

Full Length Research Paper

## Pharmacokinetics of vitexin in rats after intravenous and oral administration

Yunjiao Wang<sup>2</sup>, Chunhui Han<sup>1</sup>, Aijing Leng<sup>1\*</sup>, Wenjie Zhang<sup>2</sup>, Hefei Xue<sup>2</sup>, Yinghui Chen<sup>2</sup>,  
Jingjing Yin<sup>2</sup>, Dongrui Lu<sup>2</sup> and Xixiang Ying<sup>2</sup>

<sup>1</sup>First Affiliated Hospital of Dalian Medical University, Dalian, 116011, China.

<sup>2</sup>School of Pharmacy, Liaoning University of Traditional Chinese Medicine, Dalian 116600, China.

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Vitexin was isolated from the leaves of *Crataegus pinnatifida* Bge. var *major*, and its pharmacokinetics and bioavailability were carried out via validated high-performance liquid chromatography (HPLC) method using hesperidin as internal standard in healthy rats after intravenous and oral administration at a dose of 10 mg/kg and 30 mg/kg, respectively. The pharmacokinetic parameters were calculated by both compartmental and non-compartmental approach. When intravenous administration was used, the elimination half-life ( $t_{1/2\beta}$ ), the mean residence ( $MRT_{0\rightarrow t}$ ), the total body clearance (CL) were  $46.01 \pm 0.810$  min,  $26.23 \pm 1.51$  min and  $0.031 \pm 0.035$  L/kg-min. When oral administration was used, the  $t_{max}$  and  $C_{max}$  were  $15.82 \pm 0.172$  min and  $0.51 \pm 0.015$   $\mu$ g/ml, the  $MRT_{0\rightarrow t}$  and CL were  $60.41 \pm 5.41$  min and  $0.71 \pm 0.056$  L/kg-min. The result showed that vitexin was rapidly eliminated and presented a low absolute bioavailability (F),  $4.91 \pm 0.761\%$ .

**Key words:** Bioavailability, high-performance liquid chromatography (HPLC), pharmacokinetics, rat plasma, vitexin.

### INTRODUCTION

Flavonoids are polyphenolic compounds occurring in many plants, which have shown many biological and pharmacological activities, such as the prevention of coronary heart disease (Gambelunghe et al., 2003), inhibition of tumor promotion (Bomser et al., 1999), anti-oxidation (Hollman and Katan, 1999), and anti-inflammation (Rahman et al., 2006). Vitexin being a flavonoid component was found with many pharmacological actions, such as anti-hypotensive, anti-spasmodic, anti-inflammatory properties (Prabhakar et al., 1981), and antithyroid effect (Gaitan et al., 1995).

Recently, Tong and Liu, 2007 reported the pharmacokinetics in rats after multi-dose intravenous administration of vitexin (Tong and Liu, 2007). However, the pharmacokinetics and bioavailability after intravenous and oral administration of pure vitexin have never been reported until now, and therefore an HPLC method herein

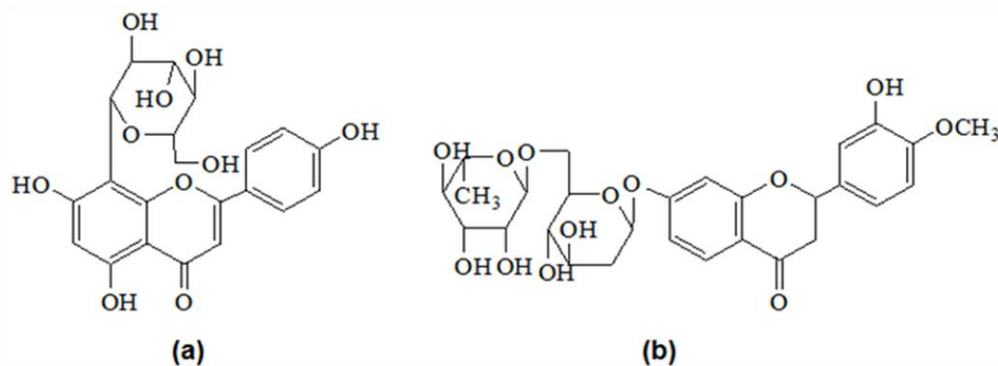
will be developed and validated for the determination of vitexin in rat plasma to investigate the pharmacokinetics of pure vitexin. To the best of our knowledge, this is the first report on the development, validation and application of HPLC method with internal standard for the determination of vitexin in rat plasma and its pharmacokinetic study after intravenous and oral administration, and its bioavailability can also be calculated.

### MATERIALS AND METHODS

#### Chemicals

Vitexin (Figure 1a) was isolated from the hawthorn leaves in our laboratory, and its purity was over 99% by HPLC analysis. The internal standard, hesperidin (Figure 1b) was provided by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol and acetonitrile (HPLC grade) were obtained from Xinxing (Chemical Reagent Plant, Shanghai, China), and the water used in all experiments was purified by a Milli-Q® Biocel Ultrapure Water System (Millipore, Bedford, MA, USA). All other chemicals were of analytical reagent grade

\*Corresponding author. Email: [aijingleng@hotmail.com](mailto:aijingleng@hotmail.com). Tel: +86 (0)411 87586005. Fax: +86 (0)411 87586078.



**Figure 1.** Chemical structures of vitexin (a) and hesperidin (b).

purchased from Sinopharm Chemical Reagent Co., Ltd.

### Chromatographic equipment and conditions

The analyses were carried out on an Agilent 1100 series HPLC system (Agilent technology, Palo Alto, CA, USA) which consisted of a quaternary Pump (G1310A), a vacuum degasser (G1322A), a UV-VIS spectrophotometric detector (G1314A) and Chemstation software (Agilent). The analytical column was a Kromasil C18 column (150 mm × 4.6 mm i.d., 5 μm, Dalian Sanjie, China) protected by a KR C18 guard column (35 mm × 8.0 mm, i.d., 5 μm, Dalian Create Science and Technology Co., Ltd., China). The mobile phase for HPLC analysis consisted of a mixture of methanol-acetonitrile-0.3% formic acid (3:1:6, v/v/v), was passed under vacuum through a 0.45 μm membrane filter and degassed before use. All the chromatographic measurements were performed at room temperature and a flow rate of 1 ml/min with the detection wavelength of 330 nm.

### Animals

Male Wistar 300-330 g were obtained from the Laboratory Animal Center of Liaoning University of Traditional Chinese Medicine (Shenyang, China). They were kept in an environmentally controlled breeding room for 1 week before the experiments and fed with standard laboratory food and water ad libitum and fasted overnight before the experiment. All animal studies were performed according to the Guidelines for the Care and Use of Laboratory Animals that was approved by the Committee of Ethics of Animal Experimentation of Liaoning University of Traditional Chinese Medicine.

### Drug administration and sample collection

To the pharmacokinetic study, 10 rats were randomly assigned to 2 groups. Vitexin was prepared with 20% propylene glycol-water (v/v) to the concentration of 2 mg/ml for intravenous injection and 3 mg/ml for oral administration. Vitexin solution was administered intravenously at a dosage of 10 mg/kg, orally at a dosage of 30 mg/kg. Blood samples (0.3 ml) were collected into heparinized tubes from the vena orbitalis at times of 2, 5, 8, 11, 15, 20, 30, 45, 60, 90 and 120 min for intravenous administration, and at times of 3, 5, 10, 15, 20, 30, 45, 60, 80, 120 and 180 min for oral administration, and then centrifuged at 3500 r/min. The obtained plasma was stored at -20°C until analysis.

### Extraction of analyte from plasma

To 200 μl plasma, 20 μl acetic acid, 30 μl hesperidin, and 1 ml acetonitrile were added, followed by vortex mixing for 1 min and centrifuged at 3500 r/min for 15 min. The supernatant was collected and evaporated to dryness at 50°C under a gentle stream of nitrogen. The dried residue was then reconstituted in 200 μl of mobile phase, and centrifuged at 10000 r/min for 10 min, and an aliquot (20 μl) of the supernatant was injected into the HPLC system for analysis.

### Preparation of calibration standards and quality control samples

Stock standard solutions of vitexin and hesperidin were prepared with methanol, and the concentrations of them were 237 and 248 μg/ml, respectively. All solutions were stored at 4°C and were found to be stable for at least 1 month. Seven calibrators (0.1, 0.2, 0.5, 1, 2.5, 5 and 20 μg/ml) of vitexin and hesperidin (124 μg/ml) were prepared by dilution of stock solutions followed by spiking with drug free plasma. Quality control (QC) samples were prepared at three concentrations (low, middle and high) representing the whole range of the calibration curve. The low concentration (0.3 μg/ml) was 3 times of lower limit of quantitation, high concentration (16 μg/ml) was 80% of the upper limit of quantitation, and middle concentration (1.5 μg/ml) was near the geometric mean of low and high concentration. All of the QC samples were stored at -20°C until analysis.

### Method validation

Selectivity was investigated by comparing chromatograms of blank plasma obtained from rats prior to dosing with those of corresponding standard plasma sample spiked with vitexin and hesperidin, and plasma sample from rats after intravenous doses of them.

The linearity was evaluated over the concentration range of 0.1-20 μg/ml at seven levels of vitexin. The calibration curves for vitexin in plasma were generated by plotting the peak area ratio of vitexin to hesperidin versus the nominal concentrations in the standard plasma samples. The regression equation was obtained by weighted ( $1/c^2$ ) least square linear regression. The limit of detection (LOD) was determined by a signal-to-noise ratio (S/N) of 3. The lower limit of quantification (LLOQ) was defined as the lowest concentration of vitexin in the calibration curves, giving an acceptable accuracy (RE) within ±20% and a precision (RSD) that

did not exceed 20%.

The accuracy and precision of the method were evaluated with QC samples at three concentrations by five replicates on three consecutive days. The intra- and inter-assay precisions were assessed by determining the quality control samples at three concentration levels of vitexin (0.3, 1.5 and 16 µg/ml). For the intra-day validation, five replicates of the QC plasma samples were analyzed on the same day. For the inter-day validation, five replicates of the QC plasma samples were analyzed on three different days. The precision was expressed as the RSD which should be less than 15%, except at the LLOQ where it should not exceed 20%, and the accuracy of the assay was determined by comparing the means of the determined vitexin concentrations with the nominal concentrations. The mean percentage deviation from the nominal values expressed as the RE which should be within ±15% of the nominal value, except at the lower limit of quantification where it should not exceed ±20%.

The extraction recoveries of vitexin were determined by comparing the peak area of QC samples that were at low, middle and high three concentrations to that of the unextracted standard solutions containing the equivalent amount of analyte ( $n = 5$ ).

Five aliquots of QC samples at low, middle and high concentration unextracted QC samples were subjected to the conditions below. Short-term stability was determined by analyzing QC plasma samples kept at ambient temperature (25°C) for 4 h. Long-term stability was assessed by analyzing the extracted QC plasma samples stored at -20°C for 1 month. Freeze-thaw stability was investigated after three freeze (-20°C) -thaw (room temperature) cycles. Then, the samples were processed and analyzed. The concentrations obtained were compared with the nominal values of QC samples.

### Pharmacokinetics and bioavailability study

Pharmacokinetic analysis of the data was performed using both compartmental and non-compartmental methods, with the help of the practical program (3P97) of the Chinese Society of Mathematical Pharmacology (Beijing, China). The compartmental parameters were generated, such as volume of distribution of the central compartment ( $V_c$ ),  $\alpha$  half-life ( $t_{1/2\alpha}$ ),  $\beta$  half-life ( $t_{1/2\beta}$ ), clearance rate (CL). The area under the plasma concentration-time curve ( $AUC_{0 \rightarrow t}$ ) was calculated by the trapezoidal method. The area under the plasma concentration-time curve from zero to infinity ( $AUC_{0 \rightarrow \infty}$ ) was calculated by means of the trapezoidal rule with extrapolation to infinity with terminal elimination rate constant. The peak plasma concentration ( $C_{max}$ ) and the peak time were directly obtained from the drug plasma concentration-time profiles. The noncompartmental based on statistical moment theory, the parameters such as; the terminal elimination rate ( $\lambda_z$ ) was estimated by the least-square regression analysis of at least last four time points of the semilogarithmic plasma concentration-time curves. The mean residence time (MRT) was determined as  $(AUMC)/AUC$ . The absolute bioavailability (F) was calculated as  $(AUC_{oral}/AUC_{i.v.}) \times (dose_{i.v.}/dose_{oral})$ .

The data were statistically analyzed (ANOVA). The differences were considered significant when  $P < 0.05$ . All data are presented as means  $\pm$  standard errors.

## RESULTS

### Method validation

Typical chromatograms obtained from blank plasma, blank plasma spiked with vitexin standard analytes and

hesperidin, plasma samples from rats after intravenous and oral administration of vitexin in rats have been shown in Figure 2. The chromatograms showed that there were no interfering peaks in the region of the peaks of the vitexin and hesperidin, which present a complete baseline resolution of analyte and internal standard with adjacent peaks. The evaluation of the linearity was performed with a seven-point calibration curve over the concentration range of 0.1-20 µg/ml. The slope and intercept of the calibration graphs were calculated by weighted ( $1/c^2$ ) least squares linear regression. The regression equation of the calibration curves was typically:  $y = 0.2031x - 0.0158$ , and  $r$  was 0.9993, where  $y$  is the peak area ratio of vitexin to hesperidin, and  $x$  is the plasma concentration of vitexin. The limit of detection (LOD) was 0.035 µg/ml determined by a signal-to-noise ratio ( $S/N=3$ ). The lower limit of quantification (LLOQ) was 0.1 µg/ml ( $S/N=10$ ). The RSD of three concentrations ranged from 4.8 to 7.4% for intra-day assay and from 6.3 to 7.3% for inter-day assay, respectively. The REs of intra- and inter-day accuracy were within -6.9 to 6.9%. The RSD determined at each concentration level is required not exceeding 15% and RE within ±15% of the actual value which conforms to the criteria for the analysis of biological sample according to guidance of USFDA (USFDA, 2001). These results in Table 1 suggested that the procedures described as above were satisfactory with respect to both accuracy and precision, and the extraction recoveries of vitexin at three concentrations (0.3, 1.5 and 16 µg/ml) were no less than  $83.83 \pm 0.157\%$  (Table 1), and that of hesperidin was  $98.81 \pm 0.165\%$ , of which the precision and accuracy of this method were acceptable. The results of short-term stability, long-term stability and freeze-thaw stability indicated that no significant degradation occurred during chromatography, extraction and sample storage processes for analytes plasma samples.

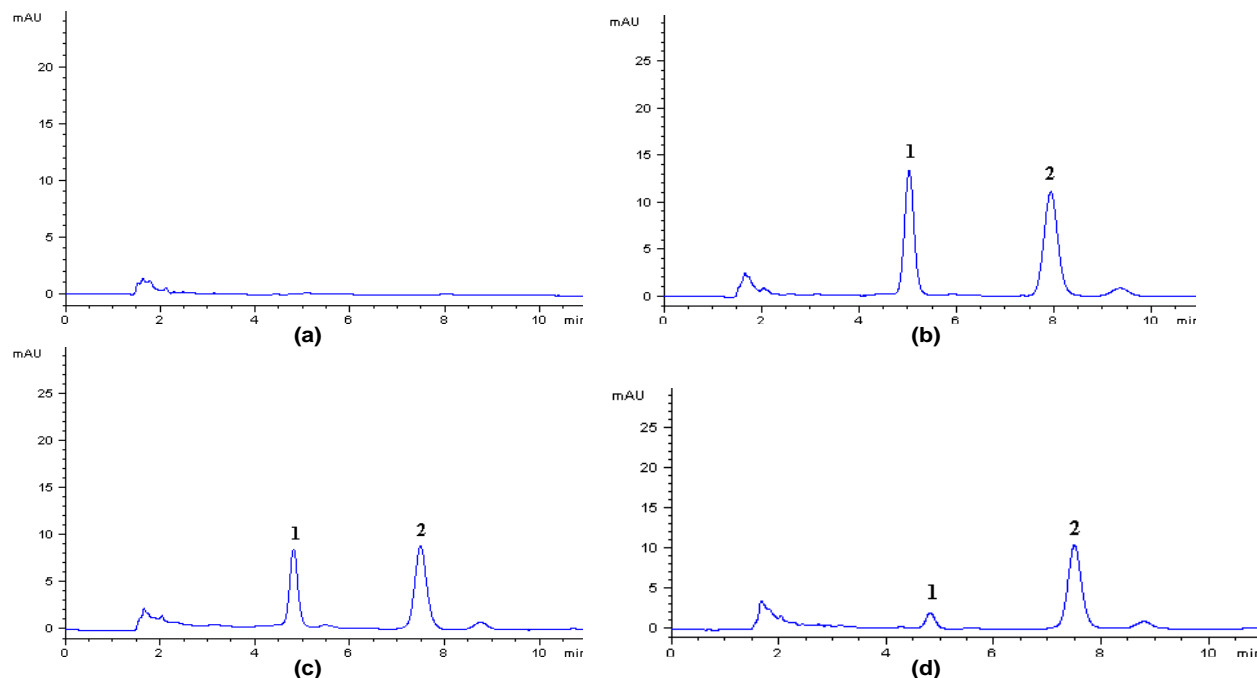
### Pharmacokinetic studies

The validated method was successfully applied to monitor the concentrations and pharmacokinetics of vitexin after intravenous and oral administration at a dose of 10 mg/kg and 30 mg/kg, respectively. The plasma concentration-time profile of vitexin was shown in Figure 3. The main pharmacokinetic parameters of vitexin were listed in Table 2.

## DISCUSSION

### Method development

To obtain solution for intravenous injection and oral administration, some auxiliary solvent, such as dimethyl sulfoxide (DMSO) and propylene glycol were tried to add



**Figure 2.** Representative chromatograms of blank plasma (a), plasma spiked with vitexin and hesperidin (b), plasma sample at 10min after intravenous administration of vitexin at dose of 10 mg/kg (c) and plasma sample at 20 min after oral administration of vitexin at dose of 30 mg/kg (d). Peak 1: vitexin; Peak 2: hesperidin.

**Table 1.** Precision and accuracy of vitexin determination in rat plasma (intra-day: n = 5; inter-day: n = 3 days with 5 replicates per day).

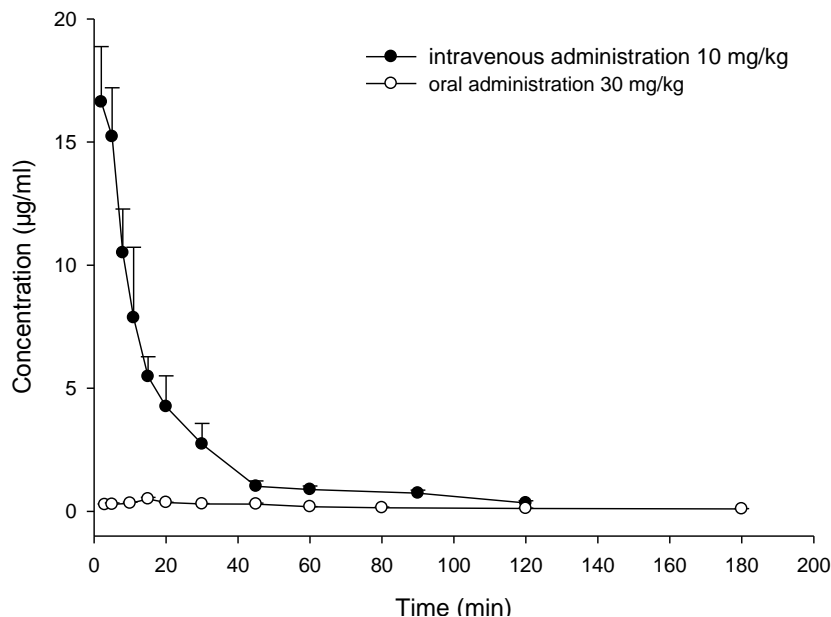
Added Conc. (µg/ml)	Intra-day			Inter-day			Recovery (%)	RSD (%)
	Conc. (µg/ml) (mean ± SD)	RSD (%)	RE (%)	Conc. (µg/ml) (mean ± SD)	RSD (%)	RE (%)		
0.3	0.32 ± 0.124	7.4	6.9	0.32 ± 0.119	6.3	5.9	94.68±0.132	3.3
1.5	1.40 ± 0.110	7.9	-6.9	1.46 ± 0.107	7.3	-2.6	83.83±0.157	6.8
16	15.11 ± 0.720	4.8	-5.9	15.41 ± 0.781	5.1	-3.8	90.45±0.320	3.5

in because of the poor solubility of vitexin in water. When DMSO > 1% (Ma et al., 2010), it presents toxicity for animals, and different concentration of propylene glycol were therefore selected, 20% of propylene glycol with good solubility for vitexin was finally chosen as the auxiliary solvent.

To obtain suitable retention time and good separation for the analysis, the mobile phase was chosen after several trials in various proportions with methanol-water (30:70-45:55), methanol-acetonitrile-water (15:15:70-30:10:60). In addition, to improve the peak shape, 0.1-0.5% formic acid were added. however, only when 0.3% formic acid was applied to the mobile phase, a good peak shape and good separation were obtained, and thus the mobile phase consisting of methanol-acetonitrile-water containing 0.3% formic acid (30:10:60, v/v/v) was chosen in our study.

The UV absorption spectrum of vitexin has two maximum absorptions at 269 and 331 nm, and hesperidin at 204 and 284 nm. The interferences from endogenous substances in the plasma were observed when the wavelength was set at 269 nm, especially for low concentration samples, and the peak area of hesperidin was weak at 269 nm, which is not conducive to the determination of vitexin. Considering the above factors, 330 nm was chosen as the detection wavelength and suitable for the analysis of both of them.

To simultaneously acquire high extraction recovery and precision of vitexin and hesperidin, acetonitrile was selected as the precipitant after several extraction solvents including methanol, acetonitrile, in different ratios being tried to precipitate the protein. In addition, 10, 20, 30 µl of acetic acid were respectively tried and added in the plasma to avoid the dissociation of polyphenols.



**Figure 3.** Plasma concentration-time profile of vitexin after intravenous (10 mg/kg) and oral administration (30 mg/kg).

**Table 2.** Pharmacokinetic parameters of vitexin in rats (mean  $\pm$  SD, n = 5) after intravenous administration and oral administration at a dose of 10 and 30 mg/kg, respectively.

Parameters	Route of administration	
	Intravenous (10 mg/kg)	Oral (30 mg/kg)
$C_{max}$	16.61 $\pm$ 2.32	0.51 $\pm$ 0.015
$T_{max}$ (min)	—	15.82 $\pm$ 0.172
$V_c$ (L/kg)	0.47 $\pm$ 0.106	0.62 $\pm$ 0.16
$t_{1/2\alpha}$ (min)	6.78 $\pm$ 0.771	—
$t_{1/2\beta}$ (min)	46.01 $\pm$ 0.810	—
$t_{1/2}(k_a)$ (min)	—	3.68 $\pm$ 0.085
$t_{1/2}(k_e)$ (min)	—	59.81 $\pm$ 2.31
$^aAUC_{0 \rightarrow \infty}$ ( $\mu\text{g} \cdot \text{min}/\text{ml}$ )	327.11 $\pm$ 26.6	42.70 $\pm$ 6.35
$^aCL$ (L/kg $\cdot$ min)	0.031 $\pm$ 0.035	0.71 $\pm$ 0.16
$MRT_{0 \rightarrow t}$ (min)	26.23 $\pm$ 1.51	60.42 $\pm$ 5.41
$MRT_{0 \rightarrow \infty}$ (min)	32.30 $\pm$ 2.92	127.3 $\pm$ 6.59
$^bAUC_{0 \rightarrow t}$ ( $\mu\text{g} \cdot \text{min}/\text{ml}$ )	324.21 $\pm$ 26.1	35.38 $\pm$ 3.56
$^bAUC_{0 \rightarrow \infty}$ ( $\mu\text{g} \cdot \text{min}/\text{ml}$ )	335.61 $\pm$ 32.4	49.34 $\pm$ 3.32
F(%)	—	4.91 $\pm$ 0.761

<sup>a</sup> and <sup>b</sup> were the compartmental and non-compartmental approach, respectively.

Eventually, 20  $\mu\text{l}$  of acetic acid was added in the plasma.

#### Pharmacokinetics and bioavailability of vitexin in rats

The pharmacokinetics relative parameters were

calculated by both compartmental and non-compartmental approach. Comparing the parameters of AIC and  $R^2$ , a two-compartment open model (weight=1/ $C^2$ ) is best fit to intravenous administration and a one-compartment open model (weight=1) is best fit to oral administration, respectively.

VIT is rapidly removed from the blood by the obtained values for the total body clearance ( $0.0298 \pm 0.23 \text{ L}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ), the  $\text{MRT}_{0\rightarrow t}$  ( $26.17 \pm 1.53 \text{ min}$ ) after intravenous administered to rats. And the pharmacokinetic parameters  $t_{1/2\beta}$  ( $46.0 \pm 0.81 \text{ min}$ ),  $V_c$  ( $0.47 \pm 0.06 \text{ L}\cdot\text{kg}^{-1}$ ) and CL ( $0.0306 \pm 0.035 \text{ L}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ) is similar from the previous reference,  $t_{1/2\beta}$  ( $43.53 \pm 4.23 \text{ min}$ ),  $V_c$  ( $0.62 \pm 0.16 \text{ L}\cdot\text{kg}^{-1}$ ) and CL ( $0.011 \pm 0.005 \text{ L}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ) (Tong and Liu, 2007). When administered orally to the rats, the plasma concentration was very low,  $C_{\text{max}}$  ( $0.413 \pm 0.015 \mu\text{g/ml}$ ). Vitexin was rapidly absorbed with peak concentrations occurring at around  $15.82 \pm 0.172 \text{ min}$ , and rapidly eliminated from plasma with  $t_{1/2}$  ( $59.81 \pm 2.31 \text{ min}$ ),  $\text{MRT}_{0\rightarrow t}$  ( $60.42 \pm 5.41 \text{ min}$ ), CL ( $0.71 \pm 0.156 \text{ L/kg}\cdot\text{min}$ ).

The absolute bioavailability calculated *via* the formula of  $(\text{AUC}_{\text{oral}}/\text{AUC}_{\text{i.v.}}) \times (\text{dose}_{\text{i.v.}}/\text{dose}_{\text{oral}})$  was very low, only 4.91%, indicating that vitexin exhibits a prominent first-pass effect, corresponding to the report of the flavonoids having poor bioavailability and the native structure of flavonoids, many common metabolites or biotransformation products (the methyl, sulfate or glucuronide conjugates) appeared by the action of enzyme in liver and small intestine (Hu, 2007; Chen et al., 2005; Zhang et al.; 2006). Taken together, the results of the pharmacokinetics and bioavailability of pure vitexin in rats were first investigated, which will provide the reliable scientific data for its clinic application, and the reason leading to the lower bioavailability of vitexin will be investigated in further studies.

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