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A rapid and sensitive HPLC–MS/MS analysis and preliminary pharmacokinetic characterization of sibiricaxanthone F in rats

Xiaojuan Yang, Guanshen Zhou, Pingping Zou, Ying Ning, Ke Zan, Pengfei Tu, Yong Jiang*

State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, Beijing 100191, China

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ABSTRACT

A simple, rapid and sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed and validated for quantifying sibiricaxanthone F (SF) in rat plasma following oral and intravenous dosings. After addition of the internal standard (IS) kaempferol and the antioxidant, 20% ascorbic acid, plasma samples were precipitated with acetonitrile and separated on an Aglient Zorbax XDB-C_{18} column (50 mm × 4.6 mm I.D., 2.1 μm) with gradient acetonitrile and water (both containing 0.01% formic acid) as the mobile phase. The detection was performed on a Sciex API 4000 LC–MS/MS with electrospray ionization (ESI) inlet in the negative multiple reaction monitoring (MRM) mode. Good linearity was achieved over the concentration range of 0.5–500.0 ng/mL ($r > 0.996$). Intra- and inter-day precisions were less than 7.60%, and accuracy ranged from 97.18% to 99.84%. The lower limit of quantification for SF was 0.5 ng/mL, and analytes were stable under various conditions (during freeze-thaw, at room temperature and under deep-freeze conditions). This validated method was successfully applied to the preliminary pharmacokinetic study of SF in rats for the first time, and the absolute bioavailability of SF was found to be only 0.22 ± 0.15%.

1. Introduction

Polygala sibirica L. is one of the authorized sources of Polygalae Radix (Yuanzhi) in Chinese Pharmacopoeia, which is a popular traditional Chinese medicine (TCM) used as an expectorant, anti-inflammatory and anti-bacterial agent. It is also classically mentioned as an anti-dementia drug in TCM and Japanese traditional medicine. Pharmacological studies demonstrated that Yuanzhi water extract has significant effects for improving the learning memory of mice [1] and neuroprotection in cultured cell model [2]. Yuanzhi ethanol extract is capable of reversing scopolamine-induced cognitive impairment by inhibiting acetylcholinesterase activity [3,4], and of improving stress-induced memory impairments through the increment of glucose utilization and the total neural cell adhesion molecule levels in rat brains [5].

Xanthones, a particular class of natural products from plant medicines including *Polygala* genus plants [6–12], display various biological activities, such as antioxidant, anti-inflammatory, CNS depression or stimulant, antidiabetic, antitumor actions, and cardiovascular protective effects [13–15]. This might explain the growing interests in this class of compounds, demonstrated by a large number of newly isolated and synthesized derivatives during the last decade [6–12,16]. However, there are few reports for the pharmacokinetic studies concerning xanthones or xanthone derivatives, except those of mangiferin and several simple xanthones up to now [17–19]. Due to their various biological activities, it is worthwhile to perform more metabolic experiments for other active xanthones in the future.

Sibiricaxanthone F (SF, Fig. 1) is one of the new active xanthone glycosides, isolated from *P. sibirica* during our search for new bioactive compounds from the *Polygala* genus plants [8–12]. In *in vitro* tests, this compound exhibited some effects on agitating the peroxisome proliferator-activated receptors (PPARs) and accelerating the differentiation of 3T3-L1 preadipocytes cell (data not shown here), and the further study is being conducted in our laboratory. To the best of our knowledge, xanthones are one type of the main active constituents of *Polygala* genus plants, however, up to now, there has been no report on the analysis of SF in biological samples. Consequently, in order to investigate the physiological disposition of this type of compounds and promote their rational applications, SF was chosen as a representative active xanthone to study the pharmacokinetic process in rats due to its large quantity available from the plant through our study. Thus a novel, rapid and sensitive LC–MS/MS method was then developed and validated here for the quantification of SF in rat plasma for the first time.

* Corresponding author. Tel.: +86 10 82802719; fax: +86 10 82802719.
E-mail address: yongjiang@bjmu.edu.cn (Y. Jiang).

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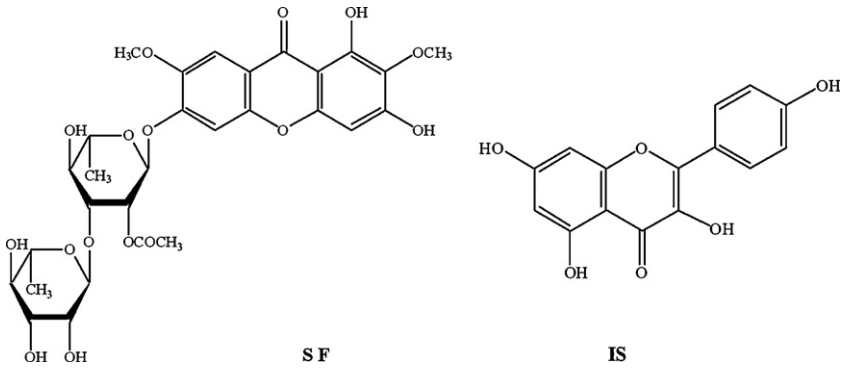

Fig. 1. Chemical structures of SF (sibiricaxathone F) and IS (kaempferol, internal standard).

2. Experimental

2.1. Chemicals and reagents

Sibiricaxanthone F (99.2%, purity) was isolated from the root of *Polygala sibirica* L. in our laboratory. Kaempferol standard (99.0%, purity, Fig. 1) as the internal standard (IS) was purchased from Tianjing Yifang Co. Ltd. (Tianjing, China). Methanol, acetonitrile and formic acid were of HPLC grade and purchased from Fisher Scientific (Fairlawn, NJ, USA) and J.T. Baker Company (Phillipsburg, NJ, USA), respectively. HPLC-quality water was obtained using a SZ-II water purification system (Jiapeng Co., Shanghai, China). Other reagents were of analytical grade and supplied by Beijing Chemical Works (Beijing, China).

2.2. Apparatus and chromatographic conditions

Chromatographic analysis was performed using an Agilent 1200 series LC system (Palo Alto, CA, USA) equipped with two series 200 micro-pumps, a vacuum degasser unit, and an autosampler. Separation of the analytes from plasma was achieved on an Aglient Zorbax XDB-C18 column (50 mm × 4.6 mm I.D., 2.1 μm, Palo Alto, CA, USA), which was eluted with a gradient mobile phase of acetonitrile (A) and water (B) both containing 0.01% formic acid. The solvent A was held at the initial condition of 25% (v/v) for 0.1 min, linearly increased to 80% over 0.1 min and maintained for 0.7 min, then returned to 25% by 0.1 min, and held for 4 min. The mobile phase was delivered at a flow-rate of 0.4 mL/min.

The HPLC system was coupled with an Applied Biosystem Sciex API 4000 Q-Trap mass spectrometer (Foster City, CA, USA) with an electrospray ionization (ESI) source.

The detection was performed by negative ion ionization in multiple reaction monitoring mode (MRM), monitoring the transitions from molecular ion to dominant product ion that were m/z 637.3 → 303.0 and m/z 285.1 → 92.9 for SF and IS (Fig. 2), respectively. The optimized working parameters for mass detection were as follows: the curtain gas (CUR) 10.0 psi, the nebulizer gas (Gas 1) 30 psi, the auxiliary gas (Gas 2) 50 psi, the needle voltage −4500 V, the collision gas (CAD) high and the turbo ion spray temperature 500 °C. Nitrogen was used as the collision gas. All the data were processed by the MassChrom software (version 1.5, Sciex).

2.3. Preparation of calibration standards and quality control (QC) samples

Primary stock solutions of SF and IS for preparation of standards and quality control (QC) samples were prepared from separate weighings. The primary stock solutions of SF and IS were prepared in methanol at a concentration of 1.0 mg/mL and stored at −70 °C. A series of working standard solutions of SF ranging from 5.0 to 5000.0 ng/mL and IS solution at 3.0 μg/mL were prepared by diluting their respective stock solutions with methanol. The solutions were stored at −20 °C for the assay within 2 weeks. Calibration standards were prepared using blank rat plasma (45 μL) spiked with 5 μL of SF working solutions, to yield the concentrations of 0.5, 1.0, 5.0, 10.0, 50.0, 100.0 and 500.0 ng/mL, respectively. QC samples were prepared in the same way as the calibration samples, representing low, middle and high concentrations of SF in plasma at 1.0, 10.0 and 100.0 ng/mL.

The calibration curve was constructed by plotting the peak area ratios of SF/IS *vs* nominal concentrations of SF in plasma.

2.4. Sample preparation

An aliquot of 5 μL plasma after i.v. dosing was diluted by 495 μL blank plasma. A 50 μL aliquot of plasma after i.g. administration or 50 μL of the diluted plasma prepared above, was mixed with 10 μL of IS (3 μg/mL), 20 μL of 20% ascorbic acid (v/v) and 150 μL acetonitrile. After vortexing for 2 min, the mixture was centrifuged at 4 °C for 5 min at 12,000 rpm. The supernatant was recovered and 2 μL was injected into the LC–MS/MS system.

2.5. Method validation

The analytical method was set up to quantify the novel compound SF in a preliminary explorative pharmacokinetic and bioavailability study, and validated to meet the acceptance criteria as per guidelines of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH).

The specificity of the method was established by comparing blank plasma samples from six different sources with those spiked with analytes to find out interference from endogenous components.

Linearity was assessed by assaying calibration curves in plasma at seven concentration levels ranging from 0.5 to 500 ng/mL in three separate runs. And the curves were fitted by a weighed ($1/x^2$) least-squares linear regression method through the measurement of the peak-area ratio of the analyte to IS. The acceptance criterion for a calibration curve was a correlation coefficient (r) of 0.99 or better, and each back-calculated standard concentration must be within 15% deviation from the nominal value except at LLOQ, for which the maximum acceptable deviation was set at 20%. The LLOQ established using five replicates was defined as the lowest concentration in the standard curve at which the relative standard deviation was within 20% and accuracy was within 100 ± 20%.

Accuracy and precision were evaluated by analyzing QC samples at three concentration levels (1, 10 and 100 ng/mL) in five replicates on three validation days. The assay accuracy was expressed as (observed concentration/nominal concentration) × 100%.

Table 1
Matrix effect of SF and IS.

Compound	Nominal concentration (ng/mL)	Observed concentration (ng/mL)	RSD (%)	Matrix effect (%) (mean ± SD)
SF	0.5	0.522 ± 0.040	7.63	104.4 ± 7.97
	5	4.621 ± 0.087	1.88	92.41 ± 1.74
	50	53.310 ± 0.901	1.69	106.62 ± 1.80
IS	136	125.922 ± 4.634	3.68	92.59 ± 3.41

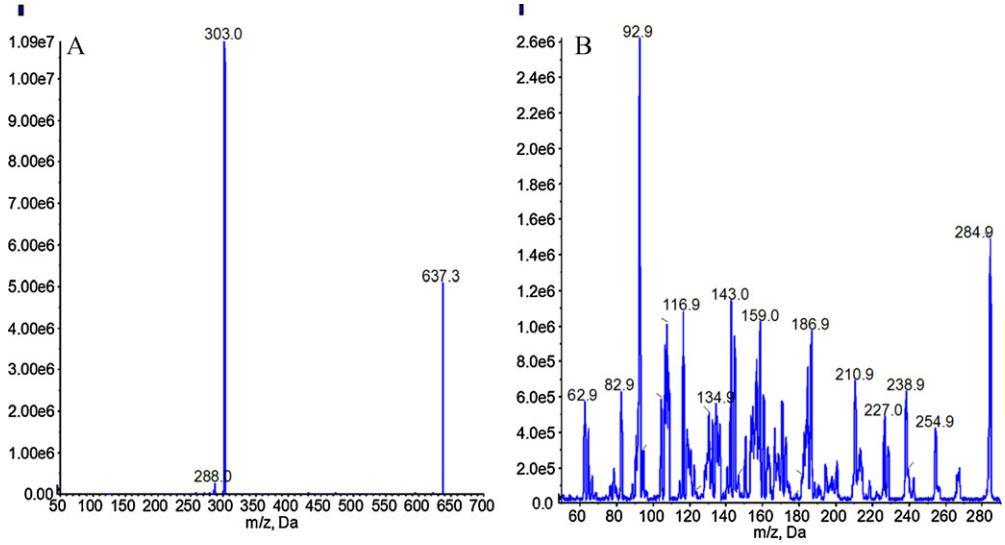

Fig. 2. MS^2 product ions spectra of the deprotonated molecular ions of SF (A) and IS (B).

Intra- and inter-day precisions were obtained by one-way analysis of variance (ANOVA) testing and were expressed as relative standard deviation (RSD). The accuracy was required to be within 85–115%, and the precision should not exceed 15%.

The extraction recoveries of SF and IS were determined at three QC levels (five samples each) by comparing the peak area of each analyte in spiked plasma samples with those of analytes in samples prepared by spiking extracted drug-free plasma samples with the same amounts of analyte at the step immediately prior to chromatography.

The stability of analytes in plasma was investigated under the following conditions: (a) 1 month storage at −20 °C; (b) three consecutive freeze-thaw cycles from −20 °C to room temperature; (c) 24 h storage at room temperature.

The matrix effect was investigated by post-extraction spike method. Peak area (A) of the analyte in spiked blank plasma with a known concentration was compared with the corresponding peak area (B) obtained by direct injection of standard in the mobile phase. The ratio ($A/B \times 100$) is defined as the matrix effect [20].

2.6. Pharmacokinetic and bioavailability study

Male Sprague-Dawley rats (190–220 g) were obtained from the Laboratory Animal Center of Peking University Health Science Center (Beijing, China). They were kept in an environmentally controlled breeding room (temperature: 20 ± 2 °C, humidity: 60 ± 5%, 12 h dark/light circle) for at least 3 days. The protocols of animal experiments were approved by the Animal Ethics Committee of Peking University Health Science Center (No. LA2011-019). The rats were fasted for 16 h before administration while water was taken *ad libitum*. Both of the formulations for intragastric and intravenous administrations of SF were prepared by mixing SF with 0.5% carboxymethyl cellulose sodium (CMC-Na) aqueous solution. All rats were randomized into two groups (six in each group): SF 150 mg/kg body weight by i.g. administration and 40 mg/kg body weight by i.v. administration, respectively. The i.v. solution injection rate was controlled within 1 min. After a single dose was administrated, blood samples (0.5 mL) were collected in heparinized tubes via the orbital vein at 0, 0.083, 0.167, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 10 h. All blood samples were centrifuged at 6000 rpm for 10 min at 4 °C to collect plasma. The plasma obtained was stored at −70 °C until analysis. To obtain the pharmacokinetic parameters of SF in rat plasma by i.v. and i.g. administrations, the concentration–time data were analyzed by non-compartmental method using the Drug and Statistics software (DAS, version 2.0, Clinical Drug Evaluation Center, Wannan Medical College, Anhui, China), or visually inspected from the pharmacokinetic profile for C_{max} and T_{max}. The parameters considered were as follows: C_{max} (maximum plasma concentration), AUC (area under the concentration–time curve from time 0 to the last detectable sample), $T_{1/2}$ (plasma half-time in the terminal phase), CL (plasma clearance defined as dose/AUC) and the bioavailability after oral administration ($F = AUC_{i.g.}/AUC_{i.v.} \times 100$).

3. Results and discussion

3.1. Method development

Since both of SF and IS are phenolic compounds, it is necessary to add some acid in the HPLC mobile phase. After screening, 0.01% formic acid was used to overcome the peak tailing effect and further improve its detection sensitivity. The gradient elution of the mobile phase was used for narrowing the peaks of SF and IS and shorting the running time of the chromatography. In our analytical method, the chromatographic run time was only 5 min.

In the present study, the clean-up of the plasma samples was achieved through a simple and fast single-step protein precipitation procedure. Different kinds of sample pre-treatment methods, including liquid–liquid extraction (LLE) with ethyl acetate and solid-phase extraction, etc., were tried during our method development, but they were limited by low or irreproducible recovery

Table 2
Accuracy and precision for the analysis of SF in QC samples ($n = 3$ days, five replicates per day).

Added concentration (ng/mL)	Found concentration (ng/mL) (mean ± SD, $n = 5$)	Accuracy (%)	Precision (%)	
			Intra-day RSD ($n = 5$)	Inter-day RSD ($n = 3$)
1	0.998 ± 0.074	99.84	7.36	0.698
10	9.718 ± 0.735	97.18	7.56	3.98
100	99.560 ± 3.956	99.56	3.97	3.86

Table 3
Recoveries of SF and IS ($n = 5$).

Compound	Concentration (ng/mL)	RSD (%)	Recovery (%) (mean ± SD)
SF	1	8.92	101.2 ± 9.07
	10	3.25	104.3 ± 3.39
	100	1.60	93.08 ± 1.60
IS	136	2.21	80.34 ± 1.77

for analytes or time-consuming procedure. Finally the method of protein precipitation with 3-fold volumes of acetonitrile was used.

It is necessary to use an IS to get high accuracy when performing MS quantitation. An appropriate IS will control variability in extraction, HPLC injection and ionization. From a list of 15 commonly used xanthone or flavones compounds, kaempferol was finally selected due to its high recovery and structural similarity with the analyte.

In addition, the stability of IS kaempferol in plasma was found to be not very good, so some anti-oxidant was added to the plasma. Finally, 20% ascorbic acid was chosen.

3.2. Method validation

3.2.1. Specificity and matrix effect

The specificity of the method was evaluated by analyzing individual blank plasma samples from six different sources. All samples were found to have no interferences from endogenous substances at the retention times of either SF or the IS. Typical chromatograms of a blank plasma, a spiked plasma sample with SF and IS, and a plasma sample after SF administration are shown in Fig. 3.

In the present study, the negative ion ionization was used for the detection of SF, due to its higher sensitivity than that of the positive mode. To investigate the matrix effect, three concentration levels of SF (0.5, 5 and 50 ng/mL) in five replicates were analyzed, and the values are shown in Table 1. The matrix effects for SF and IS ranged from 92.0 to 107.0%, and the corresponding RSDs were less than 8.0%. On the other hand, the matrix effects for the mobile phase, ascorbic acid, the dosing formulation CMC-Na and the anticoagulant heparin sodium that might exist in plasma were investigated as well, and no significant interference of these reagents was found. These results demonstrated that the

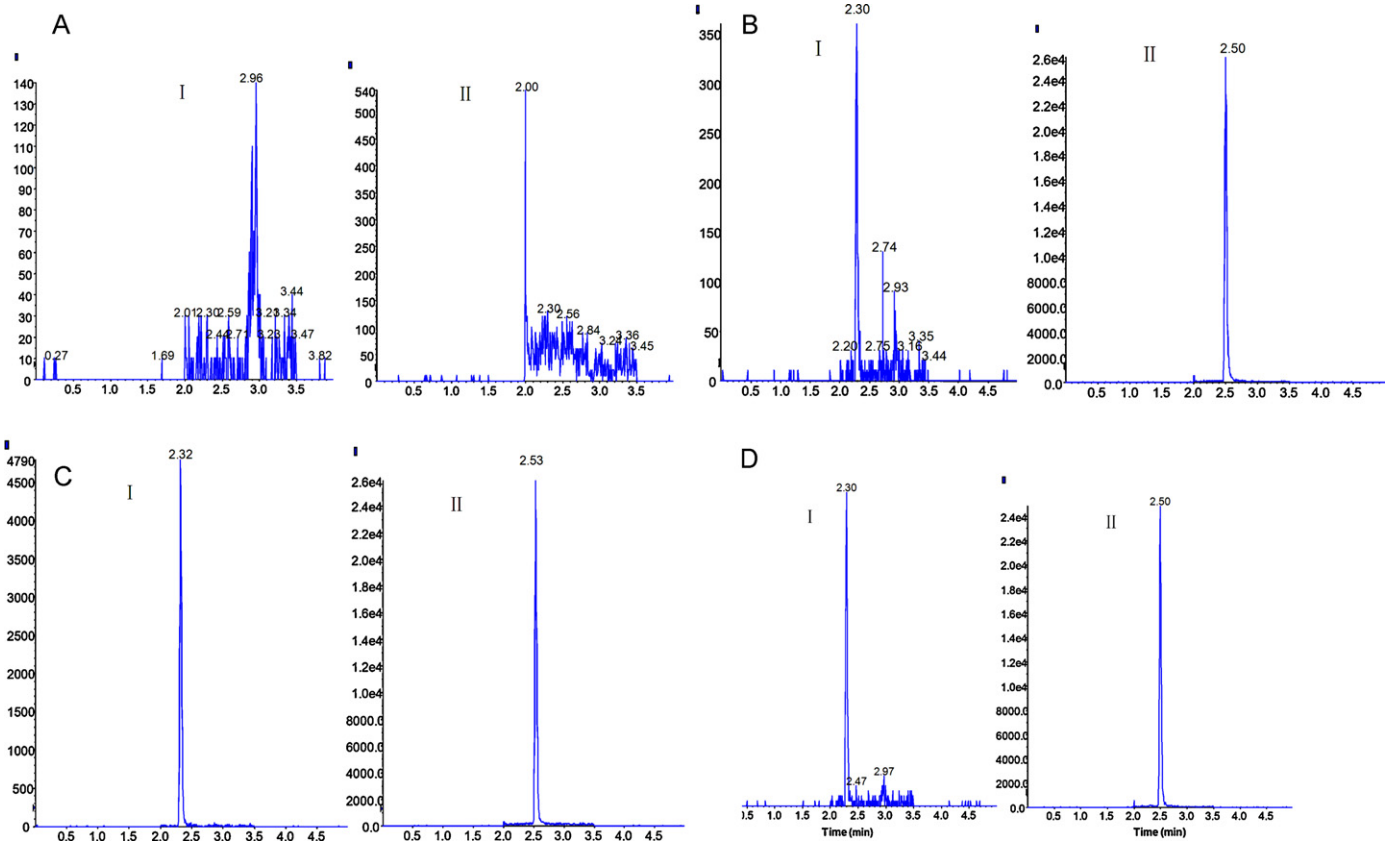

Fig. 3. Representative MRM chromatograms of: (A) a blank rat plasma sample; (B) a blank rat plasma spiked with SF (1 ng/mL) and IS (200 ng/mL); (C) a rat plasma sample at 0.5 h after an intravenous dose of SF (40 mg/kg) to rats (I: retention time, 2.30 min; m/z 637.3 → 303.0 for SF and II: retention time, 2.52 min; m/z 285.1 → 92.9 for IS); (D) a rat plasma sample at 0.5 h after an oral dose of SF (150 mg/kg) to rats.

Table 4
Stability of quality control samples ($n = 5$).

Stability tests	Time period	Theoretical conc. (ng/mL)	Found conc. (mean SD)	Precision (RSD%)	Accuracy (RE%)
Freeze/thaw stability (from −20 °C to 20 °C)	3 times	1	1.00 ± 0.04	3.63	+0.47
		10	10.56 ± 0.60	5.68	+5.63
		100	99.92 ± 1.34	1.34	−0.08
Short-term stability (20 °C)	24 h	1	0.97 ± 0.01	0.61	−3.11
		10	10.74 ± 0.91	8.46	7.63
		100	99.71 ± 3.93	3.95	−0.29
Long-term stability (−20 °C)	1 month	1	0.99 ± 0.06	5.80	−0.094
		10	10.26 ± 0.42	4.07	2.64
		100	100.03 ± 1.65	1.65	0.03

processing procedure of plasma samples was highly acceptable with no significant ion suppression or enhancement.

In addition, the "cross-talk" between the two MS/MS channels used for monitoring was assessed by separately injecting SF and IS, and monitoring the response in the other channels. No "cross-talk" between channels was observed.

3.2.2. Calibration curve, linearity and LLOQ

The method exhibited good linearity over the concentration range of 0.5–500 ng/mL with a high correlation coefficient ($r > 0.996$). A typical equation of the calibration curve was obtained as follows: $y = 1.11 \times 10^{-2} x + 1.05 \times 10^{-3}$ ($r = 0.9966$), where y is the peak-area ratio of SF to IS and x is the plasma concentration of SF, respectively.

The LLOQ for SF was 0.5 ng/mL, which is sensitive enough for the pharmacokinetic study of SF in rats. The precision and accuracy at this concentration level were acceptable, with 9.1% of the RSD and 98.3% of the accuracy, respectively.

3.2.3. Accuracy and precision

The intra- and inter-day precision and accuracy of the method were investigated by analyzing QC samples (1.0, 10.0 and 100.0 ng/mL). All the values are shown in Table 2. Intra-day RSD was below 8.0% and inter-day RSD was below 4%. The accuracy was within 97.0–100.0%. The method was proved to be highly accurate and precise.

3.2.4. Recovery and stability

The extraction recoveries of SF were 101.2 ± 9.07%, 104.3 ± 3.39% and 93.08 ± 1.60% at three concentrations of 1.0, 10.0 and 100.0 ng/mL, respectively, while the recovery of IS was 80.34 ± 1.77%. These results suggested that the recoveries of SF and IS were consistent and were not concentration-dependent. All the values are shown in Table 3.

Stability study showed that the concentrations of SF were not significantly changed in plasma stored in −20 °C for 1 month, stored at room temperature for 24 h and experienced a three-time freeze/thaw from −20 °C to 20 °C. All the values are shown in Table 4.

3.2.5. Application to a pharmacokinetic and bioavailability study

The method was successfully applied to the quantification of SF in rat plasma after intravenous and oral dosings. During the bioanalyses, no significant problems such as pressure ascend, retention time shift and interferences, were observed when the column was used continuously for at least 200 plasma samples.

The mean plasma concentration (±SD) ($n = 6$) versus time profiles for SF are depicted in Fig. 4, and their estimated pharmacokinetic parameters are presented in Table 5. Due to a significant inter-individual variability in the C_{max} and T_{max} after oral dosing, there was no statistical significance to depict the two parameters mentioned above. Other pharmacokinetic parameters were given by DAS 2.0 Software using the non-compartment model. The absolute bioavailability of SF was calculated to be 0.22 ± 0.15%. Many factors that could be responsible for the low bioavailability of this compound include the potential hydrolysis in the gastrointestinal tract, poor permeability through the intestinal epithelial membrane and first-pass effect in the liver [21]. Thus, in the next step, poly(DL-lactic-co-glycolic acid) (PLGA) nanocapsules or some corresponding salt derivatives could be tried to improve the delivery of this poorly water-soluble xanthone [18,22]. In addition, this study showed that only a small amount of SF could be absorbed in plasma by oral dosing. Therefore, the aglycone of SF or its other metabolites that might exist in plasma may be paid more attention in our further studies.

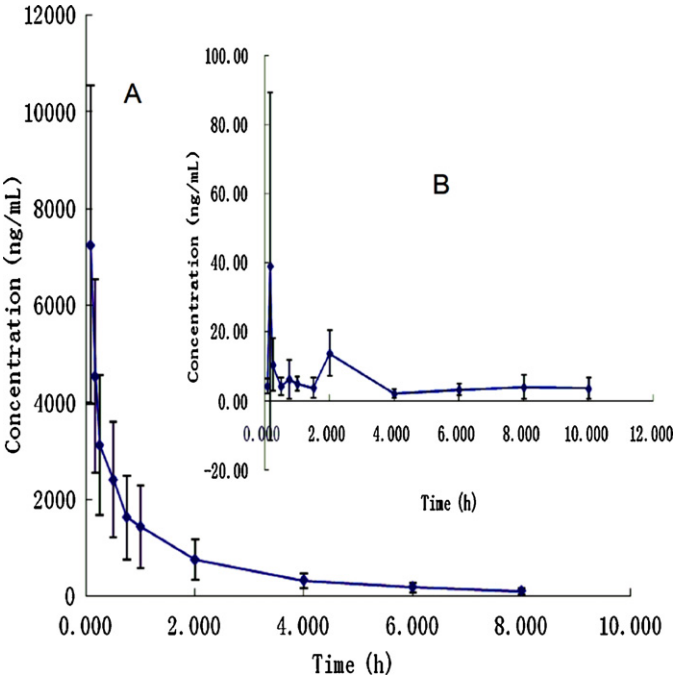

Fig. 4. Mean plasma concentration–time curves of SF in rats following i.v. (40 mg/kg, A) and i.g. (150 mg/kg, B)..

Table 5
Pharmacokinetic parameters for SF by i.v. and i.g. administrations ($n = 6$).

Parameter	SF (40 mg/kg, i.v.)	SF (150 mg/kg, i.g.)
AUC_{0-t} (ng h/mL)	6143.39 ± 2087.8	49.86 ± 7.63
$AUC_{0-\infty}$ (ng h/mL)	6426.02 ± 2150.43	60.39 ± 5.69
T_{max} (h)	–	–
$T_{1/2}$ (h)	2.08 ± 0.26	–
Cl (L/h/kg)	6.88 ± 2.55	2631.08 ± 722.06
V_d (L/kg)	21.37 ± 10.97	16,399.06 ± 12,687.9
AB %	0.22 ± 0.15	

AB (absolute bioavailability) = $(AUC_{i.g.}/AUC_{i.v.}) \times (Dose_{i.v.}/Dose_{i.g.}) \times 100$.

Notably, there were three peaks for SF following oral administration in both individual and mean plasma concentration–time curves (Fig. 4), which is similar to the curve of mangiferin in rats following single intragastric administration [17]. This might be caused by presystematic metabolism, "absorption" window along the gastrointestinal tract, enterohepatic circulation, variable gastric emptying and drug–drug interaction [23,24]. This atypical drug absorption profiles such as multiple peaks and window-type absorption profiles were also encountered in berberine, macranthoidin B, 3,6′-disinapoylsucrose, tacrolimus, etc. [25–29]. Further detailed absorption investigations are needed to elucidate the mechanism of the multiple peak phenomenon in the next step.

4. Conclusion

A sensitive and reliable LC–MS/MS method has been developed for the determination of SF in rat plasma using protein precipitation as the sample clean-up procedure. This method showed excellent sensitivity, linearity, precision, accuracy and extraction recovery. The established method was successfully applied to a preliminary pharmacokinetic study of SF, a new xanthone glycoside isolated from *P. sibirica* after intravenous and intragastric administrations to rats. The results showed that SF had a very low oral bioavailability with only $0.22 \pm 0.15\%$ of a single dose, and multiple peaks were found in both individual and mean plasma concentration–time curves of SF following oral administration. Due to the poor bioavailability of SF, its metabolites that might exist in plasma could be paid more attention in our further studies.

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