualitative liquid chromatography coupled with tandem mass spectrometry has been reported for identification of multiple ingredients in a Traditional Chinese Medicine preparation (bu-yang-huan-wu-tang) [17]. Similarly, this method is only a qualitative assay, and it doesn't meet the analytical requirements for biological fluids with respect to an efficient clean-up procedure, shorter running time and higher sensitivity. An HPLC-MS/MS method has been developed for the quantification of several components in Bu-Yang-Huan-Wu-Tang and rat plasma [18]. As a simultaneous determination method for nine main bioactive components in Bu-Yang-Huan-Wu-Tang, i.e., astragaloside I, astragaloside II, astragaloside IV, formononetin, ononin, calycosin, calycosin-7-O-b-d-glucoside, ligustilide and paeoniflorin, its running time of one injection is 18 min. However, there is only one target compound (astragaloside II) in present study, so rapid analysis is our pursuit and the reported method [18] is obviously not suitable due to its long running time.

Thus, the aim of this present work was to develop a rapid and accurate LC-MS/MS method for the quantification of AST II in rat plasma. And the developed method was further validated and then applied to a pharmacokinetic study of AST II after intravenous and oral administration of AST II in rats.

2. Experimental

2.1 Chemicals and reagents

Astragaloside II (AST II, HPLC purity ≥ 98%, chemical structure shown in Fig.1) was purchased from Shanghai Jingke Chemicals Co., Ltd (Shanghai, China). Buspirone (Internal standard, IS, batch No. 039K1325, chemical structure shown in Fig.1) was purchased from Sigma (St. Louis, MO, USA). Acetonitrile (HPLC grade) was obtained from Merck (Darmstadt, Germany). Hydroxypropyl-β-cyclodextrin (HPCD, batch No. 20130928) was purchased from Beijing Fengli Jingqiu

Commerce and Trade Co., Ltd (Beijing, China). Methylcellulose (MC, batch No. 20121018) was purchased from Beijing Fengli Jingqiu Commerce and Trade Co., Ltd (Beijing, China). DMSO (Batch No. 806077) was purchased from Tedia (Fairfield, OH, USA). Ultrapure water was produced by a Milli-Q Reagent Water System (Millipore, MA, USA). All other chemicals were of analytical grade.

2.2 Preparation of standard and quality control solutions

Appropriate amount of AST II reference standard was accurately weighed and dissolved in dimethyl sulfoxide (DMSO), and then diluted to appropriate concentrations using methanol for establishment of calibration curves in rat plasma. The concentration of stock solution of AST II was 1000 μg/mL. Working solutions of AST II were prepared at concentrations of 50, 100, 200, 500, 2000, 5000, 20000 and 50000 ng/mL. The IS (Buspirone) working solutions (10 ng/mL) were prepared by diluting with methanol:acetonitrile (50:50, v/v). All the stock and working standard solutions were stored at 4°C prior to use.

2.3 Sample preparation

After thaw at room temperature for about 30 min and vortex for 30 s, aliquots of 50 μL plasma were mixed with 5 μL of methanol (or standard or QC solution) and 150 μL of IS solution (10 ng/mL buspirone in methanol:acetonitrile (50:50, v/v)). After vortex for 1 min and then centrifugation at 12000 *g* for 10 min, aliquots of 100 μL supernatants were transferred to HPLC vials. A volume of 10 μL of this solution was then injected onto the column.

2.4 Instrumentation and analytical conditions

The HPLC system consisted of an LC-20AD pump, a DGU-20 A_3 degasser, an SIL-20AC autosampler and a CTO-20A column oven (Shimadzu, Japan). The HPLC separation was performed on an Agilent Zorbax XDB C_{18} column (2.1 mm × 50 mm, 3.5 μm) with a gradient elution by a mobile phase consisting of water

containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B) with following gradient: 0.00 min 5% B, 0.80 min 5% B, 1.20 min 98% B, 2.20 min 98% B, 2.21 min 5% B, 3.50 min 5% B, with the flow rate of 0.50 mL/min. The injection volume was set to be 10 μL.

The HPLC system was coupled with an API 4000 Qtrap mass spectrometer (Applied Biosystems/MDS Sciex, Concord, ON, Canada) via a Turbo IonSpray ionization interface. Following optimization of the setting parameters, the ESI source was operated in positive mode with the curtain, nebulizer and turbo-gas (all nitrogen) set at 20, 60 and 60 psi, respectively. The source temperature was 550ºC and the ion spray needle voltage was 5000 V. The mass spectrometer was operated at unit resolution for Q1 and low resolution for Q3 in the multiple reaction monitoring mode, with a dwell time of 150 ms per multiple reaction monitoring channel. The collision energy was set at 21 and 40 eV for AST II and buspirone, respectively. The declustering potential (DP) was set at 146 and 90 eV for AST II and buspirone, respectively. The precursor/product ion pairs were monitored at *m/z* 827.3→143.2 for AST II and 386.3→122.3 for buspirone. Data were collected and analyzed by the Analyst Data Acquisition and Processing software (Version 1.5.2, Applied Biosystems/MDS Sciex, Concord, ON, Canada).

2.5 Calibration curves

Calibration standards were prepared freshly on the day of analysis, in duplicate, by addition of 5 μL aliquots of appropriate working solutions and 150 μL of IS solution (10 ng/mL buspirone in methanol:acetonitrile (50:50, v/v)) to 50 μL aliquots of blank rat plasma (drug-free) at the following concentrations: 5.0, 10, 20, 50, 200, 500, 2000, and 5000 ng/mL. Six pools of each QC sample were prepared in blank rat plasma at concentrations of 10 ng/mL (QC Low), 200 ng/mL (QC Middle) and 4000 ng/mL (QC High). Pools of QC samples were divided into aliquots and stored at -20°C following processing.

2.6 Method validation

The method was validated for the selectivity, matrix effect, linearity, lower limit of quantification (LLOQ), accuracy, precision, recovery and stability. To evaluate the selectivity, six individual samples of blank rat plasma were analyzed by comparing with the plasma-spiked analyte for endogenous interferences. Each blank sample was tested by developed LC-MS/MS method for potential interferences. To evaluate the matrix effects, chromatographic peaks of AST II and IS from the spike-after prepared samples were compared with those of the neat standards in mobile phase at the QC concentrations.

The linearity of the method was determined by analyzing a series of standard plasma samples at concentrations of 5.0, 10, 20, 50, 200, 500, 2000 and 5000 ng/mL for AST II by least squares linear regression of the peak area ratios of AST II to IS obtained against the corresponding concentration (x) with a weighting factor of $1/x^2$. The LLOQ was defined as the lowest concentration on the calibration curve with acceptable precision and accuracy (<15%). The criteria for the calibration included a correlation coefficient (r) of 0.995 or better. Concentrations in the QCs and unknown plasma samples were quantified by using the internal standard calibration method.

The precision and accuracy of method were assessed by performing replicate analyses of QC samples spiked with low, medium and high concentrations against calibration standards. Five replicates of QC samples at each concentration level were evaluated on the same day for intra-day precision, while repeated analysis at each concentration of QC samples five times per day over five consecutive days for inter-day precision and accuracy. Standard deviations (SD) and relative standard deviations (%RSD) were calculated from the QC values and used to estimate the inter- and intra-day precision.

The matrix effect was determined by examining the ratio of the peak areas of AST II dissolved in blank

matrix extract to that in standard solution containing equivalent amounts of the compounds. The procedure was repeated five times. The matrix effect of IS was determined in the same way.

Recoveries of AST II and IS from rat plasma were determined by comparing of the responses of AST II and IS in plasma carried through the complete preparation procedure to those spiked into the prepared blank plasma of the same concentration as those of QC samples, respectively.

The stability of AST II in rat plasma was assessed by analyzing QC samples at two concentrations exposed to different time and temperature conditions. The long-term stability was assessed after the QC samples had been stored at -80°C for 1 month. The freeze-thaw stability was determined after three freeze-thaw cycles (-20°C to 20°C) on 3 consecutive days. The amount of AST II in plasma samples was determined using a newly prepared calibration curve. Stability of AST II was expressed as a percentage of nominal concentration. Deviation of the stability results should be within ±15% of the nominal values.

2.7 Application to a pharmacokinetic study

Six male Sprague-Dawley rats (weighing 220 ± 30 g, 8 weeks) were purchased from Beijing Vital River Laboratories Co., Ltd (Beijing, China). All experimental procedures were approved by the Experimental Animal Care and Use Committee of Capital Medical University (Beijing, China). The animals were housed in an air-conditioned room at a temperature of 23 ± 2°C, with a relative humidity of 55 ± 10%, an illumination intensity of 150 - 300 lx, a frequency of air ventilation of 15 - 20 times/hr and a 12 hr illumination. Food and water were supplied *ad libitum*.

Polyethylene cannulas were implanted in the femoral vein 2 days before the experiment while the rats were anesthetized with pentobarbital (50 mg/kg, intravenous). The cannulas were externalized at the back of the neck and filled with heparinized saline (20 units/mL). The rats were fasted for 16 hr before experiments with the exception of free access to water. The intravenous dosing solution with AST II

concentration of 1.0 mg/mL was prepared by dissolving appropriate amount of AST II in DMSO:30% HPCD (5:95, v/v) and filtering through 0.22 μm Millipore filter prior to use. The oral dosing suspension with AST II concentration of 4.0 mg/mL was prepared by dissolving appropriate amount of AST II in 0.5% MC solution. The intravenous and oral doses of AST II were 1.0 mg/kg and 20 mg/kg, and the intravenous and oral dose volumes were 1.0 mL/kg and 5.0 mL/kg, respectively. After intravenous administration of 1.0 mg/kg AST II through tail vein, aliquots of 0.20 mL blood samples were collected in heparinized polyethylene tubes at different time intervals post-dosing (0.033, 0.083, 0.25, 0.50, 1.0, 2.0, 4.0, 6.0 and 8.0 hr). After oral administration, aliquots of 0.20 mL blood samples were collected in heparinized polyethylene tubes at different time intervals post-dosing (0.25, 0.50, 1.0, 2.0, 4.0, 6.0 and 8.0 hr). Heparinized blood was centrifuged at 12000 *g* at room temperature for 5 minutes to obtain plasma, which was stored at -80°C until analysis.

Pharmacokinetic parameters including half-life ($t_{1/2}$), maximum plasma time (t_{max}) and concentration (C_{max}), area under concentration–time curve (AUC_{0-t} and $AUC_{0-\infty}$), clearance (CL), steady-state volume of distribution (V_Z), mean residence time (MRT) of EG were analyzed by non-compartmental method using DAS Version 2.0 (Chinese Pharmacological Society, Beijing, China). All results were expressed as arithmetic mean ± standard deviation (SD).

3. Results and discussion

3.1 Mass spectrometry and chromatography

This study describes the development of a rapid LC-MS/MS assay for the determination of AST II concentrations in rat plasma. The full-scan product ion mass spectra of AST II and buspirone (Internal standard) are shown in Fig.2. In the full-scan Q1 mass spectrum, the parent positive ion peak of AST II appeared at *m/z* = 827.3, and the abundance of this ion peak was sufficient for the quantification of AST II.

In the reported method [18], *m/z* 844.5 $[M+NH_4]^+$ was utilized to be the parent ion, and the mobile phase consisted of methanol (0.1% formic acid) and NH_4OAc (0.1% formic acid). However, it is concerned that the adduct ion (*m/z* 844.5 $[M+NH_4]^+$) would be not stable enough for accurate quantification. Therefore, *m/z* 827.3 $[M+H]^+$ was finally utilized to the parent ion in present study. For AST II, several product ions with similar abundances presented in the full-scan product ion mass spectra of AST II, including *m/z* 629.3, 455.3, 175.0 and 143.2, in which product ion *m/z* 143.2 was most stable and abundant. For buspirone, the most abundant peak was the protonated molecular ion $[M+H]^+$ found at *m/z* = 386.1. Thus, protonated AST II and buspirone were targeted for fragmentation, and the most stable and abundant ions in the product ion scan of AST II and buspirone were *m/z* 143.2 and 122.3, respectively. Subsequently, the mass transitions were monitored at *m/z* 827.3→143.2 for AST II and *m/z* 386.3→122.3 for buspirone. Other conditions such as ion spray voltage, curtain gas pressure, nebulizer gas pressure, heater gas pressure, source temperature and collision energy were further optimized to improve the sensitivity and response stability of AST II.

During the optimization of chromatographic conditions, it was investigated that AST II was extensively retained on several kinds of columns due to its strong lipophilicity. To achieve symmetric peak shapes and short chromatographic running times, the mobile phase consisting of acetonitrile with 0.1% formic acid and water with 0.1% formic acid was used on a Zorbax XDB C_{18} column. And finally a gradient elution program was utilized as follows: 0.00 min 5% B, 0.80 min 5% B, 1.20 min 98% B, 2.20 min 98% B, 2.21 min 5% B, 3.50 min 5% B. Under the present chromatographic conditions, symmetric peak shapes of AST II and buspirone were obtained, and the running time is only 3.5 min.

Mass chromatograms of AST II and IS obtained by extraction of blank rat plasma, blank plasma spiked with AST II and IS, and actual unknown plasma samples obtained in rats after intravenous and oral

injection of AST II are shown in Fig.3. AST II and buspirone were eluted at 2.34 and 2.28 min, respectively. Each analytical run was achieved within 3.5 min. No endogenous or extraneous peaks interfering with the analytes were observed.

An internal standard is usually required in LC-MS/MS analysis in order to eliminate the effects from matrix and the extraction efficiency. Usually a radio-labeled internal standard is the optimal choose, however, it is not available during the period of drug discovery. In this study, buspirone, a readily available compound, was selected as the IS, which displays similar chromatographic retention behavior (t_R = 2.28 min) with AST II and high extraction efficiency (>80%). Most importantly, as a chemically synthesized compound, buspirone will not exist in traditional Chinese medicines and animal feed. Thus, there were no interferences of IS from AST II and endogenous substances.

3.2 Assay validation

LC-MS/MS results may be adversely affected by lack of specificity and selectivity due to ion suppression caused by the sample matrix effect. In present assay, the mean absolute matrix effect value obtained was 91.5% for AST II (Table 1), which indicated that ion suppression or enhancement from plasma matrix was acceptable for this current analytical method.

The recoveries were determined in five replicates at low, medium and high concentrations of AST II. The results are summarized in Table 1. The mean recoveries of AST II were all above 80%. The data indicated that the recoveries of AST II from rat plasma were concentration-independent in the concentration range evaluated and the recoveries were acceptable for the pharmacokinetic analysis.

The calibration curves ranging from 5.0 to 5000 ng/mL were linear for the analysis of AST II in rat plasma. The slopes, intercepts, and correlation coefficients of the regression equations were determined by least squares linear regression using a weight-factor of $1/x^2$. Typical equations for the standard curves

were y = 6.36E-06 x + 9.68E-06 (r=0.9973). Deviations were within ±15% for all regression equations. The lower limit of quantification (LLOQ) was 5.0 ng/mL for AST II in rat plasma.

Table 2 summarized the intra-and inter-day precisions and accuracies of AST II at different concentration levels. As shown in Table 2, the intra- and inter-day accuracies of AST II (% RE) were within the range of ±15%. The intra- and inter-day precisions (% RSD) were all less than 15%. The results demonstrated that the values were all within the acceptable range and the method was proved to be accurate and precise.

The stability results were presented in Table 3. The results indicated that AST II at the three concentrations tested had acceptable stabilities after three cycles of freeze-thaw, at room temperature for 24 hr and at -80°C for 1 month with the % RE values being within ± 15%.

In conclusion, the present method was demonstrated to have satisfactory performance in agreement with international guideline for the separation and determination of AST II in rat plasma [19]. The LLOQ of the developed assay (5.0 ng/mL) was sufficient to characterize the pharmacokinetics of AST II at intravenous dose of 1.0 mg/kg and oral dose of 20 mg/kg.

3.3 Application of method

The developed assay was applied to an intravenous and oral study of the pharmacokinetics of AST II in rats. The plasma concentration-time profiles of AST II in rats are shown in Fig.4 and the main pharmacokinetic parameters of AST II after intravenous and oral administration are presented in Table 4. After intravenous administration of AST II at the dose of 1.0 mg/kg, the elimination half-life ($t_{1/2}$) value was estimated to be 2.19 ± 0.31 hr, and the mean area under the plasma concentration-time curve from time zero to the last measurable plasma concentration point (AUC_{0-t}) and the mean area under the plasma concentration-time curve from time zero to time infinity ($AUC_{0-\infty}$) values were 2182 ± 872 and

2202 ± 873 hr*ng/mL, respectively. Clearance (CL), mean residence time (MRT) and steady-state volume of distribution (V_{ss}) values were estimated to be 0.51 ± 0.20 mL/min/kg, 0.71 ± 0.09 hr and 1.62 ± 0.78 L/kg, respectively. After oral administration of AST II at the dose of 20 mg/kg, AST II was rapidly absorbed, reaching mean C_{max} of 118 ± 21.8 ng/mL at T_{max} of 1.00 ± 0.00 hr. The mean AUC_{0-t} and $AUC_{0-\infty}$ values were 329 ± 73.7 and 349 ± 72.1 hr*ng/mL, respectively. The oral absolute bioavailability (F) of AST II was calculated to be 0.79 ± 0.16% with an elimination half-life ($t_{1/2}$) value of 1.92 ± 0.30 hr, suggesting its poor absorption and/or strong metabolism *in vivo*.

Shaw *et al* reported that the plasma concentration-time curve of astragaloside II in rats after oral administration of Bu-Yang-Huan-Wu-Tang presented the phenomenon of a double-peak absorption phase in the plasma profile [18]. They had given several possible factors in order to explain the phenomenon of double-peak behavior: 1) entero-hepatic recycling, 2) the presence of absorption sites along the stomach and different gastrointestinal segments, 3) variable gastric emptying. In addition, it is well known that herbal preparation is a complex formulation which contains several herbs and ingredients. It is possible that the different characteristics of physical-chemical properties for individual ingredients may affect each other. However, in present study, the plasma concentration-time curve of astragaloside II in rats after oral administration presents the phenomenon of a single-peak absorption phase in the plasma profile. It should be noted that the rats in present study were single dosed with astragaloside II via oral gavage. And there are some differences in the main pharmacokinetic parameters, such as T_{max} (1.00 ± 0.00 hr *vs* 0.62 ± 0.11 hr), $t_{1/2z}$ (1.92 ± 0.30 hr *vs* 3.53 ± 0.23 hr), CL_z (59.0 ± 13.0 L/hr/kg *vs* 7.74 ± 0.90 L/hr/kg) between our research (the former values) and Shaw's (the latter values) [18]. It is mostly possible due to the dosing differences of astragaloside II *vs* Bu-Yang-Huan-Wu-Tang. The coexisting ingredients might greatly affect each other on their dissolution, absorption, mechanism, transportation and permeability *in vivo*.

Overall, the oral absolute bioavailability (F) of AST II (0.79 ± 0.16%) is very low, suggesting that astragaloside II shows poor absorption and/or strong metabolism *in vivo*. As a result, parenteral administration route is suggested for AST II in order to improve its efficacy.

4. Conclusion

An accurate and sensitive LC-MS/MS assay was developed for the determination of AST II in rat plasma. The sample preparation involved a simple protein precipitation (PPT) procedure with methanol:acetonitrile (50:50, v/v). The LLOQ was 5.0 ng/mL using 50 μL of rat plasma. The assay showed a wide linear dynamic range of 5.0–5000 ng/mL, with acceptable intra- and inter-day accuracy and precision. The developed and validated method has been successfully applied in the quantification and pharmacokinetic study of AST II in rats after intravenous and oral administration. The oral absolute bioavailability (F) of AST II was calculated to be 0.79 ± 0.16% with an elimination half-life ($t_{1/2}$) value of 1.92 ± 0.30 hr, suggesting its poor absorption and/or strong metabolism *in vivo*.

References

[1] Pharmacopoeia Committee of P. R. China, 2010. Pharmacopoeia of the People's Republic of China, Vol. I. China Medical Science and Technology Press: Beijing.

[2] J. Sun, Z.Z. Jiang, R.Q. Yan, O. Olaleye, X.L. Zhang, X. Chai, Y.F. Wang. Quality Evaluation of Astragali Radix Products by Quantitative Analysis of Multi-components by Single Marker, Chin. Herb. Med. 5 (2013) 272-279.

[3] X.D. Cheng, C.H. Hou, X.J. Zhang, H.Y. Xie, W.Y. Zhou, L. Yang, S.B. Zhang, R.L. Qian. Effects of Huang-qi (Hex) on inducing cell differentiation and cell death in K562 and HEL cells, Acta Biochem. Biophys. Sin. 36 (2004) 211-217.

[4] W. Xie, L. Du. Diabetes is an inflammatory disease: Evidence from traditional Chinese medicines,

Diabetes Obes. Metab. 13 (2011) 289-301.

[5] J. Liu, Z.Z. Zhao, H.B. Chen. Review of Astragali Radix, Chin. Herb. Med. 3 (2011) 90-105.

[6] M. Ozipek, A.A. Dönmez, I. Caliş, R. Brun, P. Rüedi, D. Tasdemir. Leishmanicidal cycloartane-type triterpene glycosides from Astragalus oleifolius, Phytochemistry. 66 (2005) 1168-1173.

[7] M. Hirotani, Y. Zhou, H. Rui, T. Furuya. Cycloartane triterpene glycosides from the hairy root cultures of Astragalus membranaceus, Phytochemistry. 37 (1994) 1403-1407.

[8] R.M. Abdallah, N.M. Ghazy, N.A. El-Sebakhy, A. Pirillo, L. Verotta. Astragalosides from Egyptian Astragalus spinosus Vahl, Pharmazie 48 (1993) 452-454.

[9] E. Polat, E. Bedir, A. Perrone, S. Piacente, O. Alankus-Caliskan. Triterpenoid saponins from Astragalus wiedemannianus Fischer, Phytochemistry. 71 (2010) 658-662.

[10] X.H. Kong, Y.B. Niu, X.M. Song, D.D. Zhao, J. Wang, X.L. Wu, R. Zhang, Q.B. Mei. Astragaloside II induces osteogenic activities of osteoblasts through the bone morphogenetic protein-2/MAPK and Smad1/5/8 pathways, Int. J. Mol. Med. 29 (2012) 1090-1098.

[11] Q. Zhang, W.Y. Gao, Y. Zhang, B.Y. Chen, Z. Chen, W.S. Zhang, S.L. Man. Protective effects of astragalus extract against intermittent hypoxia-induced hippocampal neurons impairment in rats, Chin. Med. J. (Engl) 126 (2013) 1551-1554.

[12] C.P. Wan, L.X. Gao, L.F. Hou, X.Q. Yang, P.L. He, Y.F. Yang, W. Tang, J.M. Yue, J. Li, J.P. Zuo. Astragaloside II triggers T cell activation through regulation of CD45 protein tyrosine phosphatase activity, Acta Pharmacol. Sin. 34 (2013) 522-530.

[13] C. Huang, D. Xu, Q. Xia, P. Wang, C. Rong, Y. Su. Reversal of P-glycoprotein-mediated multidrug resistance of human hepatic cancer cells by Astragaloside II, J. Pharm. Pharmacol. 64 (2012) 1741-1750.

[14] I. Ionkova, G. Momekov, P. Proksch. Effects of cycloartane saponins from hairy roots of Astragalus membranaceus Bge., on human tumor cell targets, Fitoterapia. 81 (2010) 447-451.

[15] F. Hong, W. Xiao, G. Ragupathi, C.B. Lau, P.C. Leung, K.S. Yeung, C. George, B. Cassileth, E. Kennelly, P.O. Livingston. The known immunologically active components of Astragalus account for only a small proportion of the immunological adjuvant activity when combined with conjugate vaccines, Planta Med. 77 (2011) 817-824.

[16] L.W. Qi, Q.T. Yu, P. Li, S.L. Li, Y.X. Wang, L.H. Sheng, L. Yi. Quality evaluation of Radix Astragali through a simultaneous determination of six major active isoflavonoids and four main saponins by high-performance liquid chromatography coupled with diode array and evaporative light scattering detectors, J. Chromatogr. A. 1134 (2006) 162-169.

[17] L.H. Shaw, W.M. Chen, T.H. Tsai. Identification of multiple ingredients for a Traditional Chinese Medicine preparation (bu-yang-huan-wu-tang) by liquid chromatography coupled with tandem mass spectrometry, Molecules. 18 (2013) 11281-11298.

[18] L.H. Shaw, L.C. Lin, T.H. Tsai. HPLC-MS/MS analysis of a traditional Chinese medical formulation of Bu-Yang-Huan-Wu-Tang and its pharmacokinetics after oral administration to rats, PLoS One. 7 (2012) e43848.

[19] Guidance for Industry, Bioanalytical Method Validation, US Department of Health and Human Services Food and Drug Administration, Center for Drug Evaluation and Research (CDER), 2001 Center for Veterinary Medicine (CV), May 2001.

Figure Captions:

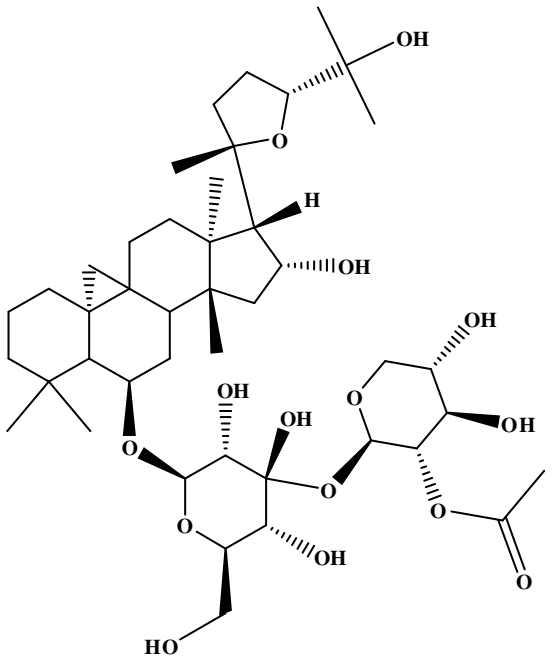
Fig.1. Chemical structures of AST II (A) and internal standard (B)

Fig.2. Full-scan product ion spectra of $[M+H]^+$ ions for (A) AST II and (B) buspirone (Internal standard)

Fig.3. Typical MRM chromatograms of (A) blank rat plasma; (B) blank rat plasma spiked with AST II (5 ng/mL, LLOQ) and IS; (C) an unknown rat plasma sample collected at 15 min after intravenous administration of 1.0 mg/kg AST II; and (D) an unknown rat plasma sample collected at 30 min after oral administration of 20 mg/kg AST II

Fig.4. Mean plasma concentration-time profiles of AST II determined by LC-MS/MS method after intravenous and oral administration of AST II to rats. Each point represents mean ± SD (n=3)

(A)



(B)

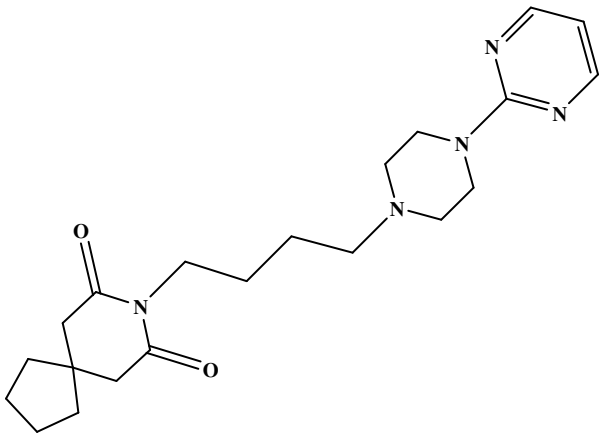


Fig.1. Chemical structures of AST II (A) and internal standard (B)

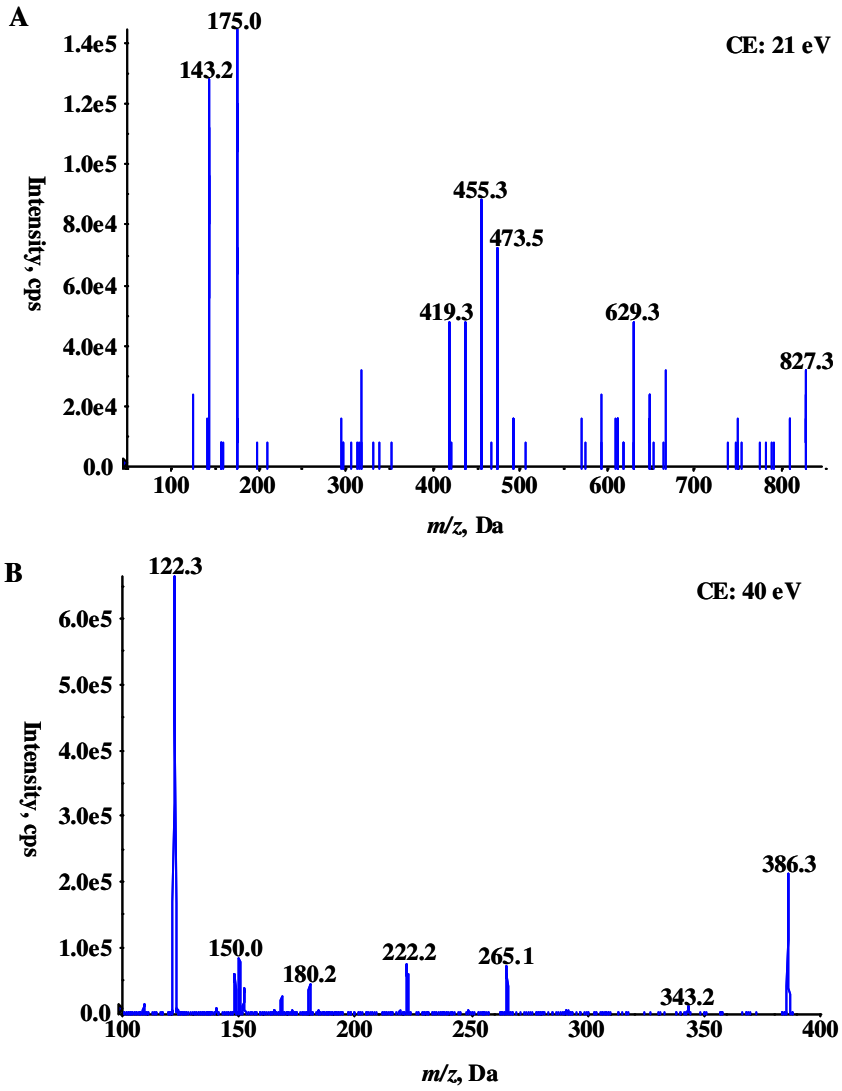
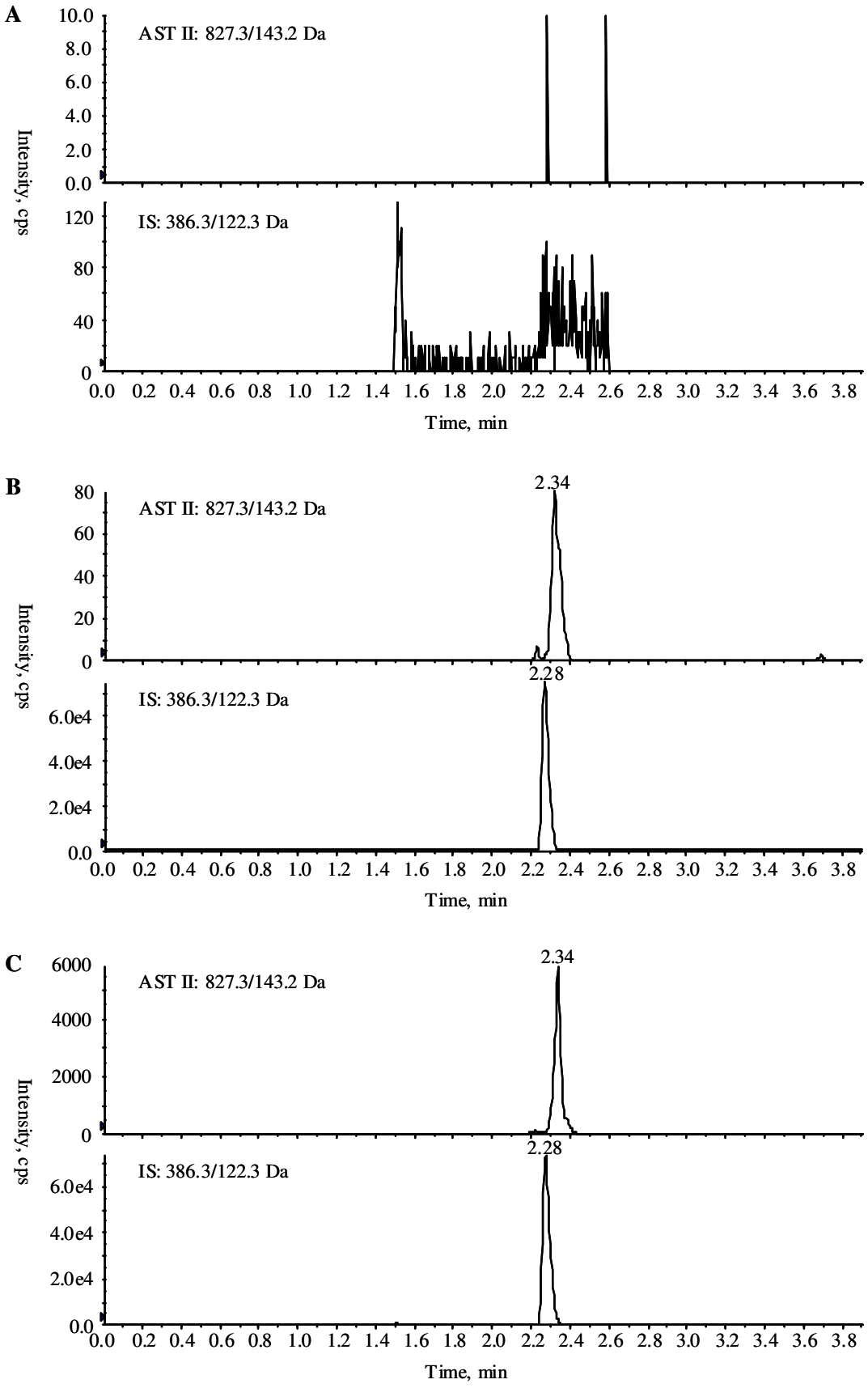


Fig.2. Full-scan product ion spectra of $[M+H]^+$ ions for (A) AST II and (B) buspirone (Internal standard)



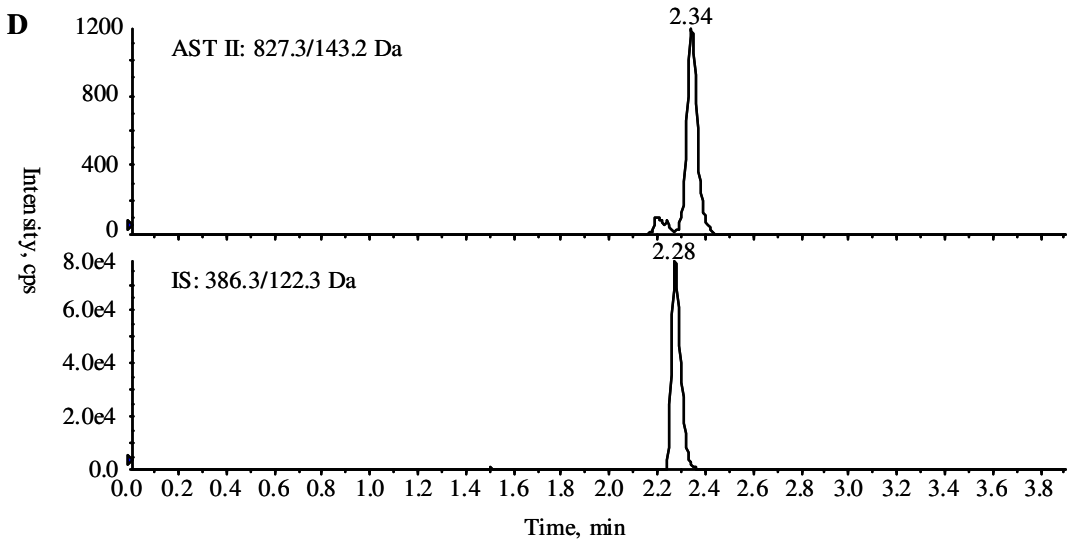


Fig.3. Typical MRM chromatograms of (A) blank rat plasma; (B) blank rat plasma spiked with AST II (5 ng/mL, LLOQ) and IS; (C) an unknown rat plasma sample collected at 15 min after intravenous administration of 1.0 mg/kg AST II; and (D) an unknown rat plasma sample collected at 30 min after oral administration of 20 mg/kg AST II

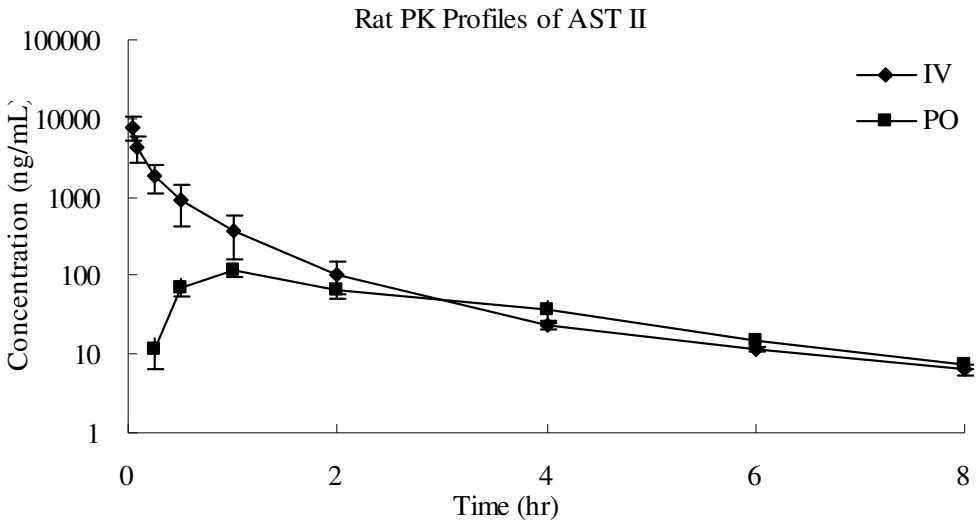


Fig.4. Mean plasma concentration-time profiles of AST II determined by LC-MS/MS method after intravenous and oral administration of AST II to rats. Each point represents mean ± SD (n=3)

Table Captions:

Table 1 Matrix effects and recoveries of AST II in rat plasma determined by LC-MS/MS (n=5)

Table 2 Intra- and inter-day accuracy and precision data (mean ± SD) for AST II in rat plasma determined by LC-MS/MS (n=5)

Table 3 Stability results of AST II in rat plasma under three different conditions determined by LC-MS/MS (n=5)

Table 4 Main pharmacokinetic parameters of AST II in rats determined after intravenous and oral administration of AST II (n=3, mean ± SD)

Table 1 Matrix effects and recoveries of AST II in rat plasma determined by LC-MS/MS (n=5)

Spiked concentration (ng/mL)	Matrix effect (%)	RSD (%)	Recovery (%)	RSD (%)
10.0	90.2	3.48	88.9	1.28
200	92.5	2.68	88.2	2.15
4000	91.7	3.07	85.8	2.71

Table 2 Intra- and inter-day accuracy and precision data (mean ± SD) for AST II in rat plasma determined by LC-MS/MS (n=5)

Concentration (ng/mL)	Intra-day (n = 5)		Inter-day (n=5)	
	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
10.0	102.6 ± 6.71	6.54	102.4 ± 6.16	6.02
200	101.8 ± 7.10	6.98	100.1 ± 5.73	5.73
4000	100.3 ± 5.93	5.91	100.2 ± 5.88	5.87

Table 3 Stability results of AST II in rat plasma under three different conditions determined by LC-MS/MS (n=5)

Condition	Concentration level	10.0	200	4000
Three freeze–thaw cycles	**Concentration (mean ± SD, ng/mL)**	9.52 ± 0.10	190 ± 2.08	3850 ± 45.5
	RE (%)	-4.77	-4.83	-3.75
Storage at −80°C for 1 month	**Concentration (mean ± SD, ng/mL)**	9.73 ± 0.09	183 ± 5.86	3809 ± 46.4
	RE (%)	-2.73	-8.67	-4.78
Room temperature for 24 hr	**Concentration (mean ± SD, ng/mL)**	9.57 ± 0.10	182 ± 2.65	3885 ± 33.4
	RE (%)	-4.33	-9.00	-2.88

Table 4 Main pharmacokinetic parameters of AST II in rats determined after intravenous and oral administration of AST II (n=3, mean ± SD)

PK Parameters	Unit	IV	PO
$AUC_{(0-t)}$	ng/mL*hr	2182 ± 872	329 ± 73.7
$AUC_{(0-\infty)}$	ng/mL*hr	2202 ± 873	349 ± 72.1
$MRT_{(0-t)}$	hr	0.60 ± 0.03	2.54 ± 0.09
$MRT_{(0-\infty)}$	hr	0.71 ± 0.09	3.05 ± 0.29
$t_{1/2z}$	hr	2.19 ± 0.31	1.92 ± 0.30
T_{max}	hr	0.03 ± 0.00	1.00 ± 0.00
CL_z	L/hr/kg	0.51 ± 0.20	59.0 ± 13.0
V_z	L/kg	1.62 ± 0.78	166 ± 62.6
C_{max}	ng/mL	7873 ± 2591	118 ± 21.8
F	%		0.79 ± 0.16