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A highly sensitive and robust UPLC–MS with electrospray ionization method for quantitation of taxifolin in rat plasma

Xiaodan Wang, Hongjun Xia, Feng Xing, Guifeng Deng, Qi Shen, Su Zeng*

College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, Zhejiang Province, PR China

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ABSTRACT

A sensitive ultra performance liquid chromatography–mass spectrometry method has been developed and validated for the quantification of taxifolin in rat plasma. Following liquid/liquid extraction by ethyl acetate, the analytes were separated on a Sunfire™ (2.1 mm × 50 mm, 3.5 μm) column and analyzed in the selected ion recording with a negative electrospray ionization mode. The method was linear over the concentration range of 6–6750 ng/mL. Intra- and inter-day precisions were all within 8% and accuracy ranged from 92.9% to 105.1%. The lower limit of quantification was 6 ng/mL. The present method was successfully applied to the estimation of the pharmacokinetic parameters of taxifolin following intravenous and oral administration to rats. The absolute bioavailability of taxifolin was 0.17% in rat.

1. Introduction

Flavonoids comprise the most common group of plant polyphenols and provide much of the flavor and color to fruits and vegetables and widely distribute in many fruits, vegetables, beverages and some drink, such as tea and wine [1]. Research interests on flavonoids have increased since the low cardiovascular mortality rate observed in mediterranean populations in association with red wine consumption and a high saturated fat intake. Now more than 5000 different flavonoids have been reported and are most commonly known for their potent antioxidant, free-radical scavenging activities observed *in vitro* and their inhibitory role in various stages of tumor development in animal studies [2]. At one time, it was thought that a deficiency of citrus flavonoids, such as rutin, quercetin, and taxifolin, may increase the brittleness of the capillary blood vessels. Those flavonoids were named "bioflavonoids".

Taxifolin ((2R,3R)-dihydroquercetin) belongs to the class of flavonone antioxidants, and is derived from the pulp of the deciduous tree such as *Pseudotsuga taxifolia* Britt, as well as in many fruits, especially grape fruits and oranges [3,4]. Today, taxifolin is used as an additive in food and health care products. Taxifolin has been studied for multiple pharmacologic actions, such as anti-cardiovascular disease activity, anti-oxidation, inactivation of cytotoxic substances, and anti-diabetes effect. Taxifolin can effectively prevent osmotic stress in hyperglycemia, decrease the tetracycline and tetrachloride methane-induced lipid peroxidation of liver microsome, and reduce the content of low-density lipoproteins in liver and serum [5–8]. It has been found that taxifolin can help to provide essential neuroprotection against the free-radical-induced oxidative damage that often occurs when the brain does not receive enough blood and oxygen [9]. Taxifolin was effective for protecting subcellular systems and red blood cells against oxidative stress *in vitro* [10].

In previous pharmacokinetic study of taxifolin, the concentration of taxifolin in rabbit plasma was analyzed by high performance liquid chromatography (HPLC) with UV detection [11]. Although this HPLC–UV method was sensitive enough to detect taxifolin after enzymatic hydrolysis with β-glucuronidase and sulfatase in rabbit plasma, it was not suitable to detect taxifolin without any enzymatic hydrolysis because of the low concentration of taxifolin in rabbit plasma. In this study, we developed a rapid and sensitive ultra performance liquid chromatography–mass spectrometry (UPLC–MS) method to determine taxifolin in rat plasma using a simple procedure of liquid–liquid extraction. According to our previous studies, the apparent permeability of taxifolin across Caco-2 cell monolayers was less than 1×10^{-6} cm/s, which indicated that oral bioavailability of taxifolin may be poor (data not published). So this UPLC–MS method was employed to study the pharmacokinetic and absolute bioavailability of taxifolin in rats to test the correlation of *in vitro* and *in vivo* studies. The method described exhibited excellent performance in terms of high selectivity, rapid, sensitive, accurate and robustness.

Abbreviations: LLOQ, lower limit of quantification; ULOQ, upper limit of quantification; LOD, the limit of detection; IS, internal standard; ESI^-, negative-ion electron spectroscopic imaging; SIR, selected ion recording; MRM, multiple reaction monitoring; QC, quality control.

* Corresponding author. Tel.: +86 571 88208407; fax: +86 571 88208407.
E-mail address: zengsu@zju.edu.cn (S. Zeng).

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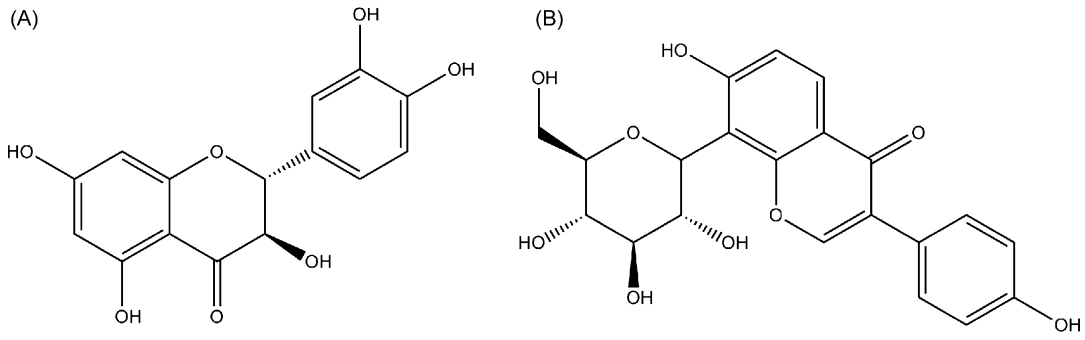

Fig. 1. Chemical structures of taxifolin (A) and puerarin (B).

2. Experimental

2.1. Chemicals and reagents

Taxifolin (Fig. 1A, $C_{15}H_{12}O_7$, M_w = 304.25) with a purity of 98.0% as determined by HPLC was purchased from Nanjing Sulang Medical Technology Development Co., Ltd. (Nanjing, Jiangsu, China). Puerarin (Fig. 1B, $C_{21}H_{20}O_9$, M_w = 416.38, the internal standard, IS) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). DMSO was purchased from Sigma Chemical Co. (Saint Louis, MO, USA). HPLC-grade acetonitrile and acetic acid were purchased from TEDIA Inc. (Fairfield, USA). All other reagents were of analytical grade or HPLC grade when appropriate. Ultra-pure water was obtained from an ELGA-purelab Ultra system (High Wycombe, UK).

2.2. Instrument and analytical conditions

A Waters ACQUITY^{TM} TQD with the power of ultra performance liquid chromatography (Waters, Milford, MA, USA) was used in the study. A chromatographic separation was achieved by using a C_{18} column (Sunfire^{TM} C_{18}, 2.1 mm × 50 mm, 3.5 μm, Waters, Milford, MA, USA) maintained at 30 °C. The mobile phase consisted of acetonitrile–water containing 0.3% acetic acid with a gradient elution starting at 10% acetonitrile and progressing linearly to 35% acetonitrile over 6.5 min then returning to 10% acetonitrile. The mobile phase was directly delivered into the negative-ion mode (ESI^-) source at 0.2 mL/min. Injection volume was 7 μL. The temperature of the sample manager was maintained at 20 °C.

2.3. Mass spectrometric conditions

Mass spectrometric analysis was performed in the ESI^- mode and set up in the selected ion recording (SIR) mode. Nitrogen was used as desolvation gas (500 L/h). The source and desolvation gas temperature was kept at 120 and 350 °C, respectively. The optimized MS parameters are listed in Table 1. The system was collection by Masslynx^{TM} V 4.1 software (Micromass, Manchester, UK). The analytes were assayed by quantifying the $[M-H]^-$ ions of taxifolin at *m*/*z* 303.0, and puerarin at *m*/*z* 415.2. The scans for the taxifolin and puerarin are shown in Fig. 2.

2.4. Preparation of standard and quality control samples

The stock standard solutions of taxifolin and puerarin were prepared by dissolving accurately weighed individual compounds in DMSO to give a final concentration of 3.0 and 1.2 mg/mL, respectively. A series of standard working solutions at concentrations over 60–67,500 ng/mL for taxifolin were obtained by further dilution of the standard stock solution with acetonitrile–water containing 0.3% acetic acid (10:90; v/v), and DMSO was less than 2.3% (v/v) in the working solutions. Internal standard working solution (1.2 μg/mL) was prepared by diluting the internal standard stock solution with the mobile phase, and the concentration of DMSO in the working solution was 0.1% (v/v). All solutions were stored at −20 °C and brought to room temperature (20 °C) before use.

Blank rat plasma was collected from six rats and pooled to get a sufficient volume to prepare calibration and quality control (QC) samples. The analytical standard and QC samples were prepared by spiking the standard working solutions to blank rat plasma during validation and each experimental run for the pharmacokinetic studies. Calibration samples were made at the concentration of 6–6750 ng/mL. QC samples were prepared at the concentrations of 6, 9, 900 and 5400 ng/mL. The dilution test samples above the upper limit of quantification (>ULOQ sample) were prepared by directly spiking the stock standard solutions of taxifolin (3.0 mg/mL) into blank rat plasma, yielding spiked plasma samples containing 67.5 μg/mL taxifolin.

2.5. Sample preparation

Standard working solutions (8 μL taxifolin and 10 μL puerarin) and 2 μL acetic acid were added into 72 μL blank rat plasma in 0.5 mL centrifuge tubes for preparing the calibration standards and QC samples (the preparation process was described in Section 2.4). Aliquot of 80 μL of each plasma sample was spiked with 10 μL internal standard working solution (1.2 μg/mL) and 2 μL acetic acid. All calibration standards, QC samples and unknown samples were vortex-mixed (Eppendorf, Hamburg, Germany) for 30 s and extracted with 240 μL ethyl acetate by a vortex mixing for 1 min. Following centrifugation at 15,700 rcf for 10 min, the upper organic phase was transferred into clean tubes and evaporated to dryness with vacuum at room temperature. The residues were dissolved in 50 μL mobile phase before UPLC–MS analysis.

Table 1
Selected ion transitions (*m*/*z* values) and optimized mass spectrometric parameters for the UPLC/MS analysis of taxifolin and puerarin in selected ion recording (SIR) mode.

	m/*z*	Capillary voltage (kV)	Cone voltage (V)	RF lens voltage (V)	Extractor (V)
Taxifolin	303.0	2.6	30	0.1	3
Puerarin	415.2	2.6	48	0.1	3

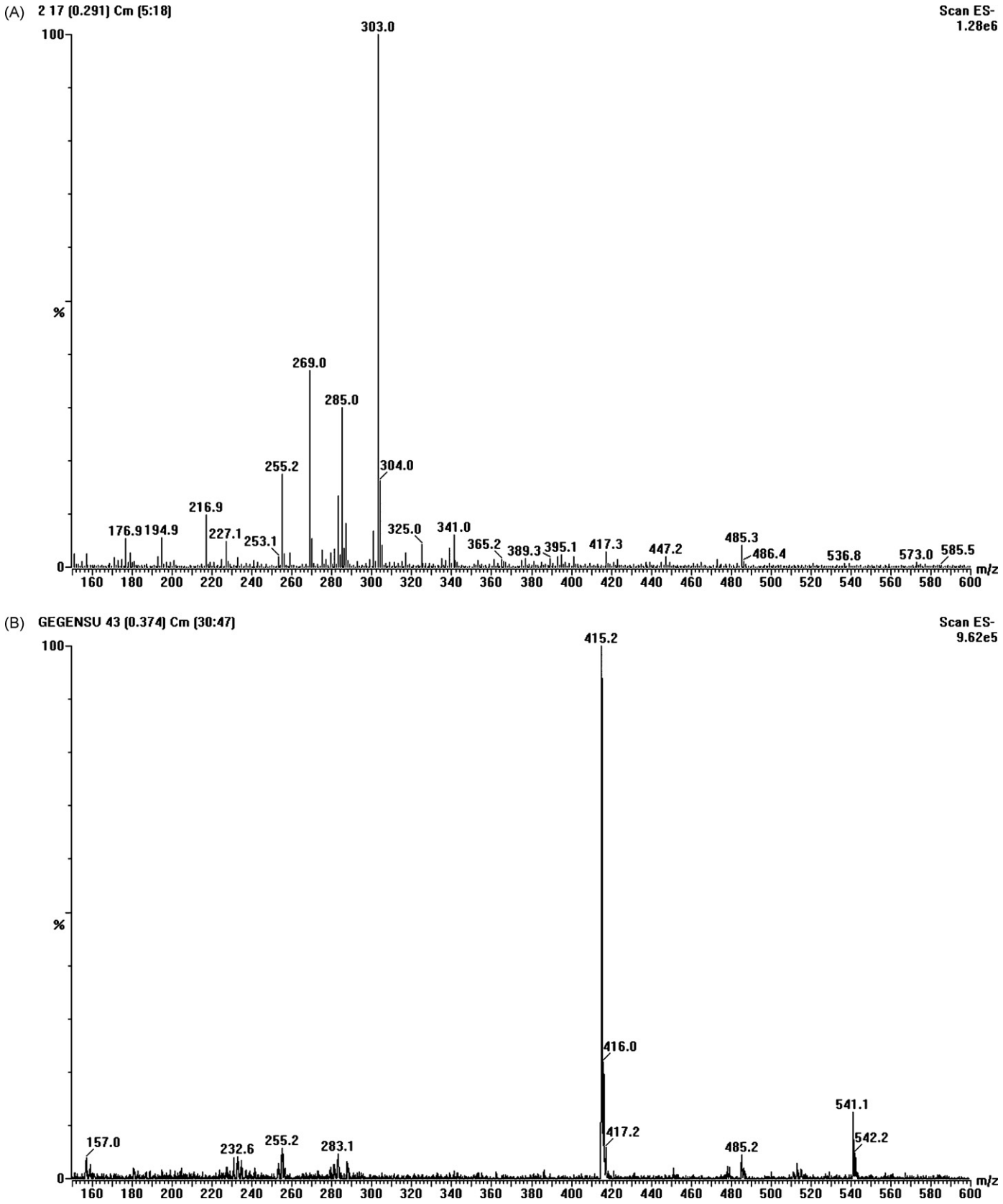

Fig. 2. Full-scan ion spectra of taxifolin (A, *m/z* 303.0) and puerarin (B, *m/z* 415.2).

2.6. Method validation

A thorough and complete method validation of taxifolin in rat plasma was done following the FDA guidelines [13]. The method was validated for selectivity, sensitivity, accuracy, precision, recovery, linearity, stability, reproducibility and matrix effect.

The specificity of the method was assessed in six batches of rat plasma samples by analyzing blank and spiked samples at the lowest concentration on the calibration curve (LLOQ) level. The limit of detection (LOD) was estimated as the amount of taxifolin which caused a signal three times to noise.

Calibration curve of taxifolin was used in each run with concentrations at 6, 30, 45, 120, 300, 450, 3000 and 6750 ng/mL. Peak-area ratios of the taxifolin to IS were calculated and the calibration curve was established by fitting these ratios to the corresponding concentrations by a linear regression method.

Assay accuracy and precision were assessed by determining QC samples at four concentration levels. The accuracy was determined by comparing the calculated concentration to the theoretical concentration of the QC samples. The precision was determined by intra- and inter-day relative standard deviation (RSD%) of the QC samples. The capacity to dilute samples originally above the ULOQ of the calibration curve was demonstrated by analyzing validation samples containing 10-fold the concentration of the ULOQ of taxifolin (67.5 μg/mL, >ULOQ sample). This dilution test sample was diluted 12.5 times using blank rat plasma to obtain samples with a final nominal concentration of 5400 ng/mL and were analyzed in one analytical run.

The matrix effects on the ionization of taxifolin and puerarin were assessed as described by Li et al. [14]: comparing the peak areas dissolved in the blank sample (the final solution of blank plasma after extraction and dissolution) with that dissolved in the mobile phase. Three different concentration levels of taxifolin were evaluated by analyzing the five samples at each level.

The recoveries (extraction efficiency) of taxifolin and puerarin from rat plasma after the extraction procedure were determined by comparing the peak areas of extracted taxifolin or puerarin with the area of taxifolin or puerarin of the same concentration level dissolved in the blank sample (the final solution of blank plasma after extraction and dissolution) with the mobile phase.

The stability of taxifolin in human plasma was evaluated using QC samples (9, 900 and 5400 ng/mL) with five samples for each concentration. The stability of taxifolin was tested under the following conditions: (1) freeze–thaw stability of taxifolin in rat plasma through three freeze–thaw cycles; (2) short-term stability of taxifolin in rat plasma at room temperature for 6 h; (3) long-term stability of taxifolin in rat plasma stored at −20 °C for 45 days; (4) post-preparative stability of taxifolin during storage in the auto sampler at 20 °C for 24 h.

2.7. Pharmacokinetic studies

The studies were approved by the Animal Ethics Committee of Zhejiang University. Male Sprague–Dawley rats (180–200 g) were obtained from the Zhejiang Laboratory Animal Center (Hangzhou, China). The rats were maintained in an air-conditioned animal quarter at a temperature of 22 ± 2 °C and a relative humidity of 50 ± 10%, having free access to water, and fed with a laboratory rodent chow (Hangzhou, China). The rats were fasted and given free access to water for 12 h prior to experiment. Twenty-four rats were divided into four groups at random. Three groups were given single dose of taxifolin solution at 10, 50 or 100 mg/kg of body weight by gavage into the stomach using a blunt ended needle [12]. One group was given single dose of taxifolin solution at 10 mg/kg by intravenous administration. Taxifolin solution for oral administration was prepared by dissolving the powder of taxifolin with isotonic sodium chloride containing 0.5% carboxymethylcellulose sodium. Taxifolin solution for intravenous administration was prepared by dissolving the powder of taxifolin with isotonic sodium chloride containing 5% ethanol. The rats were fasted for the first 2 h with free assess to water after dosing. About 200 μL blood samples were collected by scissoring rat tails into heparinized tubes at 3, 6, 10, 15, 20, 25, 30, 40, 50, 60, 90, 120, 150, 180, 240 and 300 min after dosing. After plasma collection, each blood sample was immediately centrifuged at 1500 rcf for 10 min at 4 °C and 80 μL plasma was transferred into 0.5 mL centrifuge tube and then stored at −20 °C until analysis. During routine analysis, each analytical run included six blank plasma, a set of calibration samples, a set of QC samples and unknowns.

Table 2
Matrix effects on taxifolin and puerarin ($n = 5$).

Concentration (ng/mL)	Matrix effects (Mean ± SD, %)	RSD (%)
Taxifolin		
9	103.5 ± 7.8	7.5
900	111.8 ± 1.0	0.9
5400	104.4 ± 1.8	1.7
Puerarin		
150	99.4 ± 2.9	2.9

2.8. Statistical analysis

To determine the pharmacokinetic parameters of taxifolin, the concentration–time data were analyzed by DAS Software (ver. 2.0, China State Drug Administration). Data were expressed as mean ± SD. Statistical analysis was performed using the statistical software package SAS (v8.2; SAS Institute, Inc., Cary, NC).

3. Results

3.1. Selectivity

Selectivity was assessed by comparing the chromatograms of six different batches of blank rat plasma. Typical chromatograms of the blank and spiked plasma are given in Fig. 3. The retention times of taxifolin and puerarin were about 4.3 and 2.5 min, respectively. Endogenous peaks at the retention time of the analytes were not observed for any of the blank rat plasma batches indicating no significant endogenous interference in the SIR mode for the analytes during the detections.

3.2. Matrix effects

The absolute matrix effect was determined by comparing the peak areas obtained from mobile phase spiked with low, mid and high concentrations of taxifolin (9, 900, 5400 ng/mL, $n = 5$, respectively) and puerarin (150 ng/mL, $n = 5$) with post-extraction blank rat plasma spiked samples. The ratios of the peak area resolved in the post-extraction blank sample with that resolved in the mobile phase of taxifolin and puerarin were shown in Table 2. The results showed that there were no significant matrix effects.

3.3. Linearity and calibration curve

The calibration curve of taxifolin was constructed by plotting the peak-area ratio of taxifolin to puerarin (y) versus analyte concentration (ng/mL) in spiked blank rat plasma (x). The peak-area ratios of taxifolin to puerarin in rat plasma varied linearly with concentration over the range: 6, 30, 45, 120, 300, 450, 3000 and 6750 ng/mL. The regression equation of the curve and the correlation (r) were calculated as follows: $y = 0.02174x + 0.01103$ ($r = 0.9999$). The standard error of slope was 0.00109, and the standard error of intercept

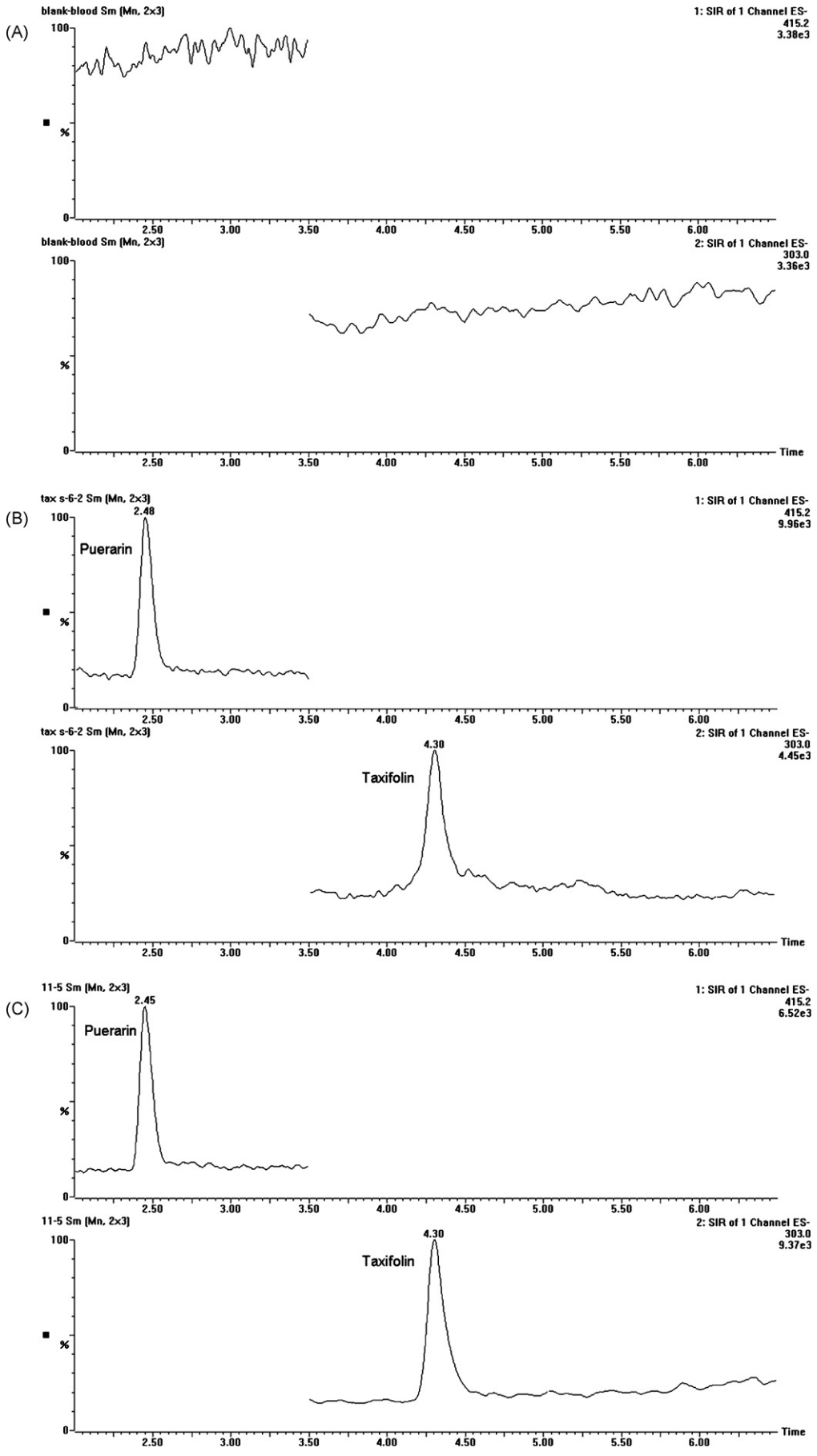
(A)
blank-blood Sm (Mn, 2x3)
1: SIR of 1 Channel ES-
415.2
3.38e3
blank-blood Sm (Mn, 2x3)
2: SIR of 1 Channel ES-
303.0
3.36e3
Time
(B)
tax s-6-2 Sm (Mn, 2x3)
2.48
Puerarin
1: SIR of 1 Channel ES-
415.2
9.96e3
tax s-6-2 Sm (Mn, 2x3)
4.30
Taxifolin
2: SIR of 1 Channel ES-
303.0
4.45e3
Time
(C)
11-5 Sm (Mn, 2x3)
2.45
Puerarin
1: SIR of 1 Channel ES-
415.2
6.52e3
11-5 Sm (Mn, 2x3)
4.30
Taxifolin
2: SIR of 1 Channel ES-
303.0
9.37e3
Time

Table 3
Calibration standards of taxifolin in rat plasma calibration curves ($n = 5$).

Nominal concentration (ng/mL)	Measured concentration (ng/mL) (Mean ± SD)	Deviation (%)	RSD (%)
6	6.3 ± 1.7	5.6	2.7
30	28.0 ± 0.5	−6.6	1.8
45	44.6 ± 2.5	−0.9	5.6
120	113.3 ± 6.1	−5.6	5.4
300	297.4 ± 8.5	−0.9	2.9
450	466.8 ± 14.3	3.7	3.1
3000	2982.1 ± 46.0	−0.6	1.5
6750	6656.2 ± 356.9	−1.4	5.4

was 0.00687 ($n = 5$). The LLOQ of taxifolin was 6 ng/mL (RSD% = 5.0%, $n = 5$, signal-to-noise >20/1). The mean LOD of taxifolin in our assay is estimated at 1.2 ng/mL which produced a signal-to-noise of 3/1. Concentrations were back-calculated from nominal concentrations and were summarized in Table 3. Deviations of the back-calculated concentrations from the nominal concentrations were between −6.6% and 5.6% with RSD between 1.5% and 5.6% for all concentrations in rat plasma.

3.4. Accuracy and precision

The intra- and inter-day precision and accuracy data at four concentrations of taxifolin were listed in Table 4. The intra-day precision (RSD%) ranged from 1.8% to 7.2%, and the inter-day precision ranged from 1.9% to 4.5%. The intra-day accuracy (expressed as percent of nominal values) ranged from 92.9% to 105.1% and the inter-day accuracy ranged from 99.6% to 103.9%. The results above demonstrated that the method established has a satisfactory accuracy, precision and reproducibility.

Samples with a concentration above the ULOQ of 6750 ng/mL can be safely diluted 12.5 times, as the intra-assay accuracy for diluted >ULOQ samples (67.5 μg/mL taxifolin in blank rat plasma diluted with a factor 12.5 using blank rat plasma resulting in a concentration of 5400 ng/mL) was −1.2% with a precision of 4.3% (RSD%).

3.5. Recovery

The recovery of taxifolin and puerarin from rat plasma was shown in Table 5. The mean recoveries of taxifolin were more than 75% at four concentration levels.

Table 4
Accuracy and precision for the analysis of taxifolin ($n = 5$).

QC (ng/mL)	Precision		Accuracy
	Mean ± SD	RSD (%)	Deviation (%)
Intra-day			
6	5.9 ± 0.3	5.0	−1.3
9	8.4 ± 0.6	7.2	−7.1
900	942.4 ± 38.9	4.1	4.7
5400	5675.3 ± 99.2	1.8	5.4
Inter-day			
6	6.2 ± 0.2	3.7	2.9
9	9.0 ± 0.4	4.5	−0.4
900	935.0 ± 17.7	1.9	3.9
5400	5493.4 ± 121.5	2.2	1.7

Table 5
Recovery of taxifolin and puerarin from rat plasma ($n = 5$).

Concentration (ng/mL)	Recovery (Mean ± SD, %)	RSD (%)
Taxifolin		
6	75.9 ± 7.9	9.9
9	83.1 ± 5.8	6.9
900	86.3 ± 3.8	4.4
5400	91.2 ± 1.4	1.6
Puerarin		
150	75.3 ± 1.8	2.3

3.6. Stability

QC samples of taxifolin at three concentrations (9, 900, 5400 ng/mL) were used for stability experiments. The stability of taxifolin was tested under different conditions. The results (Table 6) indicated that taxifolin was stable under routine laboratory conditions and no specific procedure was needed to stabilize the compounds for pharmacokinetic study. Based on our previous studies, taxifolin in DMSO or acetonitrile–water containing 0.3% acetic acid (10:90; v/v) was stable at least for six months at −20 °C.

Table 6
The stability of taxifolin in rat plasma at different store conditions ($n = 5$).

Nominal concentration (ng/mL)	Calculated concentration		
	Mean ± SD	Deviation (%)	RSD (%)
Freeze–thaw stability			
9	9.5 ± 0.2	5.6	1.8
900	935.3 ± 11.0	3.9	1.2
5400	5492.2 ± 52.9	1.7	1.0
Short-term stability			
9	9.5 ± 0.5	5.5	5.7
900	927.1 ± 20.3	3.0	2.2
5400	5519.0 ± 203.5	2.2	3.7
Long-term stability			
9	9.0 ± 0.4	1.1	3.9
900	947.5 ± 12.4	5.3	1.3
5400	5346.9 ± 181.0	−1.0	3.4
Post-preparative stability			
9	8.8 ± 0.4	−2.2	4.1
900	936.6 ± 20.5	4.1	2.2
5400	5455.7 ± 310.7	1.0	5.7

Fig. 3. Representative selected ion recording (SIR) chromatograms of taxifolin and puerarin (IS) in rat plasma. (A) Blank rat plasma sample, (B) rat plasma sample spiked with taxifolin (6 ng/mL) and puerarin (IS, 150 ng/mL), and (C) a rat plasma sample 40 min after oral administration (100 mg/kg), the concentration of taxifolin of this sample was 73.7 ng/mL. The retention times of puerarin and taxifolin were 2.5 and 4.3 min, respectively.

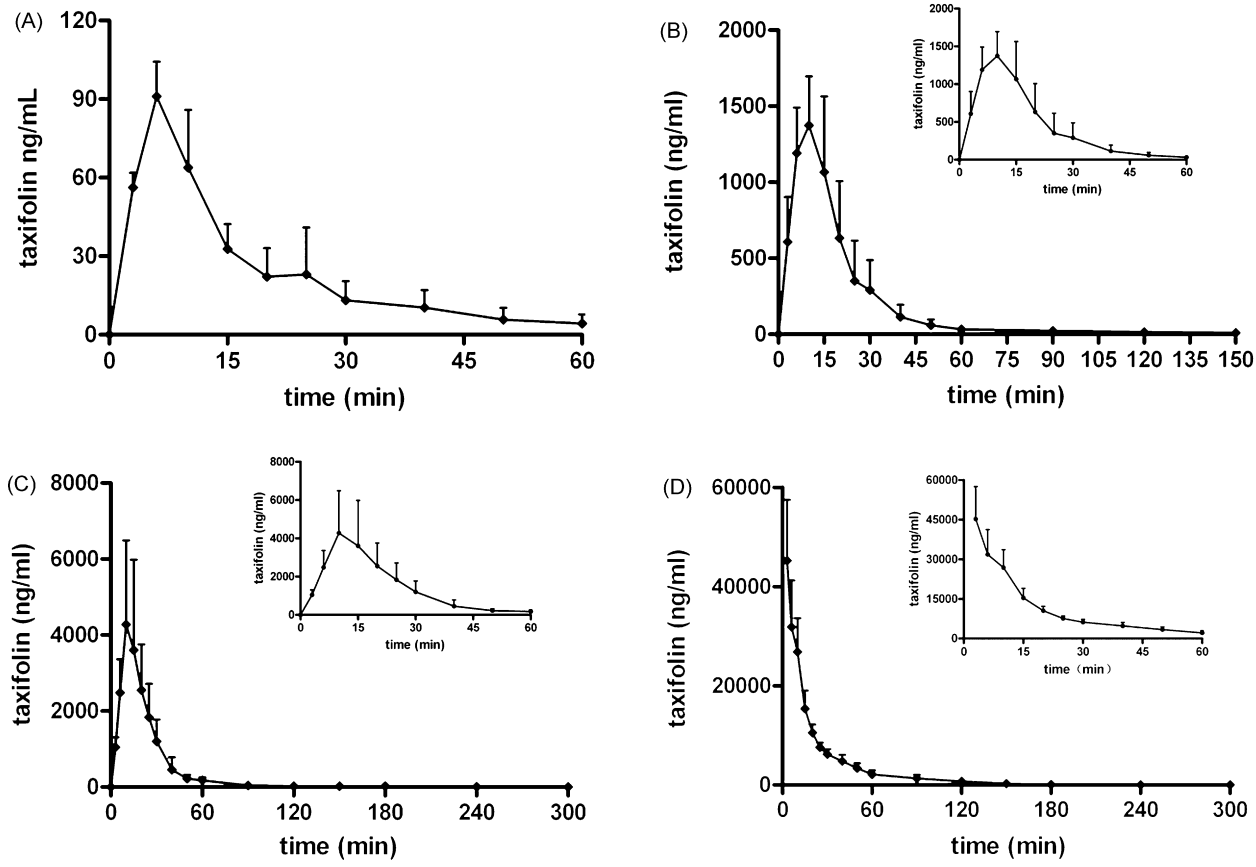

Fig. 4. Mean plasma concentration of taxifolin vs. time in rat plasma. (A) Oral administration of 10 mg/kg, (B) oral administration of 50 mg/kg, (C) oral administration of 100 mg/kg, and (D) intravenous administration of 10 mg/kg. Each point and bar represent the mean ± SD (n = 6).

Table 7
Pharmacokinetic parameters of taxifolin following a single oral dose of 10, 50 or 100 mg/kg (Mean ± SD, n = 6).

Pharmacokinetic parameters	Oral administration		
	10 mg/kg	50 mg/kg	100 mg/kg
C_{max} (ng/mL)	91.1 ± 13.2	1438.8 ± 359.7	4352.4 ± 2229.3
T_{max} (min)	6.0 ± 0.0	9.5 ± 3.3	10.2 ± 2.9
$t_{1/2}$ (min)	16.6 ± 9.3	48.0 ± 29.0	58.7 ± 12.3
MRT (min)	16.3 ± 2.4	21.4 ± 3.1	27.6 ± 5.6
AUC_{0-t} (ng min/mL)	1445.2 ± 410.8	28190.5 ± 9796.9	93548.9 ± 40190.1
$AUC_{0-\infty}$ (ng min/mL)	1575.3 ± 502.0	29256.9 ± 8874.3	93869.3 ± 40052.0
Ke (1 min^{-1})	0.039 ± 0.03	0.123 ± 0.037	0.098 ± 0.036
CL/F (L/(min kg))	6840579.0 ± 1801858.0	2120541.1 ± 1175838.1	1318941.5 ± 796076.3

3.7. Application to pharmacokinetic study

This UPLC–MS method developed has been used successfully in the pharmacokinetic studies in rats. The mean plasma concentration–time curves of taxifolin among the twenty-four rats with different dose of taxifolin (10, 50 or 100 mg/kg, after oral or intravenous administration) were shown in Fig. 4. The pharmacokinetic parameters of taxifolin were presented in Tables 7 (oral administration) and 8 (intravenous administration). For oral administration, the drug concentration was below LLOQ or LOD for the samples collected after 60 min in the 10 mg/kg group or after 150 min in the 50 mg/kg. T_{max} and $t_{1/2}$ are not proportional to the dose but they are depended to the dose with a coefficient of correlation of 0.9084 for T_{max} and 0.9425 for $t_{1/2}$.

Table 8
Pharmacokinetic parameters of taxifolin following intravenous administration of 10 mg/kg (n = 6).

Pharmacokinetic parameters	Mean ± SD	Pharmacokinetic parameters	Mean ± SD
C_{max} (ng/mL)	45211.9 ± 12284.4	K_{10} (1 min^{-1})	0.0737 ± 0.033
$t_{1/2\alpha}$ (min)	5.1 ± 2.2	K_{12} (1 min^{-1})	0.0748 ± 0.0949
$t_{1/2\beta}$ (min)	25.5 ± 2.4	K_{21} (1 min^{-1})	0.0522 ± 0.0204
$t_{1/2\gamma}$ (min)	245.8 ± 200.9	K_{31} (1 min^{-1})	0.004 ± 0.0022
CL (L/(min kg))	0.00967 ± 0.00121	K_{13} (1 min^{-1})	0.0192 ± 0.0092
AUC_{0-t} (ng min/mL)	888480.4 ± 100183.6	MRT_{0-t} (min)	26.0 ± 6.5
$AUC_{0-\infty}$ (ng min/mL)	1037486.6 ± 136804.2	$MRT_{0-\infty}$ (min)	26.3 ± 6.7

4. Discussion

At first, we used HPLC to detect taxifolin in rat plasma. However, the sensitivity was too low to detect plasma concentrations of taxifolin. LOQ of HPLC was about 52 ng/mL (about 1 ng of taxifolin in 20 μL injection volume), while for UPLC/MS, LOQ was 6 ng/mL (42 pg in 7 μL injection volume). Therefore, this UPLC/MS method established made it possible for fewer samples required and the extended calibration curve made it easy to perform animal pharmacokinetic studies.

The intensity of the molecular ion obtained in the negative mode was much stronger than that in the positive mode, so the ESI^- was chosen as the ionization source mode. It was amazing that the sensitivity of SIR mode was much higher than the multiple reaction monitoring (MRM) mode with S/N 23/1 for SIR mode and 13/1 for MRM mode at the concentration of LLOQ (6 ng/mL). This phenomenon maybe caused by the unstable daughter ions of taxifolin in MRM mode. Therefore, we chose the SIR mode as the detection mode in this study. MS detector conditions were optimized by infusing 1 μg/mL solutions of taxifolin or puerarin (acetonitrile–water containing 0.3% acetic acid, 10:90; v/v) into mass spectrometer in 150–600 m/z range. After optimizing experiments, 30 V of the cone voltage was selected for taxifolin and 48 V for puerarin, respectively. Although the source temperature and desolvation gas flow are important as they play the important roles in minimizing ion suppression and altering the sensitivity, in this study they seemed to have less important effects on the analytical method.

In order to achieve good resolution and symmetric peak shapes, it was found that the addition of 0.3% (v/v) acetic acid in water phase was an important factor for achieving optimal and stable sensitivity, a best peak shape and good ionization. As the chromatographic behaviors were different between taxifolin and puerarin, the gradient elution mode was selected as the UPLC conditions instead of isocratic elution mode. In order to detect the metabolites of taxifolin, especially the metabolites of glucuronidation, the run time was adjusted to 6.5 min (data of metabolites were not shown). A dwell time of 0.2 s was sufficient and no cross-talk was found between all SIRs. We also compared Xbridge™ C18 (2.1 mm × 50 mm, 3.5 μm, Waters) column with Sunfire™ C18 (2.1 mm × 50 mm, 3.5 μm) column, which has little differences on the chromatographic behaviors of taxifolin and puerarin. When using 200 μL weak wash solution (water–acetonitrile, 80:20; v/v) and 200 μL strong wash solution (water–acetonitrile, 20:80; v/v) as needle wash solution (with overfill), sometimes the carryover could be noticed. At concentrations of taxifolin ≥3000 ng/mL, carryover was equivalent to ~30 area units, approximately 8–10% of the area of the LLOQ peak (6 ng/mL). Then the volume of both weak and strong wash solution was increased to 800 μL, the carryover was abolished.

In order to maximize recoveries and facilitate the sample preparation, different sample preparation methods were tested. The recoveries were higher when using methanol or ethyl acetate as extraction solvent, but low for chloroform. Due to interferences, it was better to employ the liquid–liquid extraction using ethyl acetate as extraction solvent. No interference was found in the drug-free rat plasma extracted by ethyl acetate. Furthermore, the addition of 2 μL acetic acid was the key factor to obtain satisfied recovery of puerarin. The pK_{a1} was 7.20 ± 0.01 and pK_{a2} was 9.84 ± 0.08 for puerarin [15]. The recovery of puerarin was about 30% without the addition of 2 μL acetic acid.

It was found that the best fit pharmacokinetic model to estimate the pharmacokinetic parameters was single compartment model with weight of $1/C^2$ for oral administration, and three compartment model with weight of $1/C^2$ for intravenous administration by using DAS software. The area under the curve (AUC) and C_{max} was non-proportional to the dose of oral administration ranged from 10 to 100 mg/kg. T_{max} and $t_{1/2}$ were depended on the doses of oral administration. Those results indicated the absorption of taxifolin in rat by oral administration was a typical nonlinear process. We found that the biotransformation of taxifolin in rat was so fast that the metabolites can be detected at the first sampling time for both oral administration and intravenous administration (data was unpublished). The fast biotransformation and the saturation of the metabolic enzyme(s) at high dose administration may be the reason to cause the nonlinear process of taxifolin in rats. We also observed a weak "double-peak" phenomenon in the higher dose group of 100 mg/kg. The second peak was about 120–180 min apart. Those phenomena were very important for the further pharmacodynamic studies of taxifolin.

In this pharmacokinetics study, the absolutely bioavailability of taxifolin in rats was 0.17%, which was according to the results of Caco-2 cells transport study. Voskoboinikova et al. had studied the pharmacokinetics of taxifolin in rats using HPLC in 1993 [17]. According to their results, taxifolin could be detected in blood plasma only in trace amounts after oral administration. Nonlinear pharmacokinetic behavior was demonstrated for taxifolin when administered intravenously to rats. Pozharitskaya et al. reported the pharmacokinetics of taxifolin in rabbit recently [11]. In their previous papers, they found that solubility and bioavailability of taxifolin was increased in the form of self-microemulsifying system *in vitro* [18,19]. Therefore, taxifolin dissolved in Labrasol was used as dosing solution for oral administration. The AUC after oral administration at dose of 80 mg/kg was 2.5 times more of the AUC after oral administration at dose of 8 mg/kg. They found that taxifolin showed a relatively high bioavailability of 36%, which was different with the result of our study (0.17%). They detected the total of conjugated and free taxifolin *in vivo* because the blood samples were analyzed after enzymatic hydrolysis with β-glucuronidase and sulfatase. The results of their studies suggested that the bioavailability of taxifolin from lipid solution for oral administration was higher than that by oral administration of taxifolin tables, which may result from the more efficient absorption of lipid solution owing to the improvement of permeability. Grimm et al. also did the pharmacokinetics study of taxifolin in the maritime pine bark extract in healthy volunteers [16]. The pharmacokinetic parameters were determined from healthy volunteers plasma after oral administration of pycnogenol tablets at the dose of 14.35 μg taxifolin per mg pycnogenol. They found that taxifolin was not detected before 2 h after ingestion of the pine bark extract and maximal plasma concentrations were recorded after 8 h, and they supposed that the compound's late appearance in plasma was due to metabolic degradation processes. Those studies indicated that the species differences between rabbit, rat and human were obviously.

5. Conclusion

A simple, rapid and specific UPLC–MS method has been established for investigating pharmacokinetics of taxifolin in rats. The method results in high sensitivity with a lower limit of quantitation of 6 ng/mL, wide linearity, specificity and no interferences from endogenous substances. The absolute bioavailability of taxifolin was 0.17%.

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