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Short communication

HPLC study of pharmacokinetics and tissue distribution of morroniside in rats

Xiaona Li [a,b], Qiao Wang [a], Xiaowei Zhang [c], Xiaona Sheng [c], Yanan Zhou [a], Min Li [a], Xiujuan Jing [a], Deqiang Li [a], Lantong Zhang [a,*]

[a] *Department of Pharmaceutical Analysis, School of Pharmacy, Hebei Medical University, Shijiazhuang 050017, PR China*
[b] *Department of Pharmacy, North China Coal Medical College, Tangshan 063000, PR China*
[c] *The Second Affiliated Hospital of Hebei Medical University, Shijiazhuang 050000, PR China*

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Abstract

Morroniside is an important constituent of traditional Chinese medicines Fructus Corni with several bioactivities. An HPLC method for determination of morroniside in rat plasma and tissues was developed and the pharmacokinetic and tissue distribution characteristics of morroniside after intravenous and oral administrations were investigated. The bio-samples were prepared based on a simple protein precipitation and the separation of morroniside was achieved on a C_{18} column with a mobile phase consisted of acetonitrile–methanol–0.1% formic acid (10:10:80, v/v) at a flow rate of 1.0 ml/min. Chromatograms were monitored at 239 nm and the temperature of column was kept at 25 °C. Pharmacokinetic study found that morroniside was absorbed and eliminated rapidly in rat and manifested linear dynamics at 10–40 mg/kg range and absolute bioavailability of morroniside was lower. Tissue distribution showed the highest level was observed in small intestine, then in kidney and stomach, but no morroniside was detected in brain, which indicated that small intestine, kidney and stomach were major distribution tissues of morroniside in rats, and morroniside had difficulty in crossing the blood-brain barrier. It was also found there was no long-term accumulation of morroniside in rat tissues.

Keywords: Fructus Corni; Morroniside; Pharmacokinetics; Tissues distribution; HPLC

1. Introduction

Fructus Corni has been used for thousands of years as an important folk medicine in China, and it is also considered as one of 25 vegetable drugs that were most frequently applied in PR China, Japan and Republic of Korea. It exhibits a number of biological activities, including immunological regulation, reducing blood glucose, antishock, antiarrhythmia and antibiosis [1,2]. It was reported that the total iridoid glycosides is the main active constituents of Fructus Corni. Morroniside is one of the most abundant iridoid glycoside and it has been proved to be effective in invigorating stomach and preventing diabetic angiopathies [3,4]. Its chemical structure is shown in Fig. 1.

There has been active interesting in recent years in developing and optimizing analytical methods for determination of morroniside in plant extracts, for example, high performance liquid chromatography (HPLC) with UV detection [5] and micellar electrokinetic capillary chromatography [6]. With the growing significance of a potential beneficial role of morroniside in human health, there is an increasing demand for analyzing it in vivo and researching on its pharmacokinetics. But few reports had addressed the quantification of morroniside in biological samples. Only in our previous work, pharmacokinetics of loganin and morroniside in mouse was studied with an HPLC method after intravenous administration of Fructus Corni extract [7]. And up to now, there was no research on the pharmacokinetics and tissues distribution of morroniside after oral administration of pure morroniside. Therefore, it is necessary for an intensive investigation on pharmacokinetics of pure morroniside.

To achieve this aim, we developed and validated a highly specific and rapid RP–HPLC method for determination of morroniside in bio-samples and applied it for the study of its

* Corresponding author. Tel.: +86 311 86266419; fax: +86 311 86052053.
E-mail address: zhanglantong@263.net (L. Zhang).

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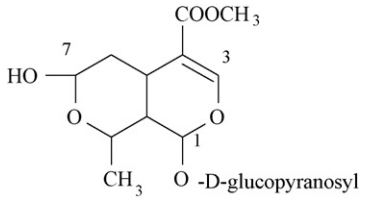


Fig. 1. Chemical structure of morroniside.

pharmacokinetic characterization after single oral administration of pure morroniside to healthy rats in the present report.

2. Experimental

2.1. Materials and reagents

The reference standard of morroniside was extracted and purified from Fructus Corni by School of Pharmacy, Hebei Medical University (Shijiazhuang, China). Its chemical structure was confirmed by ^{1}H and ^{13}C-nuclear magnetic resonance spectroscopy and its purity was over 99% by HPLC analysis. Morroniside purity administrated to rats was over 95%. Acetonitrile and methanol were of HPLC grade and obtained from Tedia (Tedia, Fairfield, USA). The distilled water was prepared from demineralized water and used throughout the study.

2.2. HPLC conditions

The essential parts of the HPLC system consisted of a Model Waters 515 pump, a Model Waters 2487 dual λ absorbance detector, Waters 2996 photodiode array detector (Waters Assoc., Milford, MA, USA). Data was collected by a HPLC chromatography workstation (Intelligence and Information Institute of Zhejiang University).

The analytic column was a Diamonsil C_{18} reversed-phase column (5 μm, 250 mm × 4.6 mm). The separation was carried out with the mobile phase consisting of acetonitrile–methanol–0.1% formic acid (10:10:80, v/v) at a flow rate of 1.0 ml/min. Chromatograms were monitored at 239 nm and the temperature of column was kept at 25 °C.

2.3. Animals, drug administration and sampling

Male Sprague–Dawley rats (350 ± 20 g) were obtained from the Hebei Laboratory Animal Center (Shijiazhuang, People's Republic of China). They were kept in an environmentally controlled breeding room for 5 days before starting the experiments and fed with standard laboratory food and water ad libitum. All rats were dosed following an overnight fast (except for water).

For pharmacokinetic study, 20 rats were randomly assigned to four groups. Each group contained five rats. Water solution of morroniside (concentrations of morroniside were 2, 4 and 8 mg/ml, respectively) was administered orally at a dose of 10, 20 and 40 mg/kg to groups 1, 2 and 3, respectively, then 0.3 ml blood samples were obtained from fossa orbitalis vein according to the specific schedule, 0, 5, 10, 15, 30, 45, 60, 90, 120, 180, 240 and 300 min. Water solution of morroniside (3 mg/ml) was administered intravenously (by vena caudalis) at a dose of 5 mg/kg to group 4 and the designated time points were at 0, 2, 7, 15, 30, 60, 120 and 240 min. Then blood samples were put into heparinized micro-centrifuge tubes and followed by centrifuging at approximately 9000 × *g* for 10 min. The resulting plasma layers were separated and stored in micro-centrifuge tubes at −20 °C until analysis performed with the procedure described below.

For study of tissue distribution, 25 rats were randomly assigned to five groups. Each group contained five rats. After water solution of morroniside (4 mg/ml) was administered orally at a dose of 20 mg/kg to each group, heart, liver, lung, spleen, kidney, brain, stomach and small intestine samples were collected at 15, 30, 60, 120 and 300 min, respectively. Tissue samples were weighed rapidly and put into normal saline solution to remove the blood or content, blotted on filter paper, and then weighed for wet weight and homogenized in saline solution (600 mg/ml). The obtained tissue homogenates were centrifuged at approximately 9000 × *g* for 10 min and stored at −20 °C until analysis, performed using the procedure described below.

2.4. Preparation of standard and quality control samples

Stock solution of morroniside was prepared in methanol to give a final concentration of 505 μg/ml. A series of standard solutions with concentration in the range of 0.0808–20.2 μg/ml was obtained by further dilution of the stock solution with methanol. All the solutions were stored at −20 °C and were brought to room temperature before use.

To prepare the standard calibration samples, 50 μl of standard solutions and 250 μl of methanol were added to 100 μl of blank plasma or tissues. The mixture was then treated following sample extraction procedure described below. The final standard plasma and tissues concentrations were 0.0404–10.1 μg/ml and 0.101–10.1 μg/ml for morroniside. The quality control (QC) samples, which were used in the validation, were prepared in the same way as the standard calibration samples.

2.5. Sample processing

To 100 μl of plasma or tissues samples, 300 μl of methanol were added. The resulting solution was thoroughly vortex-mixed for 10 s. After centrifugation at 9000 × *g* for 10 min, 20 μl of the supernatant were injected into the HPLC system for analysis.

2.6. Pharmacokinetic analysis

Non-compartmental pharmacokinetic analysis of concentration-time data was performed using Excel software. The pharmacokinetic parameters, such as maximum plasma concentration (C_{max}) and time of maximum concentration (T_{max}), were obtained directly from the plasma concentration-time plots. The elimination rate constants (k) were determined by linear regression on the logarithmic transformation of the last four data points of the curve. The elimination half-life ($T_{1/2}$) was calculated by the following equation: $T_{1/2} = 0.693/k$. The area under the plasma concentration versus time curve up to the last time (t) (AUC_{0-t})

was determined using the trapezoidal rule. The $AUC_{0-\infty}$ values were calculated by adding the value of $C_t \times k^{-1}$ to AUC_{0-t}.

Absolute bioavailability (F_{abs}) was calculated as $(AUC_{po} \times D_{iv})/(AUC_{iv} \times D_{po}) \times 100\%$, where po and iv, respectively, express oral and intravenous administration, and D was dosage of administration.

Statistical analysis of pharmacokinetic parameters was calculated with the SAS version 6.12 (SAS Institute Inc., Cary, NC, USA).

3. Results and discussion

3.1. Preparation of plasma and tissue samples

Morroniside is one of the iridoid glycosides with higher polarity. It is difficult to achieve good extraction recovery from plasma samples by liquid–liquid extraction. It was proved in our study that both ethyl acetate and chloroform as extraction solvents only gave the recoveries below 50%. Hence protein precipitation prior to solvent extraction was chosen as a simple and effective sample pretreatment method. Different protein precipitant such as methanol, acetonitrile, perchloric acid and trichloracetic acid were compared by detection the interference peaks and methanol was selected as the best protein precipitant.

3.2. Method validation

3.2.1. Specificity

The degree of interference by endogenous substances was assessed by inspection of chromatograms derived from processed blank and rat samples. Fig. 2 presents typical chromatograms of blank plasma and liver, blank plasma and liver spiked with morroniside, and rat plasma and liver sample after oral administration of morroniside. It was indicated that a good separation was obtained under the described condition and morroniside was eluted at 8.0 min approximately. No interfering peaks were found at the retention time of morroniside.

3.2.2. Linearity of calibration curve and lower limit of quantification

The standard curves of the peak area (Y) to the concentration (C) were constructed using $1/x^2$ weighted linear least-aquares regression model. The standard curves, correlation coefficients and linear ranges of morroniside in plasma and each tissue are listed in Table 1.

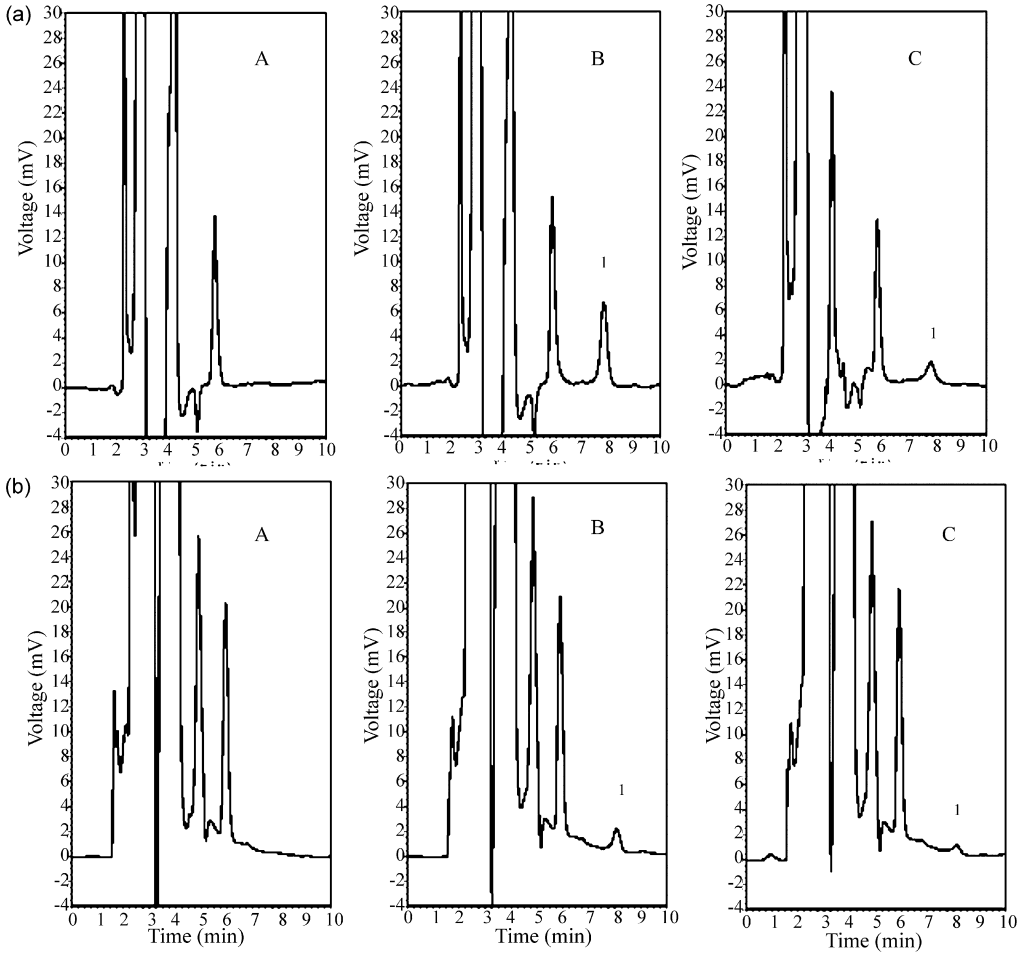


Fig. 2. Chromatograms of morroniside in rat plasma (a) and liver (b). (A) Blank plasma or liver; (B) blank plasma or liver spiked with morroniside; (C) plasma or liver samples after oral administration morroniside; (1) morroniside.

Table 1
Standard curves, correlation coefficients and linear ranges of morroniside in tissue samples

Tissues	Standard curves	Correlation coefficients	Linear ranges (μg/ml)
Plasma	$Y = 29843.20C + 2347.35$	0.9961	0.0404–10.1
Heart	$Y = 29476.88C + 141.28$	0.9974	0.101–10.1
Liver	$Y = 30202.44C + 968.37$	0.9949	0.101–10.1
Lung	$Y = 29710.27C + 2082.46$	0.9958	0.101–10.1
Spleen	$Y = 29962.77C + 155.76$	0.9969	0.101–10.1
Kidney	$Y = 30843.12C + 8070.79$	0.9957	0.101–10.1
Brain	$Y = 32049.36C + 1483.38$	0.9963	0.101–10.1
Stomach	$Y = 37067.87C + 650.77$	0.9946	0.101–10.1
Small intestine	$Y = 29266.03C–875.92$	0.9960	0.101–10.1

The lower limit of quantification (LLOQ) was defined as the lowest concentration at which both precision and accuracy were less than or equal to 20%. The current assay offered an LLOQ of 0.0404 μg/ml in plasma and 0.101 μg/ml in tissues ($n = 6$), respectively (Table 2). The limits were sufficient for studies of pharmacokinetics and tissue distribution following a single oral administration of morroniside.

The LLOQ of the method was relatively high (0.66 μg/ml) in our previous work, so it was failed to study pharmacokinetics of morroniside after oral administration in mouse [7]. By optimization of the chromatographic condition, the present method, whose the LLOQ was 0.0404 μg/ml in plasma and the mobile phase was modified from acetonitrile–methanol–0.2% formic acid (12:8:80) to acetonitrile–methanol–0.1% formic acid (10:10:80), was presented and validated for the determination of morroniside in rat plasma and tissues.

3.2.3. Precision and accuracy

Accuracy, intra- and inter-day precisions were evaluated from the results of QC samples. Six replicates of QC samples at three concentration levels were determined on three different days. The mean values and R.S.D. for QC samples were calculated over three validation days. These data were then calculated by using a one-way analysis of variance (ANOVA). The intra- and inter-day precisions were expressed by relative standard deviation (R.S.D.). The accuracy of the method was determined by calculating the percentage deviation observed in the analysis of QC samples and expressed as the relative error (R.E.). The results are shown in Table 2. It was suggested that the method was accurate and reproducible for the determination of morroniside in rat plasma and tissues.

Table 3
Stability of samples of morroniside in rat plasma and liver ($n = 3$)

Statistical variable	Theoretical concentration (μg/ml)			
	Plasma		Liver	
	0.101	8.08	0.202	8.08
Three freeze and thaw cycles				
Mean (μg/ml)	0.095	8.19	0.182	7.91
CV (%)	13.1	1.7	10.3	0.9
R.E. (%)	−6.1	1.4	−9.8	−2.1
Plasma stored at room temperature for 4 h				
Mean (μg/ml)	0.104	8.33	0.180	8.00
CV (%)	9.5	0.40	9.7	3.9
R.E. (%)	2.6	3.1	−10.7	−0.93
Pretreated rat plasma stored at room temperature for 24 h				
Mean (μg/ml)	0.098	8.255	0.194	7.38
CV (%)	8.2	2.3	2.9	5.1
R.E. (%)	−3.3	2.2	−4.1	−8.7

3.2.4. Recovery and stability

The recoveries of morroniside were tested at three QC levels by comparing the peak areas from extracted plasma or liver samples with those found by direct injection of standard solutions at the same concentration. The results showed that the mean recoveries of analyte in plasma were 99.9, 100.4 and 101.8% ($n = 6$) and R.S.D. were 10.9, 6.0 and 3.3% at concentrations of 0.101, 1.01 and 8.08 μg/ml, respectively. The mean recoveries of analyte in liver were 88.8, 100.2 and 100.0% ($n = 6$) and R.S.D. were 8.0, 5.3 and 2.7% at concentrations of 0.202, 1.01 and 8.08 μg/ml, respectively.

The stability of morroniside was investigated under a variety of storage and process conditions. The analyte was found to be stable (R.E. within ±15%) after three cycles of freeze (−20 °C) and thaw (room temperature) in rat plasmas or liver. The analyte was also shown to be stable in the reconstituted solution for 24 h at room temperature (R.E. within ±15%) and in rat plasmas or liver at room temperature for at least 4 h (R.E. within ±15%). No signs of degradation were found under the freeze condition (−20 °C) for 3 days. Results of stability are shown in Table 3.

Table 2
Intra-day and inter-day variability for the assay of morroniside in rat plasma and liver

		Intra-batch ($n = 6$)				Inter-batch ($n = 18$)		
Plasma	QC (μg/ml)	0.0404	0.101	1.01	8.08	0.101	1.01	8.08
	Mean (μg/ml)	0.0393	0.102	1.03	8.02	0.101	1.02	8.18
	CV (%)	3.2	12.5	2.2	0.82	1.2	1.5	4.3
	R.E. (%)	−2.6	0.62	1.5	−0.78	0.08	1.1	1.2
Liver	QC (μg/ml)	0.101	0.202	1.01	8.08	0.202	1.01	8.08
	Mean (μg/ml)	0.101	0.179	1.01	8.08	0.178	1.02	7.93
	CV (%)	11.2	8.0	5.3	2.7	14.7	2.0	4.9
	R.E. (%)	0.39	−11.2	0.23	0.022	−11.9	1.2	−1.8

Table 4
Pharmacokinetic parameters of morroniside in rat plasma after oral and intravenous administration of morroniside at different dosages ($n = 5$)

Pharmacokinetic parameters	Doses			
	10 mg/kg	20 mg/kg	40 mg/kg	5 mg/kg
C_{max} (μg/ml) (mean ± S.D.)	0.766 ± 0.085[a]	1.292 ± 0.346[a]	1.481 ± 0.268[a]	36.54 ± 4.33[b]
T_{max} (min) (mean ± S.D.)	54 ± 8.2	60 ± 0	60 ± 0	
$T_{1/2}$ (min) (mean ± S.D.)	69.4 ± 14.1	103.9 ± 23.4	90.2 ± 32.6	40.2 ± 8.3
k (1/min) (mean ± S.D.)	0.0103 ± 0.0021	0.0070 ± 0.0016	0.0086 ± 0.0032	0.0179 ± 0.0039
AUC_{0-t} (μg min/ml) (mean ± S.D.)	102.81 ± 17.7[a]	166.04 ± 30.42[a]	193.36 ± 45.56[a]	772.17 ± 101.79
$AUC_{0-\infty}$ (μg min/ml) (mean ± S.D.)	108.52 ± 22.21[a]	190.02 ± 29.60[a]	224.14 ± 78[a]	779.33 ± 104.23

[a] There was extraordinary significant correlation ($p < 0.01$) in analysis of variance at different dosage.
[b] Concentration of morroniside at 2 min.

Table 5
Distribution of morroniside in tissues after administration of 20 mg/kg morroniside to rats ($n = 5$)

Tissues	15 min (μg/ml)	30 min (μg/ml)	60 min (μg/ml)	120 min (μg/ml)	300 min (μg/ml)
Heart	0.101 ± 0.072	0.124 ± 0.039	0.064 ± 0.057[a]	0.046 ± 0.065[a]	ND
Liver	0.073 ± 0.104	0.258 ± 0.155	0.129 ± 0.046	0.232 ± 0.205	ND
Lung	0.052 ± 0.034[a]	0.099 ± 0.085[a]	0.165 ± 0.081	0.085 ± 0.082[a]	ND
Spleen	0.029 ± 0.064[a]	0.092 ± 0.061[a]	0.054 ± 0.084[a]	0.073 ± 0.068[a]	ND
Kidney	0.212 ± 0.153	0.489 ± 0.372	2.444 ± 1.51	0.609 ± 0.396	ND
Brain	ND	ND	ND	ND	ND
Stomach	6.573 ± 1.504	2.636 ± 1.434	0.348 ± 0.296	0.155 ± 0.043	ND
Small intestine	29.895 ± 7.898	10.822 ± 5.044	2.941 ± 1.514	0.306 ± 0.086	ND
Plasma	0.559 ± 0.049	0.769 ± 0.144	1.292 ± 0.346	0.541 ± 0.105	0.154 ± 0.045

ND: undetectable.
[a] Below limit of quantification.

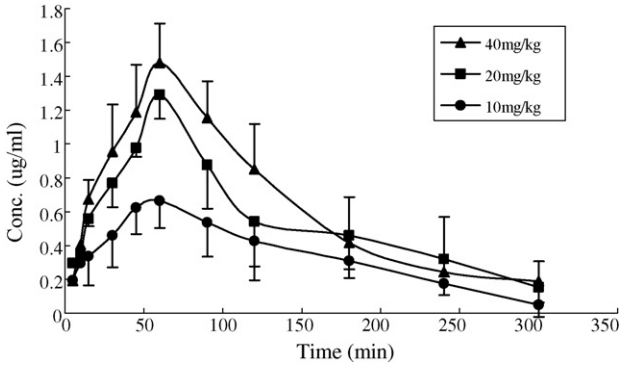


Fig. 3. Mean plasma concentration-time curves of morroniside after an oral administration of 10, 20 and 40 mg/kg morroniside.

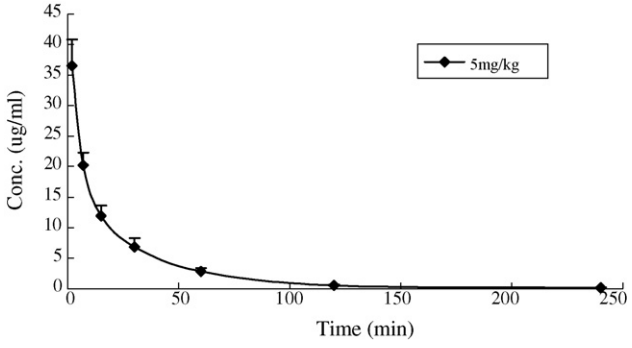
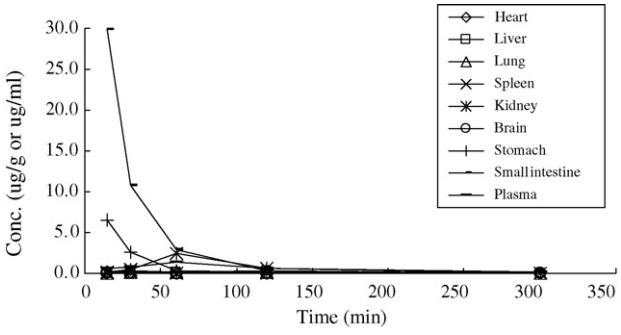


Fig. 4. Mean plasma concentration-time curve of morroniside after intravenous administration of 5 mg/kg morroniside.



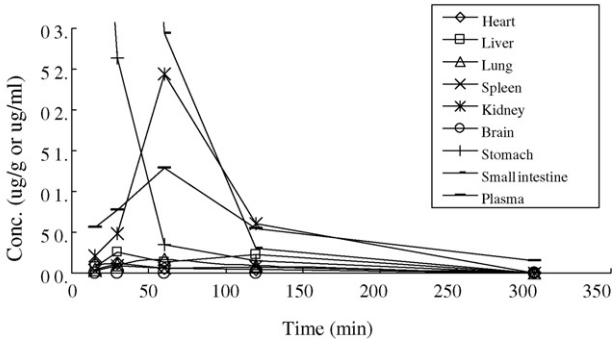


Fig. 5. Tissues and plasma concentration-time profiles of morroniside after oral administration of 20 mg/kg morroniside to rats ($n = 5$).

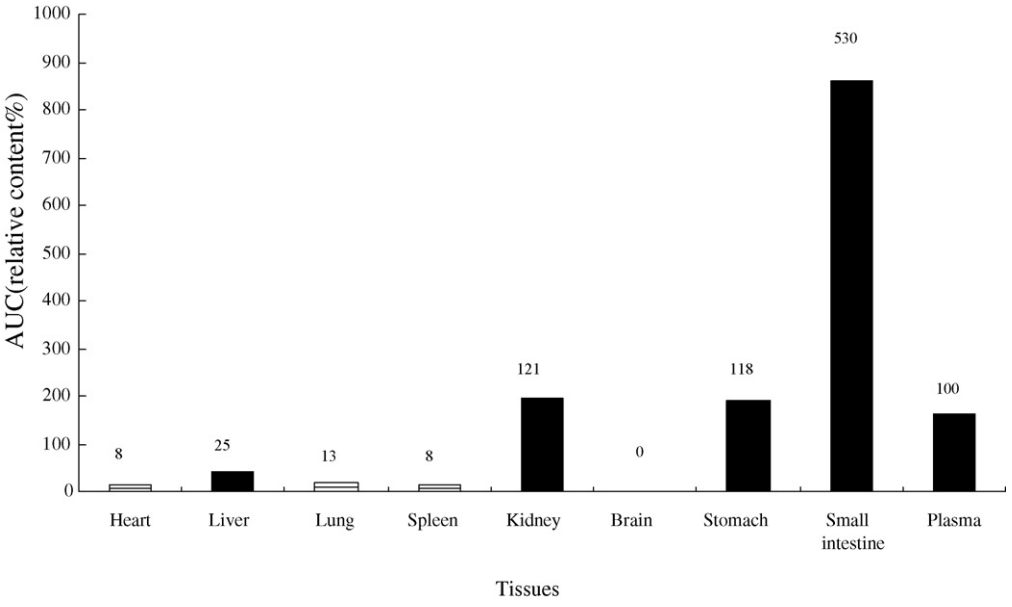


Fig. 6. AUC of morroniside tissues and plasma after oral administration of 20 mg/kg morroniside to rats ($n = 5$).

3.3. Application of analytical method in pharmacokinetic studies

The HPLC method was successfully applied to investigate the pharmacokinetics of morroniside after a single oral (10, 20 and 40 mg/kg) and intravenous (5 mg/kg) administration of morroniside. The corresponding pharmacokinetic parameters are listed in Table 4 and mean plasma concentration-time curves ($n = 5$) are presented in Figs. 3 and 4. In general, morroniside could be absorbed and eliminated rapidly in rat, for it was detected in plasma at 5 min, and T_{max} were found at 60 min approximately after orally administrated with different dose.

All statistic analysis was accomplished after logarithmic transformation of AUC_{0-t} and C_{max} of different oral dosages. Q test showed that there were significant difference in AUC_{0-t} and C_{max} between low and medium dosage, low and high dosage, and no significant difference was observed between medium and high dosage ($p > 0.05$). A good linear correlation ($p < 0.01$) was obtained in correlation and regression analysis of AUC_{0-t} -dosage and C_{max} - dosage plots, respectively. Rank sum test demonstrated that both $T_{1/2}$ and T_{max} were dose-independent. Then it could be concluded that morroniside displayed linear dynamics in dosage range of 10–40 mg/kg, and 40 mg/kg might be saturation dosage according to the results of Q test.

As shown in Fig. 4, the plasma levels of morroniside administrated intravenously were much higher than those after oral administration. The absolute oral bioavailability of morroniside was calculated to be 7.0, 6.1 and 3.6% for low, medium and high dosage, respectively. Two following reasons might be helpful to explain this fact. The one was all kinds of digestive enzymes produced by enteric microbial flora in rat digestive could convert morroniside. The other was from liver first pass effect, in other words, morroniside absorbed in blood would be metabolized by liver drug enzyme. However, exact cause is needed to be investigated further.

3.4. Application of analytical method in tissue distribution

As listed in Table 5, morroniside was detected at 15 min and undetected at 5 h in inspected tissues (except brain) after oral administration of morroniside, which indicated that morroniside could be distributed rapidly and no long-term accumulation of morroniside in tissues was proved.

Tissues and plasma concentration-time profiles of morroniside after oral administration of 20 mg/kg morroniside to rats are shown in Fig. 5. It indicated that content of morroniside in small intestine and stomach showed similar dynamic change, maximum concentration was at 15 min and then declined gradually, which might mainly be attributed to the oral administration. The peak levels in kidney and lung coincided with that in plasma, which implied that the distribution of morroniside in kidney and lung was depended on the blood flow or perfusion rate of the organ.

The AUC of morroniside in tissues and plasma after oral administration of 20 mg/kg morroniside to rats are displayed in Fig. 6. It could be seen that AUC of morroniside in tissues was in descending order of small intestine, kidney, stomach, plasma, liver, lung, spleen, heart and brain. The highest level was found in small intestine, the AUC was 530% of that in plasma, which also explained that morroniside had special affinity to small intestine except the reason of oral administration. There were all kinds of digestive enzymes and enzymes produced by enteric microbial flora in rat digestive tract, so morroniside might be converted to its metabolites by these enzymes after ingestion. The AUC in kidney which was 121% of that in plasma showed that kidney might be the primary excretion organ of prototype morroniside. The AUC value in stomach ranked the third being 118% of that in plasma, which made it necessary to further investigate whether it was concerned with the effectiveness of morroniside in invigorating stomach. Morroniside was undetected in brain. Perhaps its higher polarity resulted

in the difficulty for morroniside to cross the blood-brain barrier.

4. Conclusion

A rapid and specific RP–HPLC assay was presented and validated for the determination of morroniside in rat plasma and tissues. The method included a simple protein precipitation procedure only using 0.1 ml rat plasma or tissues homogenate. This is the first report by using HPLC to investigate pharmacokinetics and tissues distribution after oral administration of morroniside.

After a single oral administration of 10, 20 and 40 mg/kg morroniside to rats, morroniside was fast absorbed and eliminated in rat and manifested linear dynamics in dose range of 10–40 mg/kg. But absolute bioavailability of morroniside was lower, which suggested that morroniside should be prepared to suitable dosage form or given by fitting administration route.

The major distribution tissues of morroniside in rats were small intestine, kidney and stomach, but morroniside had difficulty in crossing the blood-brain barrier. There was no long-term accumulation of morroniside in rat tissues.

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