Journal of Chromatography B, 911 (2012) 34–42

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Identification and assay of 3′-O-methyltaxifolin by UPLC–MS in rat plasma

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ARTICLE INFO

Article history:
Received 9 July 2012
Accepted 2 September 2012
Available online 4 October 2012

Keywords:
3′-O-methyltaxifolin
Taxifolin
UPLC–MS
Pharmacokinetics

ABSTRACT

A new metabolite of taxifolin: 3′-O-methyltaxifolin (3′-O-MTAX) in Caco-2 cells and in rat plasma was identified. The chemical structure of 3′-O-MTAX was determined by MS and ^{1}H NMR. A rapid, sensitive and specific UPLC–MS method to determine 3′-O-MTAX in rat plasma was also developed. Following ethyl acetate extraction, 3′-O-MTAX in plasma was separated on a Sunfire^{TM} (2.1 mm × 50 mm, 3.5 μm) column and analyzed in the selected ion recording with a negative electrospray ionization mode using puerarin as the internal standard. The lower limit of quantification (LLOQ) was 2.75 ng/mL. Intra- and inter-day precisions (% RSD) were all within 7.2% and accuracy (% deviation) ranged from −5.0 to 4.7%. The overall recoveries at four concentrations were all >72.0%. This validated method was successfully applied to measure 3′-O-MTAX in rat plasma after oral administration of taxifolin.

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1. Introduction

Taxifolin (dihydroquercetin, $C_{15}H_{12}O_7$, Fig. 1A), 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 [1]. Taxifolin has many activities such as protecting cerebral ischemic reperfusion injury, inhibition of triglyceride synthesis and antimicrobial effect [2–5]. Taxifolin has been widely used as an additive in food and health care products, and in the treatment of cerebral infarction and sequel as cerebral thrombus, coronary heart disease and angina pectoris [6,7].

Booth and De Eds [8] reported the conversion of taxifoin to 3,4-dihydroxyphenylacetic, m-hydroxyphenylacetic, and 3-methoxy-4-hydroxyl-phenylacetic acids in the urine of two human volunteers using chromatographic examination. Brown and Griffiths [9] had found some new biliary metabolites of taxifolin in rats. In their study, following the i.p. administration of taxifolin, 13 metabolites were detected in bile. The metabolites were hydrolyzed by a mixed glucuronidase/aryl sulphatase preparation to give 1 of 3 aglycone products: T1, T2 and T3. Separation of these aglycones was effected by TLC and the eluted compounds were submitted to mass spectrometry and UV-spectroscopy. The mass spectrum analysis indicated that T2 was 3′ or 4′-O-methyltaxifolin. However, due to the absence of authentic standards, the authors did not determine the exact structure of the metabolite.

Seredin et al. [10] reported a number of taxifolin metabolites in the urine of rats, predominantly derivatives of diastereomers of taxifolin. Two unchanged diastereomers of taxifolin, 2R, 3R-trans and 2R, 3S-cis, and two metabolites of these compounds were detected in the urine. Mass spectrometry indicated that the two metabolites were methylated derivatives of the diastereomers of taxifolin. In addition, four more compounds of the biotransformation were detected in the urine: glucuronconjugate of diastereomer 2R, 3R-trans and glucuronconjugate of diastereomer 2R, 3S-cis and their respective methylated metabolites.

In our previous studies, the bioavailability for oral administration of taxifolin was predicted to be low by using Caco-2 cell monolayer model in vitro [11]. Those results were proven later by our pharmacokinetic studies of taxifolin: the absolutely bioavailability of taxifolin was about 0.17% in rats [12].

Using ultra performance liquid chromatography–mass spectrometry (UPLC–MS) and NMR, a new metabolite of taxifolin in rat plasma: 3′-O-methyltaxifolin ($C_{16}H_{14}O_7$, 3′-O-MTAX, Fig. 1B) was identified. 3′-O-MTAX was also found to be the major metabolite of taxifolin metabolized by Caco-2 cells in our previous studies. Further more, a rapid and sensitive UPLC–MS method to determine 3′-O-MTAX in rat plasma was developed with a simple procedure of liquid–liquid extraction. Puerarin ($C_{21}H_{20}O_9$, Fig. 1C), an isoflavone, was used as the internal standard in this analytical method. The method exhibited excellent performance with high selectivity, rapid, sensitive, accurate, robustness, and the method was successfully applied to determine the 3′-O-MTAX in rat plasma after oral administration of taxifolin.

Abbreviations: 3′-O-MTAX, 3′-O-methyltaxifolin; LLOQ, lower limit of quantification; ULOQ, upper limit of quantification; LOD, the limit of detection; RSD, relative standard deviation; IS, internal standard; DMEM, Dulbecco's modified eagle's medium; ESI$^-$, negative-ion electrospray ionization; SIM, selected ion monitoring; QC, quality control.

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http://dx.doi.org/10.1016/j.jchromb.2012.09.006

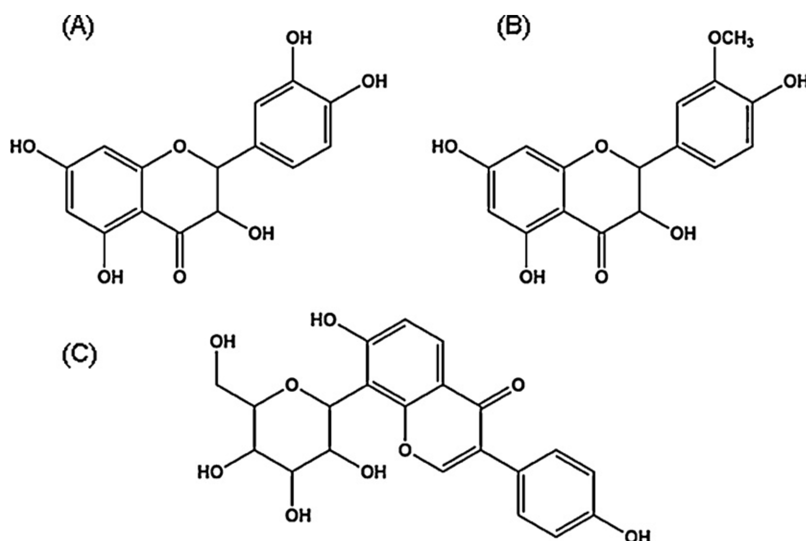

Fig. 1. Chemical structures of taxifolin (MW = 304) (A), 3′-*O*-methyltaxifolin (MW = 318) (B) and puerarin (MW = 416) (C).

2. Materials and methods

2.1. Chemicals and reagents

Taxifolin (98%) was purchased from Nanjing Sulang Medical Technology Development Co., Ltd (Nanjing, Jiangsu, China). Puerarin was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). 3′-*O*-MTAX with the purity of 99.1% was prepared in our laboratory. DMSO was purchased from Sigma Chemical Co. (Saint Louis, MO, USA). HPLC-grade acetonitrile and acetic acid were bought from TEDIA Inc. (Fairfield, USA). DMSO-D_6 was bought from Apllichem GmbH (Gatersleben, Saxony-Anhalt, Germany). All other reagents were of analytical grade or HPLC grade. Ultra-pure water was obtained from an ELGA-purelab Ultra system (High Wycombe, UK).

Caco-2 cells were obtained from Chinese Academy of Medical Sciences (Beijing, China). Dulbecco's modified eagle's medium (DMEM, high-glucose), fetal bovine serum, nonessential amino acids, 0.25% trypsin–EDTA solution and antibiotic–antimycotic were purchased from Gibco BRL Life Technology (Grand Island, NY, USA). Cell culture dishes were purchased from Corning Costar Corp. (Bedford, MA, USA).

2.2. Cell culture

Caco-2 (passage 50–60) cells were cultured in DMEM with 10% fetal bovine serum, 1% nonessential amino acids, and 100 U/mL antibiotic–antimycotic. Cells were grown in a humidified atmosphere of 5% CO_2 at 37 °C. After reaching 80% confluence, Caco-2 cells were harvested with 0.25% trypsin–EDTA solution and seeded in culture dishes (∅, 9 cm) at a density of 1.0×10^5 cells/cm^2.

2.3. Metabolite of taxifolin in Caco-2 cells and the preparation of 3′-O-MTAX

After overnight culture, the medium was aspirated and replaced by the fresh culture medium containing 80 μg/mL taxifolin. The culture medium was collected after incubation with taxifolin for 48 h and then extracted using acetic ether. The upper organic phase was transferred into clean tubes and evaporated to dryness with vacuum at room temperature.

The separation of the metabolites and repeated column purification of 3′-*O*-MTAX were performed using HPLC. The HPLC condition was as follows: an Agilent 1200 HPLC system composed of a quaternary pump with a degasser, a thermostatted column compartment, a variable wavelength detector operated at 291 nm, an auto-injector, and Agilent ChemStation software. The HPLC separation was performed on a Waters Symmetry C18 column (5 μm, 150 mm × 4.6 mm, Waters, Milford, MA, USA) with a guard C18 column maintained at 30 °C. The mobile phase consisted of methanol–water containing 0.3% acetic acid (33:67, v/v) at a flow rate of 1.0 mL/min.

2.4. Identification of metabolites

The metabolites were identified by using a Waters ACQUITY^{TM} TQD mass spectrometer with direct loop injection and negative-ion electrospray ionization (ESI$^-$) MS analysis. ^{1}H NMR and ^{1}H–H NEOSY NMR spectrums of one major metabolite were recorded on Bruker Avance III (500 MHz). The purity of the major metabolite was determined by HPLC. The conditions of HPLC determination was as follows: an Agilent 1200 HPLC system composed of a quaternary pump with a degasser, a thermostat column compartment, a variable wavelength detector operated at 291 nm, an auto-injector, and Agilent ChemStation software. The mobile phase consisted of methanol–water containing 0.3% acetic acid (33:67, v/v) at a flow rate of 1.0 mL/min.

2.5. UPLC–MS analytical conditions

A Waters ACQUITY^{TM} TQD with the power of Ultra Performance Liquid Chromatography (Waters, Milford, MA, USA) was used in this 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. A gradient elution was employed with a starting at 10% acetonitrile and 90% water containing 0.3% acetic acid, then progressing linearly to 35% acetonitrile and 65% water containing 0.3% acetic acid over 6.5 min. The mobile phase was directly delivered into the ESI$^-$ source at 0.2 mL/min. Injection volume was 7 μL. The temperature of the sample manager was kept at room temperature (20 °C).

MS analysis was performed in the ESI$^-$ mode and using the selected ion monitoring (SIM) mode. Nitrogen was used as desolvation gas at 500 L/h flow rate. The temperature of ion source and desolvation gas was set at 120 and 350 °C, respectively. The

Table 1
Selected ion m/z values and optimized mass spectrometric parameters for the UPLC–MS analysis of 3′-*O*-MTAX and puerarin in selected ion monitoring (SIM) mode.

	m/z	Capillary voltage (kV)	Cone voltage (V)	RF lens voltage (V)	Extractor (V)
3′-*O*-MTAX	317.0	2.6	30	0.1	3
Puerarin	415.2	2.6	48	0.1	3

optimized MS parameters were listed in Table 1. The analytes were assayed by quantifying the $[M-H]^-$ ions of 3′-*O*-MTAX at m/z 317.0, and puerarin at m/z 415.2.

Masslynx™ V 4.1 software (Micromass, Manchester, UK) was employed for instrument control, data acquisition and processing.

2.6. Preparation of standard and quality control samples

The stock standard solutions of 3′-*O*-MTAX and puerarin were prepared by dissolving accurately weighed individual compounds in DMSO to give a final concentration of 1.1 and 1.2 mg/mL, respectively. A series of standard working solutions at concentrations over 27.5–30,000 ng/mL for 3′-*O*-MTAX were obtained by further dilution of the standard stock solution with acetonitrile–water containing 0.3% acetic acid (10:90, v/v). Internal standard working solution (1.2 μg/mL) was prepared by diluting the internal standard stock solution with acetonitrile–water containing 0.3% acetic acid (10:90, v/v). All solutions were stored in the dark at −20 °C and brought to room temperature (20 °C) before use.

Blank rat plasma was collected from six drug-free 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 the validation and each experimental run of the pharmacokinetic studies. Standard working solutions (8 μL 3′-*O*-MTAX, 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. Calibration samples were made at the concentration of 2.75–3000 ng/mL. QC samples were prepared at the concentrations of 2.75, 5.5, 550 and 2200 ng/mL. The dilution test samples above the upper limit of quantification (ULOQ) were prepared by spiking the stock standard solutions of 3′-*O*-MTAX into blank rat plasma, yielding spiked plasma samples containing 27.5 μg/mL 3′-*O*-MTAX.

2.7. Animal and administration

The study was approved by the Animal Ethics Committee of Zhejiang University. Male Sprague-Dawley rats (180–200 g) were bought from 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 before experiment. Twelve rats were divided into two groups at random. One group was given single dose of taxifolin solution at 100 mg/kg by oral administration. The other 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 two hours 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, 30, 40, 60, 90, 120, 180, 240, 300 and 360 min after dosing.

2.8. Plasma sample preparation

Each blood sample was immediately centrifuged after collected at 4000 rpm for 10 min at 4 °C and 80 μL plasma was transferred into 0.5 mL centrifuge tube and stored at −20 °C until analysis. The plasma samples were spiked with 10 μL internal standard working solution and then 2 μL acetic acid. All samples were vortex-mixed (Eppendorf, Hamburg, Germany) for 30 s and extracted with 240 μL acetic ether by a vortex mixer 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.

2.9. Method validation

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 concentration of 3′-*O*-MTAX with a signal three times to noise.

Calibration curve of 3′-*O*-MTAX was gained by analyzing the standards samples and used in each run. Peak-area ratios of the 3′-*O*-MTAX to internal standard were calculated and the calibration curve was established by fitting these ratios against the corresponding concentrations by a linear regression method.

QC samples were processed in five replicates at four concentration levels for 3′-*O*-MTAX. 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 27.5 μg/mL 3′-*O*-MTAX. The dilution test samples were diluted 12.5 times using blank rat plasma to yield a final nominal concentration of 2200 ng/mL and were analyzed in one analytical run.

The matrix effect on the ionization of the compounds was assessed by comparing the peak areas of the 3′-*O*-MTAX dissolved in the blank sample (the final solution of blank plasma after extraction and dissolution) with that dissolved in the mobile phase. Four different concentration levels of 3′-*O*-MTAX were evaluated by analyzing the five samples at each level.

The recovery (extraction efficiency) of 3′-*O*-MTAX and puerarin from rat plasma after the sample preparation procedure were determined by comparing the peak areas of extracted 3′-*O*-MTAX or puerarin with the area of the same concentration level dissolved in the blank sample (the final solution of blank plasma after extraction and dissolution) with mobile phase.

The stability of 3′-*O*-MTAX was investigated under various storage conditions using QC samples with five replicates for each concentration. Freeze–thaw stability was assessed by using QC samples stored at −20 °C and subjected to three freeze–thaw

cycles: frozen at −20 °C for 1 day per cycle and thawed (without warming) at room temperature. The long-term stability of 3′-*O*-MTAX was assessed using QC samples after storage at −20 °C for 20 days. The stability for test solution (extracted sample) was assessed in the auto sampler at 20 °C (room temperature) for 12 h. Short-term stability was assessed using QC samples at room temperature for 6 h.

During routine analysis, each analytical run included a blank plasma sample, a set of calibration samples, a set of QC samples ($n = 2$) and unknowns.

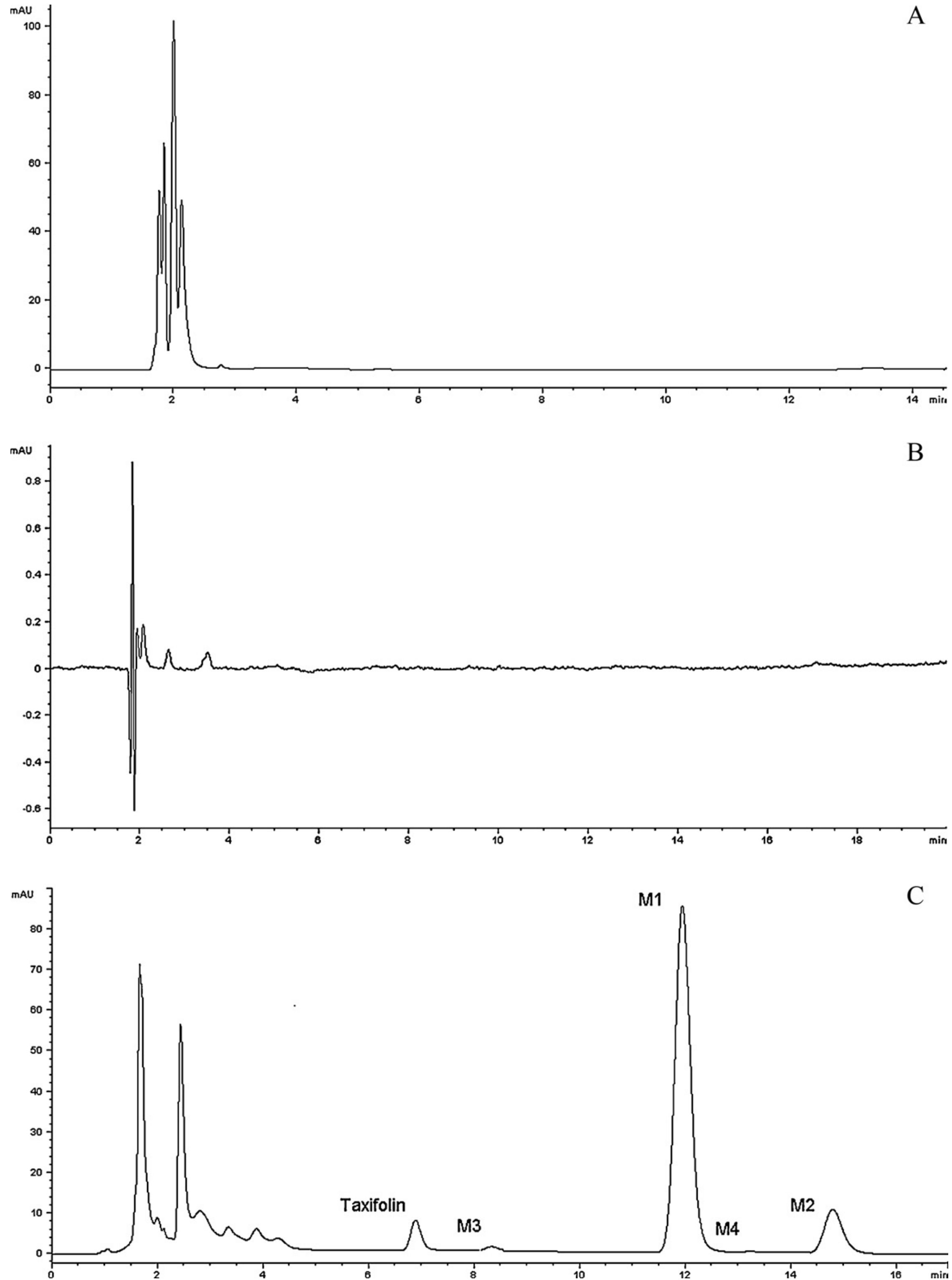

Fig. 2. Typical HPLC chromatography of the metabolite of taxifolin by Caco-2 cells. (A) blank culture medium; (B) blank culture medium extracted with acetic ether; (C) culture medium of Caco-2 cells incubated with taxifolin for 48 h; (D) culture medium of Caco-2 cells containing taxifolin and its metabolites after extracted with acetic ether; (E) M1 prepared using HPLC.

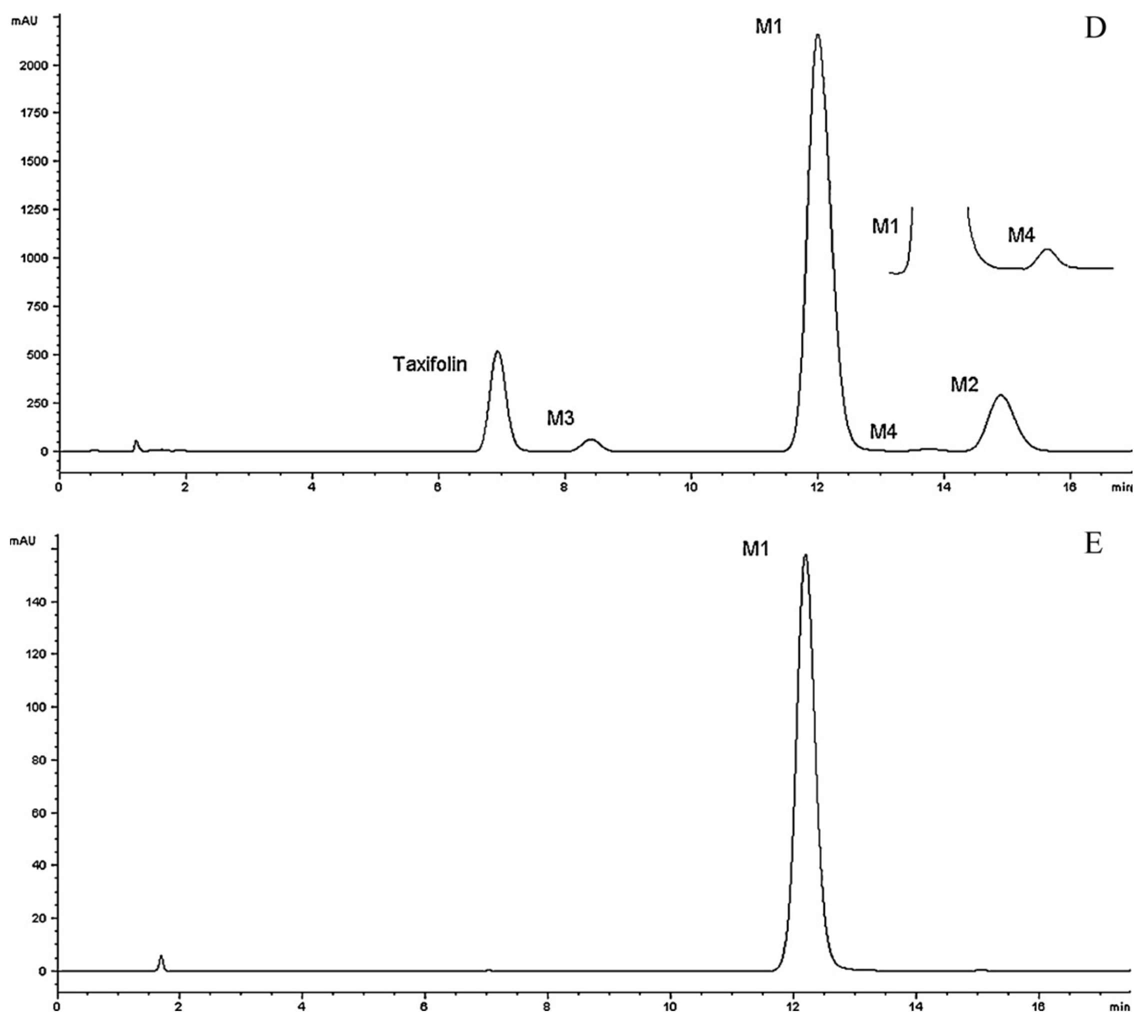

Fig. 2. (*Continued*).

3. Results and discussion

3.1. In vitro metabolism of taxifolin and identification of the metabolites

Incubation of taxifolin with Caco-2 cells led to form four new metabolites of taxifolin (Fig. 2). After repeated column purifications using HPLC, the purity of 3′-*O*-MTAX was 99.1% quantitated by area normalization method. In order to characterize the metabolic profiles of taxifolin, the possible structure of metabolites were speculated according to the rule of drug metabolism and the metabolism of astilbin, the glycoside of taxifolin. Astilbin can be metabolized into 3′-*O*-methylastilbin when incubation with rat liver microsomal/cytosolic fractions in vitro [13]. ESI$^-$ MS analysis showed that the [M−H]$^-$ of those metabolites were 317 (M1), 317 (M2), 303 (M3) and 285 (M4) (Fig. 3) indicating the molecular weight of M1 and M2 were 14 mass units higher than taxifolin.

^{1}H NMR data of taxifolin and its biosynthesized metabolite M1 were shown in Table 2. M1 showed chemical shifts and coupling patterns similar to those of taxifolin, except for the appearance of proton (δ 3.788) signal derived from a methyl group. The position of the methoxy group was confirmed by the observation of a nuclear Overhauser effect between methoxy protons and 2′-H. Irradiation of the resonance at 3.788 ppm (—OCH_3) produced a nuclear Overhauser effect at the meta-coupled doublet (d, *J* = 1.92) at 7.112 ppm (2′-H). The structure of M1 was further supported by ^{1}H–H NEOSY NMR experiment, which a correlation between the signals at 7.112 (d, *J* = 1.92, 2′-H) and 3.788 (s, —OCH_3) was observed. Thus, the M1 was identified as 3′-*O*-MTAX, the phase II metabolite of taxifolin. To the best of our knowledge, this is the first evidence for 3′-*O*-MTAX as a new flavonoid and as a metabolite of taxifolin. The enzyme responsible for this metabolism may be catechol-*O*-methyltransferase, which is an intracellular enzyme

Table 2
^{1}H NMR spectral data of taxifolin and 3′-*O*-methyltaxifolin (3′-*O*-MTAX).

Position	^{1}H NMR	
	Taxifolin	M1
—OH	11.893 (s, 1H)	11.92 (s, 1H)
—OH	10.802 (s, 1H)	10.79 (s, 1H)
—OH	9.002 (s, 1H)	9.14 (s, 1H)
—OH	8.949 (s, 1H)	
2′-H	6.879 (br, s, 1H)	7.112 (d, *J* = 1.92, 1H)
6′-H	6.748 (br, s, 2H)	6.905 (dd, *J* = 8.117, 1.935, 1H)
5′-H		6.79 (d, *J* = 8.062, 1H)
8-H	5.911 (d, *J* = 2.031, 1H)	5.918 (d, *J* = 2.062, 1H)
6-H	5.863 (d, *J* = 2.026, 1H)	5.87 (d, *J* = 2.058, 1H)
3-OH	5.734 (d, *J* = 6.176, 1H)	5.76 (d, *J* = 4.587, 1H)
2-H	4.983 (d, *J* = 11.126, 1H)	5.044 (dd, *J* = 11.43, 4.25, 1H)
3-H	4.497 (dd, *J* = 11.126, 6.176, 1H)	4.66 (d, *J* = 11.44, 1H)
—O—CH_3		3.788 (s, 3H)

Recorded in DMSO at 500 MHz; chemical shift (δ) values in ppm and coupling constant (*J*) values in Hz. Splitting patterns: s, singlet; brs, broad singlet; d, doublet; dd, double of doublets.

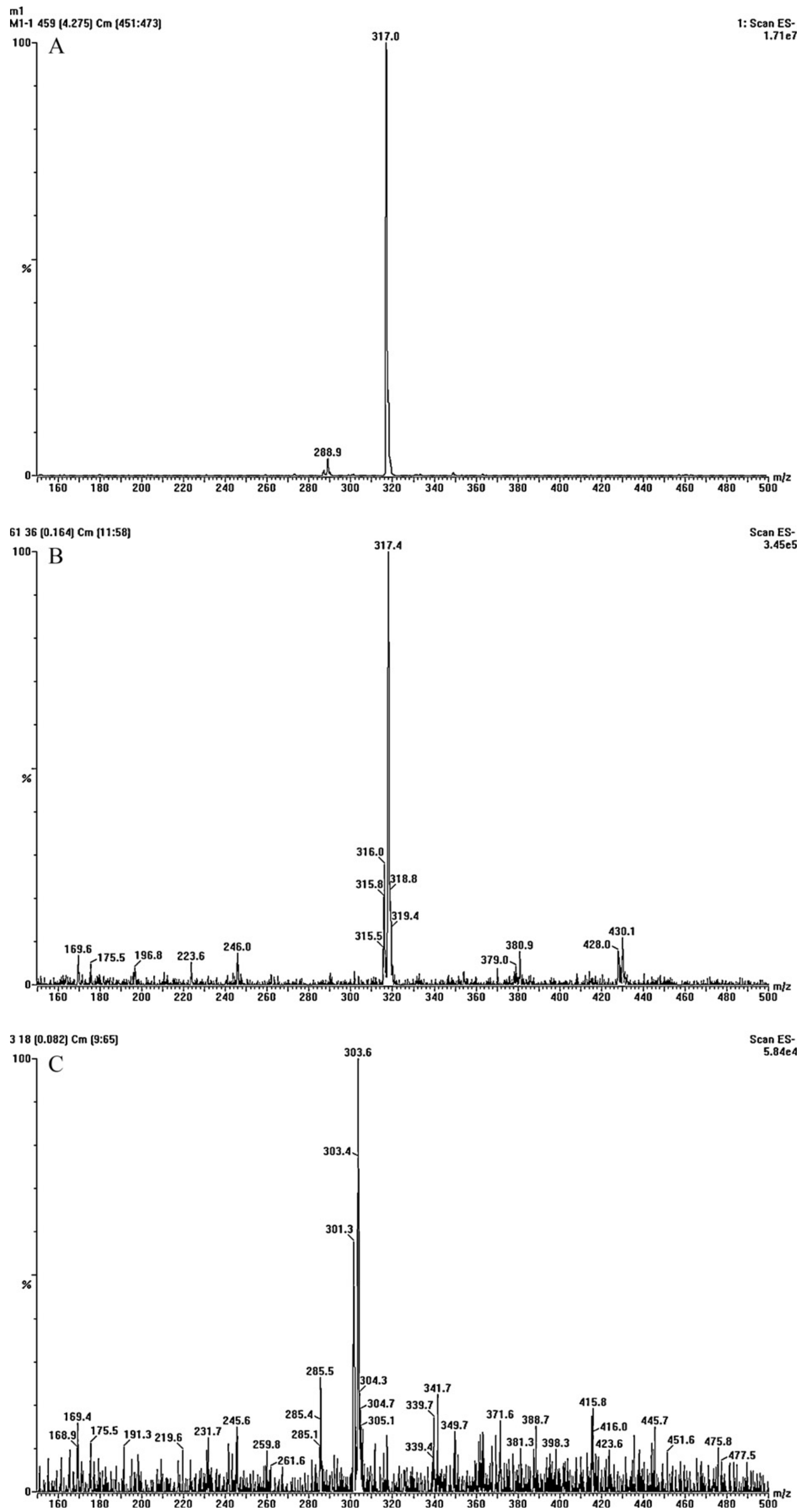

Fig. 3. Full-scan ion spectrums of M1 (A); M2 (B); M3 (C); and M4 (D).

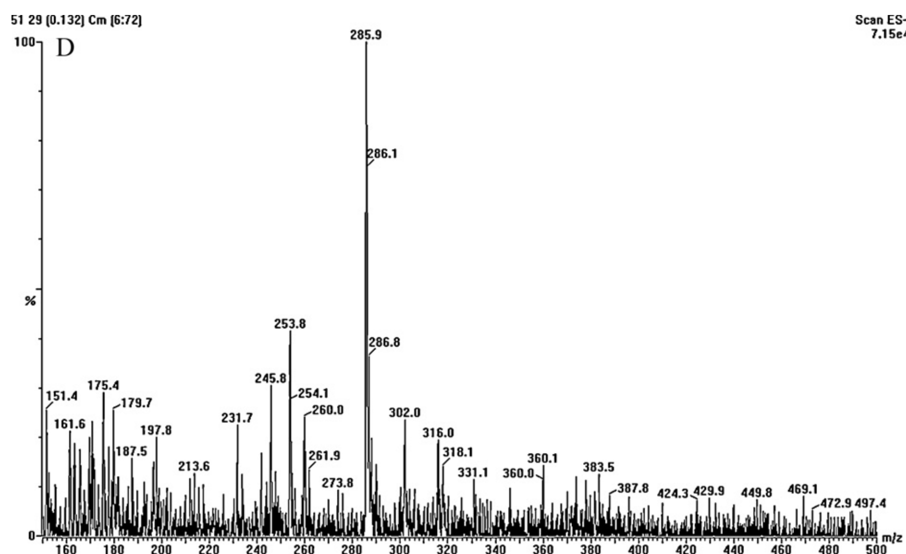

Fig. 3. (*Continued*).

widely distributed throughout the mammalian organs and is able to methylate only one of the two neighbor catechol hydroxyls [14]. According to the *O*-methylation of astilbin [13], there was a selective formation in the metabolism of taxifolin. This was another evidence for the catechol-*O*-methyltransferase involved in the transformation of flavone favored 3′-*O*-methylation over 4′-*O*-methylation. Identification of the other metabolites needed more work and was in process.

3.2. Evaluation of the UPLC–MS method

MS parameters were optimized in order to achieve the maximum response of 3′-*O*-MTAX and puerarin. Acquisition parameters were determined by direct injection of 1 μg/mL of each compound in mobile phase into the mass spectrometer in both positive ion and negative ion modes. Although 3′-*O*-MTAX and puerarin could be detected in both positive ion and negative ion modes, the response of 3′-*O*-MTAX and puerarin was much better in negative ion mode with higher sensitivity. So the negative ion mode was selected as the ionization source mode for MS analysis. The optimized MS parameters of 3′-*O*-MTAX and puerarin were shown in Table 1. The quasi-molecular ion $[M-H]^-$ peaks of 3′-*O*-MTAX appeared at m/z 317.0 (Fig. 3), and SIM mode was chose as the detection mode in this study.

Several UPLC method variables influencing the separation of 3′-*O*-MTAX and puerarin were investigated in our extensive preliminary experiments. It was found the addition of 0.3% (v/v) acetic acid in water phase was an important factor for achieving an optimal, stable sensitivity, the best peak shape and ionization of 3′-*O*-MTAX and puerarin.

3.2.1. Selectivity

The selectivity of the method was evaluated by analyzing blank rat plasma samples from six different rats. Typical chromatograms of the blank and spiked plasma were given in Fig. 4. The retention times of 3′-*O*-MTAX and puerarin were about 5.37 and 2.46 min. Endogenous peaks at the retention time of the analytes were not observed for any of the blank rat plasma batches. This indicated the UPLC–MS method with SIM acquisition was suitable for the selective detection of these compounds in rat plasma.

3.2.2. Matrix effects and recovery

The absolute matrix effect was evaluated by comparing the peak areas obtained from the spike after extraction of 3′-*O*-MTAX (2.75, 5.5, 550 and 2200 ng/mL, $n = 5$) and puerarin (150 ng/mL, $n = 5$) to those obtained from the standards in the mobile phase at equivalent concentrations. The ratios were 98.6, 104.7, 99.8, 97.1 and 98.9% for LLOQ, low, middle and high concentrations of 3′-*O*-MTAX and puerarin, respectively. Those results indicated there were insignificant matrix effects.

The recoveries of 3′-*O*-MTAX from rat plasma were 72.1, 72.2, 75.6 and 78.0% for LLOQ, low, middle and high concentrations, respectively. The recovery of puerarin from rat plasma was 78.6%. The mean recoveries were more than 72% at four concentration levels.

3.2.3. Linearity and calibration curve

The calibration curve of 3′-*O*-MTAX was constructed by plotting the peak-area ratio of 3′-*O*-MTAX to puerarin (y) versus analyte concentration (ng/mL) in spiked blank rat plasma (x).

Table 3
Calibration standards of 3′-*O*-methyltaxifolin in rat plasma calibration curves ($n = 5$).

Nominal concentration (ng/mL)	Mean measured concentration (ng/mL)	Deviation (%)	RSD (%)
2.75	3.0	9.1	3.2
11	10.6	−3.9	1.8
55	58.8	6.8	1.5
275	266.0	−3.3	3.3
440	442.6	0.6	3.5
880	881.6	0.2	3.9
3000	2999.8	−0.1	2.2

Table 4
Accuracy and precision for the analysis of 3′-*O*-methyltaxifolin ($n = 5$).

	QC (ng/mL)	Precision		Accuracy	
		Mean ± SD	RSD (%)	Mean nominal (%)	Deviation (%)
Intra-day	2.75	2.9 ± 0.1	3.5	104.7	4.7
	5.5	5.2 ± 0.1	2.5	95.1	−4.9
	550	555.7 ± 19.7	3.6	101.0	1.0
	2200	2089.7 ± 83.3	3.9	94.9	−5.0
Inter-day	2.75	2.8 ± 0.1	3.2	102.7	2.7
	5.5	5.4 ± 0.3	4.9	98.5	−1.5
	550	553.9 ± 14.0	2.5	100.7	0.7
	2200	2120.5 ± 152.9	7.2	96.4	−3.6

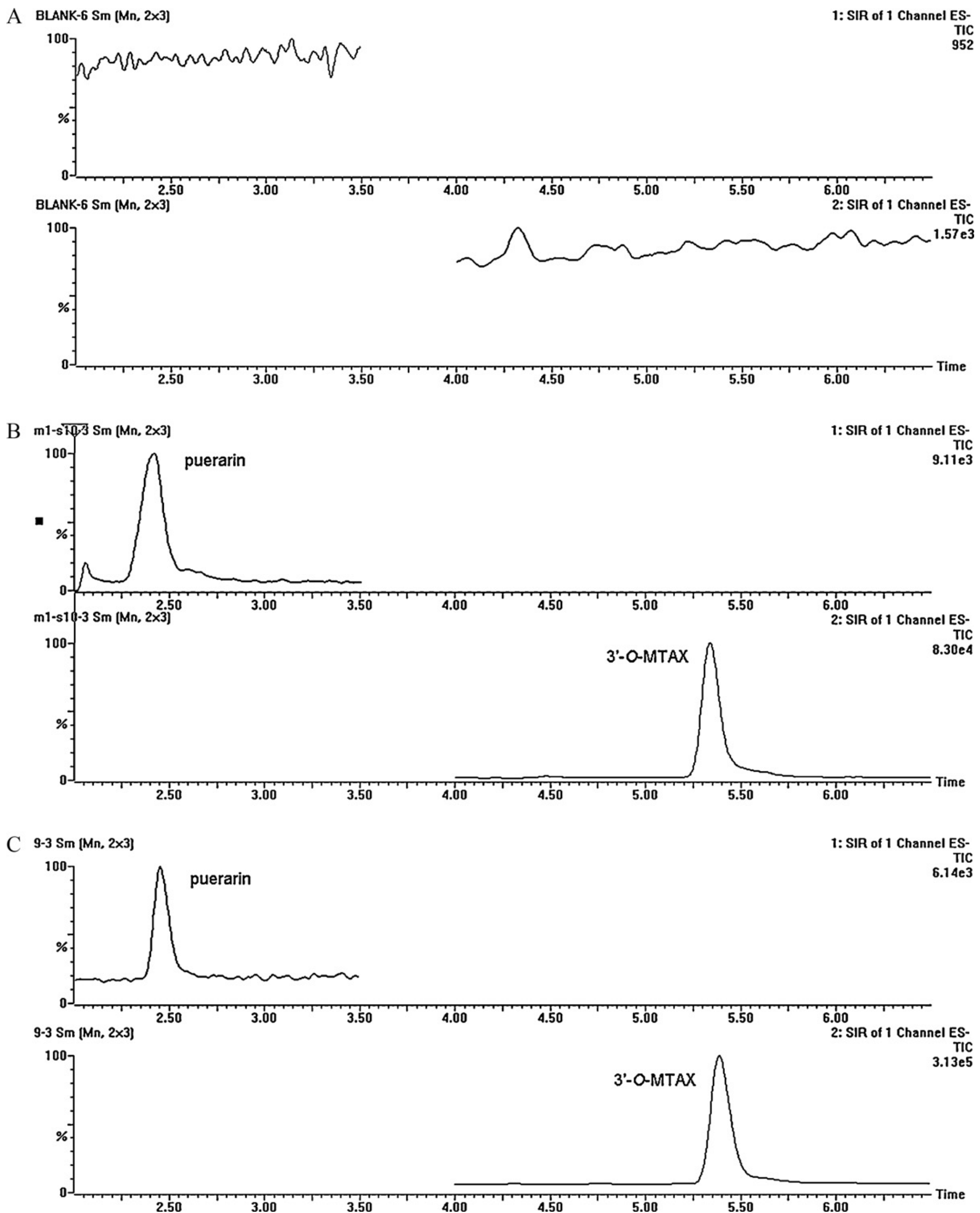

Fig. 4. Representative selected ion monitoring (SIM) chromatograms of 3′-*O*-MTAX and puerarin in rat plasma. (A) blank rat plasma sample; (B) rat plasma sample spiked with 3′-*O*-MTAX (55 ng/mL) and puerarin (150 ng/mL); and (C) a rat plasma sample 50 min after oral administration. The retention times of puerarin and 3′-*O*-MTAX were 2.46 and 5.37 min respectively.

The peak-area ratios of 3′-*O*-MTAX to puerarin in rat plasma varied linearly with concentration over the range: 2.75, 11, 55, 275, 440, 880 and 3000 ng/mL. The standard error of slope was 0.009589 (RSD = 6.82%, $n = 5$), and the standard error of intercept was 0.01202 (RSD = 10.12%, $n = 5$). The LOD of 3′-*O*-MTAX was estimated at 0.687 ng/mL which produced a signal-to-noise of 3/1. The LLOQ of 3′-*O*-MTAX was 2.75 ng/mL (RSD = 3.5%, $n = 5$, signal-to-noise > 15/1). Concentrations were back-calculated from nominal concentrations and were summarized in Table 3. The deviations of the back-calculated concentrations from the nominal concentrations were between −3.85% and 9.07% with RSD between 1.52% and 3.95% for all concentrations in rat plasma.

3.2.4. Accuracy and precision

The intra- and inter-day accuracy and precision data at four concentrations of 3′-*O*-MTAX were listed in Table 4. The intra-day precision (expressed as mean ± SD and RSD%) ranged from 2.5 to 3.9%, and the inter-day precision ranged from 2.5 to 7.2%. The intra-day accuracy (expressed as percent of nominal values) ranged from 94.9 to 104.7%, and the inter-day accuracy

Table 5
The stability of 3′-*O*-MTAX in rat plasma at different storage conditions ($n = 5$).

Concentration (ng/mL)	Calculated concentration	
	Mean ± SD	Recovery (%)
Freeze–thaw stability (3 freeze–thaw cycles)		
5.5	5.7 ± 0.1	103.6
550	556.3 ± 22.5	101.1
2200	2235.3 ± 14.3	101.6
Short-term stability (room temperature for 6 h)		
5.5	5.5 ± 0.2	100.0
550	556.7 ± 4.7	101.2
2200	2194.0 ± 21.5	99.7
Long-term stability (−20 °C for 20 days)		
5.5	5.7 ± 0.2	103.6
550	567.5 ± 11.0	103.2
2200	2188.0 ± 36.0	99.5
Post-preparative stability (20 °C for 12 h)		
5.5	5.6 ± 0.2	101.8
550	564.0 ± 7.7	102.5
2200	2191.2 ± 59.6	99.6

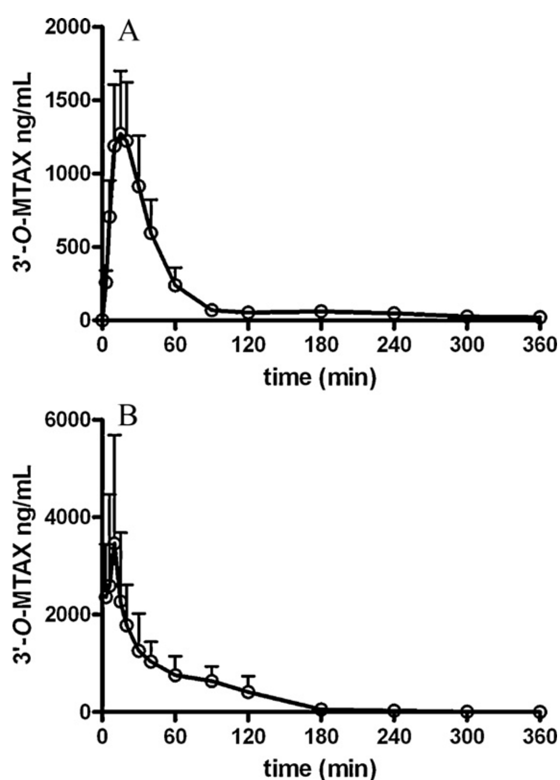

Fig. 5. Mean plasma concentration of 3′-*O*-MTAX vs. time in rat plasma. (A) Oral administration of 100 mg/kg taxifolin; (B) intravenous administration of 10 mg/kg taxifolin. Each point and bar represents the mean ± SD ($n = 6$).

ranged from 96.4 to 102.7%. The results demonstrated that the method established had a satisfactory accuracy, precision and reproducibility.

3.2.5. Stability

QC samples of 3′-*O*-MTAX at three concentrations (5.5, 550 and 2200 ng/mL) were used for stability experiments. The stability of 3′-*O*-MTAX was tested under the following conditions: (1) short-term stability of 3′-*O*-MTAX in rat plasma at room temperature for 6 h; (2) long-term stability of 3′-*O*-MTAX in rat plasma stored at −20 °C for 20 days; (3) post-preparative stability of 3′-*O*-MTAX during storage in the auto sample at 20 °C (room temperature) for 12 h; (4) freeze–thaw stability of 3′-*O*-MTAX in rat plasma through three freeze–thaw cycles. The results in Table 5 indicated the analyte could be considered stable under the various conditions investigated.

3.3. UPLC–MS analysis of 3′-O-MTAX in rat plasma

The method was applied to the analysis of 3′-*O*-MTAX in rat plasma obtained after oral administration (100 mg/kg) or intravenous administration (10 mg/kg). The mean plasma concentration–time curve of 3′-*O*-MTAX was shown in Fig. 5.

4. Conclusion

The major metabolite of taxifolin: 3′-*O*-methyltaxifolin has been identified in vitro and in vivo for the first time. A simple, rapid and specific UPLC–MS method was established for determination of 3′-*O*-methyltaxifolin in rat plasma. The method has been employed to assay 3′-*O*-methyltaxifolin in rat plasma, and allows us to study the kinetics of taxifolin and the metabolism mechanism of taxifolin in human.

Acknowledgements

This project was supported by the National Major Project of China (81230080, 2012ZX09506001-004), Special Funds of National Clinical Medical and Pharmaceutical Study (L2011336) and Key Item of Medical Technology of Wuxi Hospital Administration Center (YGZF1104).

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