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Studies on Pharmacokinetics and Liver Microsome Metabolism of Nuciferine in Different Species of Animals

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Abstract: This study aimed to compare the characteristics of pharmacokinetics and liver microsome metabolism among different species of animals including rabbit, rat and mouse and healthy New Zealand white rabbits, SPF Sprague Dawley (SD) rats and ICR mice were given intraperitoneal injection of nuciferine at a dose of 50 mg per kg body weight. Blood samples were collected via caudal vein after administration and nuciferine concentrations in different points were determined by the method of HPLC-UV. Then the plasma concentration-time curves were drawn and the pharmacokinetics parameters were calculated by DAS 3.0 software. Besides, liver microsome from rabbit, rats and mice were prepared to study the metabolism of nuciferine *in vitro*. These results showed that nuciferine fitted two-compartment model in rabbits and rats and one-compartment model in mice. The calculated results of pharmacokinetics parameters showed that the fast absorption of nuciferine exhibited in rabbits, rats and mice, while the elimination was relatively slow. Compared with rabbits and rats, the nuciferine elimination was fastest in mice. The Km value of nuciferine metabolism *in vitro* was least in mice liver microsome, which suggested that the fastest metabolism of nuciferine existed in mice liver. Therefore, the pharmacokinetics characteristics of nuciferine in different species were significantly different, the elimination of nuciferine is the fastest in mice and the slowest in rabbits, which was in line with the *in vitro* metabolism trends by liver microsome from different species.

Keywords: Nuciferine, Pharmacokinetics, Liver Microsome Metabolism, Species Difference

Introduction

According to Chinese Pharmacopoeia, Lotus leaf (*Nelumbo Nucifera Gaern)* is a traditional Chinese medicine with a long history, which is mainly cultivated in southern China. The medicinal lotus leaf can be obtained by cleaning, cutting and drying (2015). Nuciferine is one of the main active ingredients in lotus leaves (Xu, 2021), which have good effects of reducing blood lipid (Ma *et al*., 2015), inhibiting hypercholesterolemia (You *et al*., 2014; Zhang *et al*., 2018), resisting cancer (Liu *et al*., 2015), inhibiting bacteria (Li and Xu, 2007) and anti-tumor effects (Li *et al*., 2019). Lotus leaf can be used as both food and medicine, which belongs to the category of "medicine and food homology" and is widely used. Wang *et al*. (2008) studied the pharmacokinetics of nuciferine in beagle dog plasma by reverse high performance liquid chromatography. The results showed that nuciferine distributed rapidly in vivo and eliminated quickly. Liu *et al*. (2010) confirmed that nuciferine was distributed and eliminated rapidly in rat plasma by tail vein injection (Gu *et al*., 2014). Studies have shown that the pharmacokinetic parameters of nuciferine in rats, such as $t_{1/2\beta}$ (elimination half-life) and t_{max} (peak time), are less often affected by the dose, while C_{max} (peak concentration of plasma drug) and AUC_{0-t} (area under the concentration-time curve) are directly proportional to the dose. However, there is no report on the differences of the pharmacokinetic characteristics of nuciferine in different species of animals.

Various active enzymes including CYP 450 are richest in liver, which is the main organ of drug metabolism. The most common model used for drug metabolism *in vitro* is the liver microsome and the metabolism characteristics of some drugs are usually evaluated in liver microsome *in vitro* (Ma *et al*., 2016; Xia *et al*., 2019). Certain study has shown that there is no significant species difference in the P450-catalyzed metabolism of nuciferine, while the

glucuronidation product was only detected in microsome from humans and rabbits, which suggested that significant differences exited in glucuronosyltransferase-catalyzed metabolism in different species animals (Lu *et al*., 2010). However, the published experiment is only conducted from the correlation of liver microsomal enzymes in animals of different genera. As for the species-specific differences of metabolic rates in liver microsome, there is no published report. Therefore, the pharmacokinetics and *in vitro* metabolism rate of nuciferine was compared among rabbits, rats and mice, which aimed to clarify the pharmacokinetic differences of nuciferine in different species of animals from *in vivo* pharmacokinetic characteristics to *in vitro* liver microsome metabolism.

In lotus leaf, flavonoid components have also been prepared and used to pharmaceutical study except for lotus leaf-alkaloids in some literature, which suggested that pharmacological activities of lotus leaf maybe result from synthetic action including flavonoid and alkaloid products. And now, lotus leaf has always been used as dietic tea or other Chinese patent drug in China. Therefore, in order to clarify the drug safety and effectiveness in human, it would be very significant to reveal the pharmacokinetics difference of nuciferine from different species animals. And these above studies should be helpful to extrapolate the effectiveness and safety from experimental animal to human.

Materials and Methods

Animals

SD rats (300±50 g) and ICR mice (20-40 g) were purchased from Shanghai Experimental Animal Co., Ltd., with animal license number SCXK (Shanghai) 2017-0005. The New Zealand white rabbits (2.6±0.5 kg) was purchased from Hangzhou Yuhang Kelian Rabbit Industry Professional Cooperative with license number SCXK (Zhejiang) 2017-0004. Before animal treatment, all animals were housed in our experimental animal center and provided with sterilized tap water and granule feed *ad libitum*. Room temperature was maintained at 25°C±2°C with a relative humidity of 65±15% and a day/light cycle of 12 h. And these animal experiments protocol was approved according to the agreement of Laboratory Animal Ethics Committee from China Jiliang University.

Reagents and Instruments

The standard product of nuciferine was purchased from Sichuan Weikeqi Biotechnology Co., Ltd. (purity ≥98%). Isoimperatorin was purchased from Shanghai Yuanye Biotechnology Co., Ltd. (purity ≥98%). Chromatographic acetonitrile was purchased from Shanghai Spectrum Chemical Co., Ltd. Chromatographic methanol was purchased from Shanghai Macklin Biochemical Technology Co., Ltd. Ethyl acetate was analytically pure and purchased from Hangzhou Gaojing Chemical Co., Ltd. The HPLC system used in this research was SHIMADZU-20AT series equipment, with a UV detector and a Zhida N 2000 instrument workstation written by Zhejiang University.

RP-HPLC Method

The HPLC system is shimadzu 20AT (Shimadzu, Japan), the chromatographic column is Discovery SUPELCO ODS C18 column (5 μm, 150 × 4.6 mm), the guard column is Agilent ODS C18 column (5 μm, 12.5 × 4.6 mm) and the mobile phase is composed of acetonitrile and 0.1% triethylamine (50: 50, v/v). The column temperature was kept at 35 ºC and the detector wavelength was set at 270 nm. The flow rate was 1.0 mL/min. Then 20 μL were injected directly into the RP-HPLC system.

200 μL of blank plasma or liver microsomal homogenate was added with 20 μL of isoimperatorin (100 μg/mL) and 20 μL of serial nuciferine standard solutions (1.0, 5.0, 10.0, 50.0, 100.0, 500.0 mg/L). The tube was vortex-mixed for 1 min, then 3 mL ethyl acetate was added. After centrifugation (5000 r/min) for 5 min, the organic phase was transferred to a glass test-tube. All the organic phase were evaporated to dryness at 45°C under a stream of nitrogen gas. The residue was reconstituted with 100 μL methanol, centrifuged again for 5 min (10000 r/min) and 20 μL of supernatant was inlet into HPLC system.

The Quality Control samples (QC) of nuciferine plasma and liver microsome were prepared at high, medium and low concentrations (50, 5 and 0.5 mg/L). The sample was treated by the method as the same above and five repeats were performed for each concentration. The extraction recovery at different concentrations was calculated by the ratio of the peak area in the treated sample to the peak area of nuciferine directly dissolved in chromatographic methanol. Intra-day and inter-day precisions were calculated according to the nuciferine areas from the same quality control samples and the Relative Standard Deviation (RSD, %) was calculated by comparing the peak area changes measured five times. At the same time, the peak area ratio of spiked samples was substituted into the standard curve equation and the nuciferine concentration was calculated. By comparison of the calculated concentrations of spiked samples with the added concentration, the accuracy (%) of the HPLC-UV method was obtained.

The Quality Control samples (QC) with concentrations of 0.5, 5.0 and 50.0 mg/L were also prepared and stored at room temperature, 4 and -20°C, respectively and all these samples were processed according to the above-mentioned method and the samples with each concentration were injected for 5 repeats. The detected peak area ratio of these samples was substituted into the standard curve equation and the ratio of the obtained concentration to the added concentration was calculated, which was used to characterize the nuciferine stability in plasma and liver microsome.

Sample Collection

The New Zealand white rabbits were fixed on a constant temperature holding table. After depilation, indwelling needles were placed in femoral vein. The quaternized chitosan nanoparticle solution of nuciferine was injected Intraperitoneal at a dose of 50 mg/kg body weight and blood samples were collected at 5, 10, 20, 30 and 45 min, 1, 2, 3, 6, 10 and 24 h after administration, respectively. Rats and mice were also intraperitoneally administered and blood was collected at the same points as above. And the plasma was centrifuged at 5000 r/min for 5 min, 200 μL plasma was absorbed and added into a blank tube with an addition of 20 μL isoimperatorin solution as internal standard. According to the prepared method above mentioned, plasma was arrayed and 20 μL supernatant was taken for analysis.

Liver Microsome Preparation

According to published literature (Feng *et al*., 2020), the preparation method was partly changed. The liver microsome were prepared by the use of 0.15 mol/L KCL-0.2 mol/L sucrose solution (pH7.5) and 0.05 mol/L Tris-0.25 mol/L sucrose solution (pH7.5), which were labeled as solution I and solution II, respectively. Animal livers were washed with solution I in order to remove blood attached to liver surface, absorbed water with filter paper and weighed. After cutting, liver tissue was homogenized with homogenizer in ice bath. Then the homogenate centrifuged for 20 mins (10 000 ×g, 4°C) and for 60 min (105 000 ×g, 4°C), respectively and the reddish precipitate in the lower layer was liver microsome. Then the microsome were resuspended with solution II and the protein concentration was measured according to BCA kit. The protein concentrations of liver microsome were 5.81, 34.54 and 22.12 mg/mL in rabbit, rat and mouse, respectively and these micro some were diluted to 1 mg/mL with 1×PBS for use.

In vitro Metabolism in Liver Microsome

The NADP reaction system consists of 1.0 mmol/L NADP, 100 mmol/L phosphate buffer and 4.0 mmol/L $MgCl_2$. The protein concentration of liver microsomes of the three animals was 1.0 mg/mL and the concentration of nuciferine standard solution is 12.5, 25, 50, 100, 200 and 400 μM in the reaction system, respectively. After the reaction was started, it was incubated at 37°C. After reaction for one hour, 200 μL frozen acetonitrile was added to stop the reaction. Then 20 μL isoimperatorin was added into the reaction system, which was treated by the method above mentioned and analyzed by HPLC. The Line Weaver-Burk equation was used to calculate kinetic values (Vm and Km).

Data Analysis

The results of all test datas were expressed by mean standard deviation and the differences were analyzed by SPSS 18.0 software. $P<0.05$ indicated significant differences.

Results

Chromatogram and Specificity Analysis

Under the selected chromatographic analysis conditions, nuciferine and impurities are well separated by comparing the chromatograms generated from standard solution samples (Fig. 1A), blank microsome (Fig. 1B), blank microsome with standard solution (Fig. 1C) and microsome metabolism (Fig. 1D). It suggests that nuciferine and the internal standard is operator in had no interference peaks at the corresponding retention times, which are at 13.727 and 24.257 min respectively.

Standard Curve

All the nuciferine concentrations were set at 0.1, 0.5, 1, 5, 10 and 50 mg/L respectively. After being treated according to method above, 20 μL sample was used for analysis.The regression equation of the curve and correlation coefficients (*r*) were calculated as followed:

$$Plasma: Y = 0.0115X + 0.0007\ (r = 0.9999); Liver\ microsome: Y = 0.0138X + 0.0023\ (r = 0.9998)$$

Validation of Nuciferine

The average recovery of nuciferine in plasma was higher than 90.0%, the intra-day and inter-day coefficients of variation were both lower than 10.0% and the accuracy was higher than 95.0%, which indicated that the recovery of nuciferine in plasma was good, with good precision and accuracy (Table 1), which could fully meet the requirements of this experiment. At the same time, 0.5, 5.0 and 50 mg/L nuciferine solution was selected and stored at room temperature, 4 and -20°C, respectively and the stability of nuciferine under different storage conditions was calculated. The results showed that nuciferine was stable in plasma and had significant metabolism in liver microsome (Table 2).

Pharmacokinetics of Nuciferine in Different Animals

According to the HPLC quantitative analysis method established in this experiment, the ratio of the peak area of nuciferine in plasma at different time points to the peak area of the internal standard isoimperatorin was substituted into the standard curve equation and the plasma concentration of nuciferine at different time points was calculated. According to the plasma collection time and nuciferine concentration of animals of different species, draw the plasma concentration-time curve, as shown in Fig. 2.

The pharmacokinetic parameters were calculated by pharmacokinetic software, which were listed in Table 3. The absorption half-life ($t_{1/2Ka}$) of nuciferine in three animals was 0.19 h in mice, 0.14 h in rabbits and 0.12 h in rats, which indicated that nuciferine absorbed rapidly in three animals. The elimination half-life ($t_{1/2\beta}$) of nuciferine in rabbit, rat and mouse plasma were 17.41,

7.44 and 2.16 h, respectively, indicating that nuciferine was eliminated fastest in mice. The AUC values of the area under the plasma concentration-time curve in different animals were 121.00, 39.06 and 3.97 $mg \cdot h \cdot L^{-1}$ and the AUC in rabbits was much higher than that in rats and mice, indicating that the relative dwelling of nuciferine in rabbit was highest during the administration time.

Table 1: Recovery, accuracy and precision of nuciferine in plasma and liver microsome (n = 5)

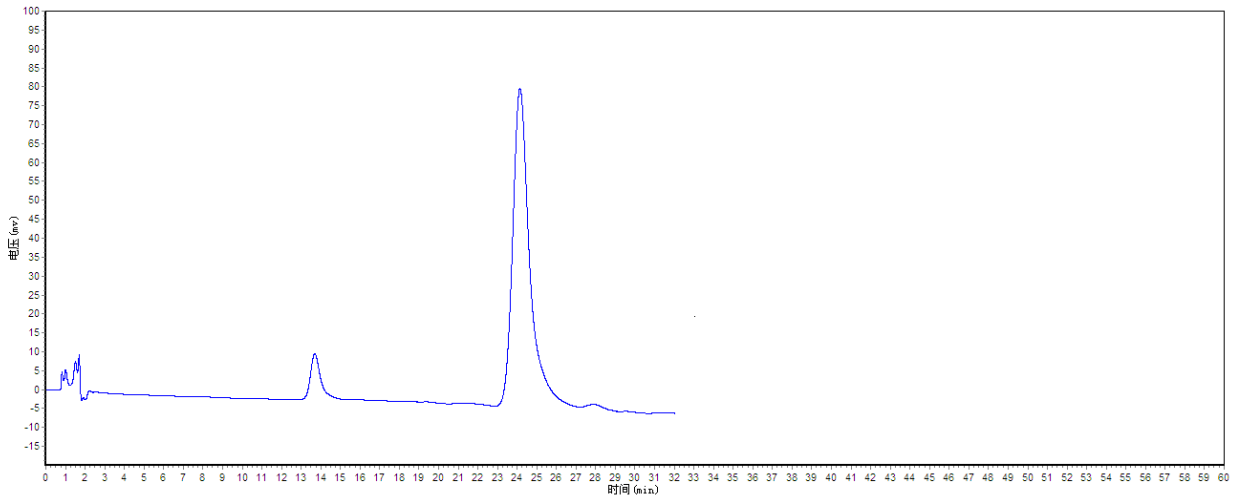
	Concentrations (mg/L)	Recovery (%)	Accuracy (%)	Precision (%) intra-day	inter-day
Plasma	0.5	96.90±1.26	97.31±5.38	4.34	6.74
	5.0	98.45±0.63	100.48±6.56	2.71	7.67
	50.0	99.12±0.66	98.71±4.66	2.22	6.67
Microsome	0.5	96.25±1.14	98.58±3.04	7.41	8.48
	5.0	98.69±0.89	98.36±6.17	3.69	8.25
	50.0	99.05±0.68	98.71±1.54	1.87	3.94

Table 2: Stability of nuciferine in plasma and liver microsome (n = 5)

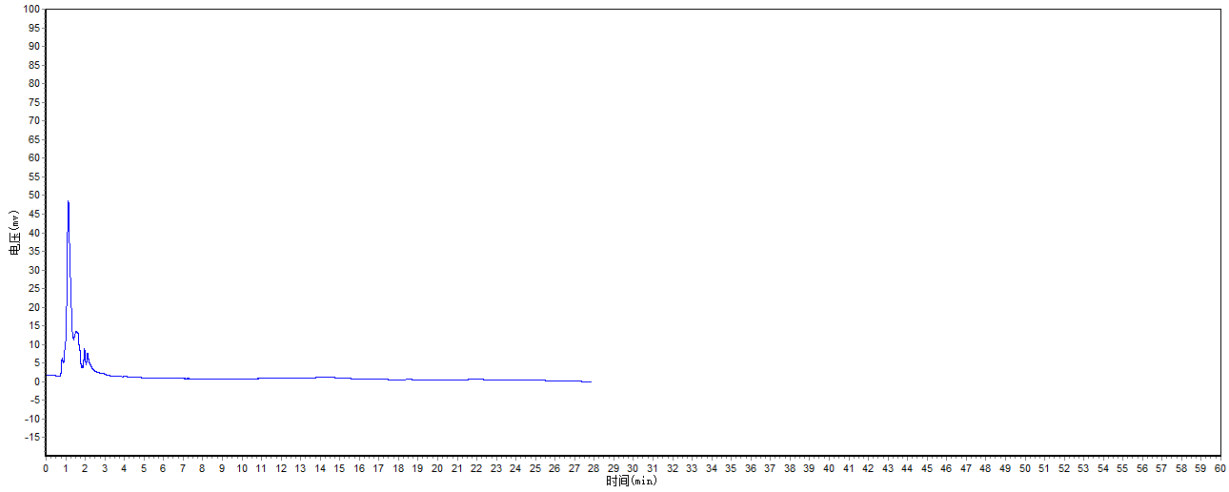
Concentrations (mg/L)	Storage conditions	Stability (%) Plasma	Microsome
0.5	Room temperature	96.16±1.72	70.91±5.82
	4℃	98.03±0.97	93.68±2.96
	-20℃	98.32±0.87	98.27±0.86
5.0	Room temperature	96.78±1.59	88.35±2.67
	4℃	98.08±1.27	97.88±1.38
	-20℃	98.67±1.6	98.60±1.13
50.0	Room temperature	98.66±0.67	89.19±6.73
	4℃	98.54±2.79	98.318±2.75
	-20℃	98.26±3.36	98.14±3.29

Table 3: Pharmacokinetics parameters of nuciferine in three animals (n = 5)

Parameters	Rabbits	Rats	Mice
$t_{1/2ka}$/h	0.14	0.12	0.19
$t_{1/2\alpha}$/h	0.21	0.15	/
$t_{1/2\beta}$/h	17.41	7.44	2.16
T_{max}/h	/	/	0.74
C_{max}/$mg \cdot L^{-1}$	/	/	1.00
AUC/$mg \cdot h \cdot L^{-1}$	121.00	39.06	3.97
$V_{d/F}$/$L \cdot kg^{-1}$	10.38	13.74	39.32
$CL_{T/F}$/$L \cdot min^{-1}$	0.41	1.28	12.60



(a)



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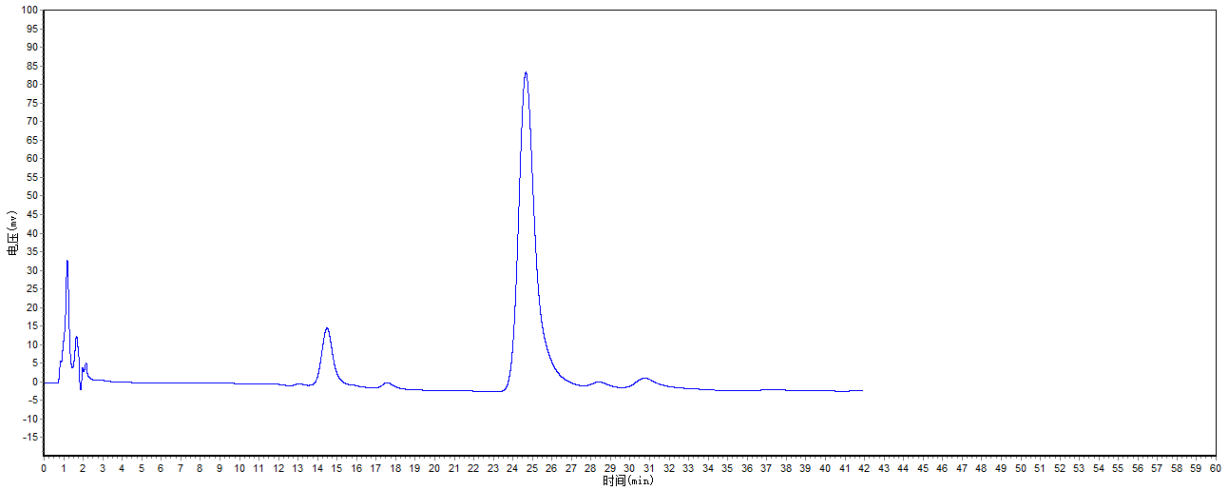
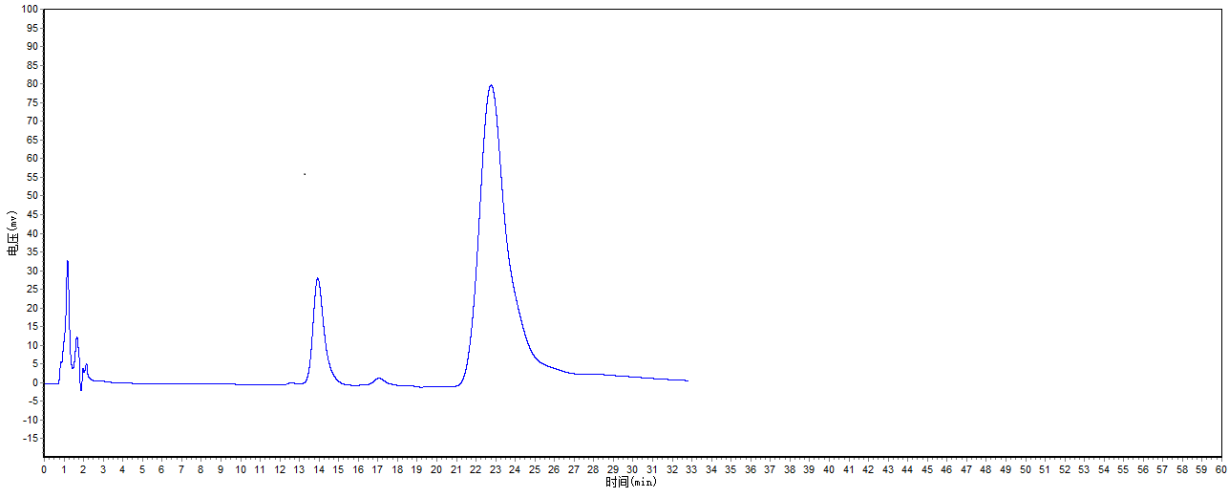
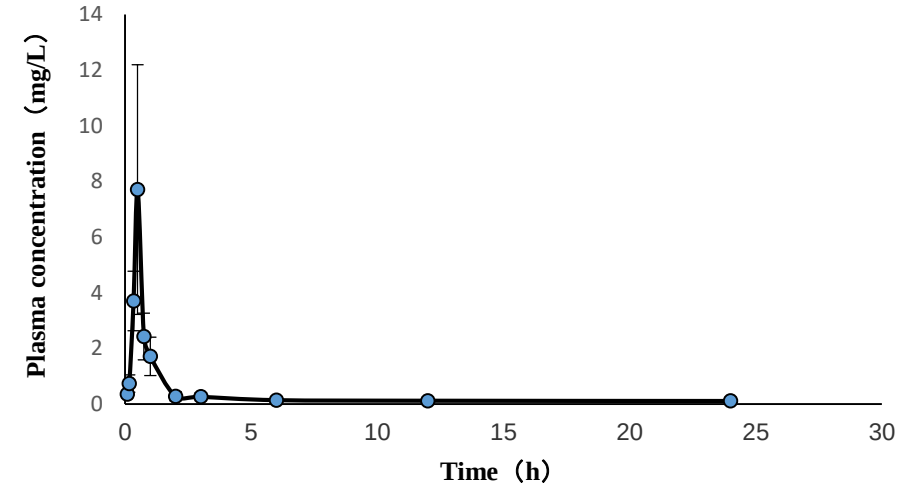


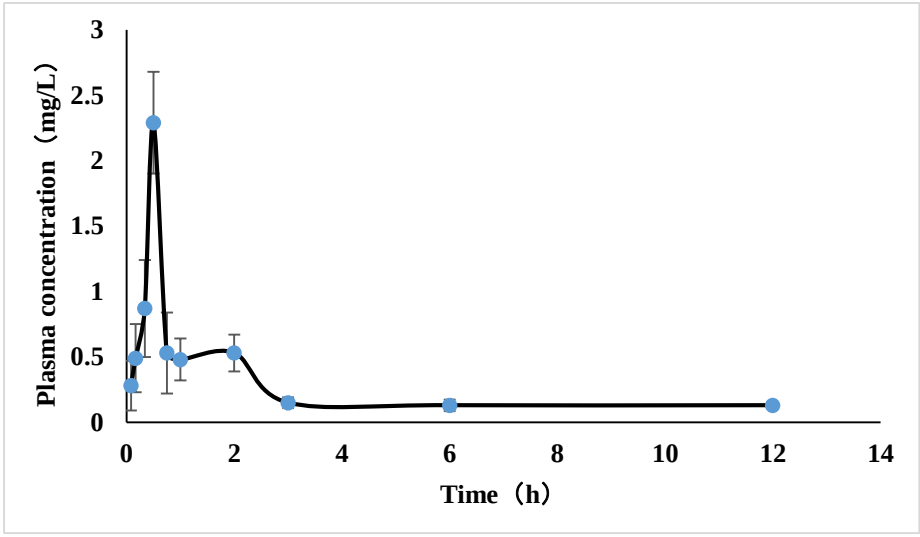
(c)



(d)

Fig. 1: Chromatograms of nuciferine generated from: (A) Standard chromatogram; (B) blank liver microsome; (C) blank microsome with standard solution of nuciferine; (D) samples from microsome metabolism. Peak 1: Nuciferine Peak 2: Internal Standard





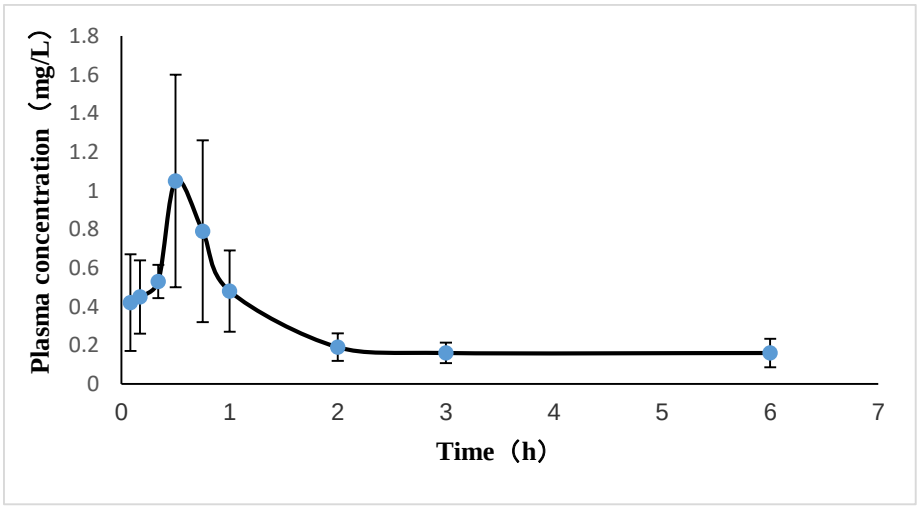


Fig. 2: Plasma concentration-time curve of nuciferine in rabbits, rats and mice A: Rabbits; B: Rats; C: Mice

Metabolic Characteristics of Nuciferine in Liver Microsome

The metabolic characteristics of nuciferine in liver microsome of different species were compared and the Vm values of nuciferine in liver microsome of rabbits, rats and mice were calculated as 1.20±0.61 mg/(h·L), 1.64±0.87 mg/(h·L) and 0.12±0.02 mg/(h·L), respectively. At the same time, the Km values of nuciferine were calculated to be 207.01±109.19 mg/L, 126.22±59.09 mg/L and 35.17±1.05 mg/L, respectively and the Km value of nuciferine in mouse liver microsome was the smallest, which indicated that nuciferine had strong binding action with metabolic enzymes in mouse liver microsome.

Discussion

According to the pharmacokinetics parameters of different animals, the absorption of nuciferine in three animals is fast and the absorption half-life is short. The result of elimination half-life was rabbit > rat > mouse and AUC values from high to low was rabbit > rat > mouse. Li *et al* has calculated the $t_{1/2\beta}$, Vd, CL and $AUC_{0\text{-inf}}$ in rats by intravenous injection, which were (1.73±0.58) h, (5.03±0.24) L/kg, (4.23±0.78) $L \cdot h^{-1} \cdot kg^{-1}$ and (2.35±0.46) $mg \cdot L^{-1} \cdot h$ respectively (Gu *et al*., 2014) and these were quite different from our experiment results, which may be due to the fact that the administration route in this study is intraperitoneal injection and the injection of nuciferine nanoparticles encapsulated by quaternized chitosan has significant slower release function.

After injection, nuciferine first pass through the liver and then distribute to the whole body. The liver is rich with a variety of drug metabolism enzymes, which is the main place for the metabolism of a variety of drugs (Xia *et al*., 2019). However, the organs and tissues of body are complex, so it is difficult to directly study and elucidate the pharmacokinetic characteristics of drugs in vivo. In vitro, liver microsome experiment has the advantages of simple operation and easy collection of samples (He *et al*., 2021). Certain published literature has compared the differences of metabolic enzymes of nuciferine in different species of liver microsome (Lu *et al*., 2010), in which only metabolic enzymes of nuciferine from liver microsome of different species animal were studied, but not involving the study of the metabolic rate of nuciferine in different liver microsome. Therefore, the in vitro metabolic differences of nuciferine were studied in rabbits, rats and mice liver microsome. The results confirmed that the *Km* values of nuciferine metabolism in liver microsome were rabbit > rat > mouse ($p<0.05$). *Km* value is an important parameter of enzyme metabolism reaction in vitro, which indicates the substrate concentration when metabolic rate of nuciferine reaches half of the maximum metabolism rate, that is to say that the smaller the *Km* value, the greater affinity between metabolic enzyme and nuciferine. In this experiment, the *Km* value of nuciferine in mouse liver microsome was the smallest, which suggested that the affinity between mouse liver microsome enzyme and nuciferine was the highest and its metabolism ability was the strongest. Then the deep metabolism mechanism needed to more study in future.

Conclusion

The calculated results of pharmacokinetics parameters showed that the fast absorption of nuciferine exhibited in rabbits, rats and mice, while the elimination was relatively slow. Compared with rabbit and rat, the nuciferine elimination was fastest in mice. The *Km* value of nuciferine metabolism *in vitro* was least in mice liver microsome, which suggested that the fastest metabolism of nuciferine existed in mice liver. Therefore, the pharmacokinetics characteristics of nuciferine in different species were significantly different, the elimination of nuciferine is the fastest in mice and the slowest in rabbits, which was in line with the *in vitro* metabolism trends by liver microsome from different species.

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Author's Contributions

Dong Xiao-Chen: Complete experiments and writing.

Liu Ming-Qi, Liu Jun and Guan Feng: Design of part experiments.

Xu Ai-Chun and Zhao Jin: Guide of part experiments.

Ge Jian: Design and guide experiments.

Ethics

The authors declare their responsibility for any ethical issues that may arise after the publication of this manuscript.

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