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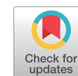
Original Article

Simultaneous determination of six bioactive components of total flavonoids of *Scorzonera austriaca* in rat tissues by LC-MS/MS: application to a tissue distribution study



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ABSTRACT

A liquid chromatography-tandem mass spectrometry method was developed and validated for simultaneous determination of six bioactive constituents including vitexin, orientin, isoorientin, 2″-*O*-β-D-xylopyranosyl isoorientin, 2″-*O*-β-D-xylopyranosyl isovitexin, and 6-*C*-L-α-arabipyranosyl vitexin in rats' various tissues using isoquercitrin as the internal standard. Biological samples were pretreated by protein precipitation with acetonitrile. Chromatographic separation was carried out on a C18 column with a gradient mobile phase consisting of acetonitrile and 0.1% aqueous formic acid. All analytes and internal standard were quantitated through electrospray ionization in negative ion selected reaction monitoring mode. The mass transitions were as follows: m/z 431 → 311 for vitexin, m/z 447 → 327 for orientin or isoorientin, m/z 579 → 459 for 2″-*O*-β-D-xylopyranosyl isoorientin, m/z 563 → 293 for 2″-*O*-β-D-xylopyranosyl isovitexin, m/z 563 → 353 for 6-*C*-L-α-arabipyranosyl vitexin, and m/z 463 → 300 for the internal standard, respectively. The lower limits of quantification for the six analytes in different tissue homogenates were 0.8–2.2 ng/ml. The validated assay was successfully applied to a tissue distribution study of the six components in rats after intravenous administration of total flavonoids of *Scorzonera austriaca* Willd; Asteraceae. The results of the tissue distribution study showed that the high concentrations of six components were mainly in the kidney.

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Introduction

Scorzonera austriaca Wild., Asteraceae, is a perennial herb widely distributed in the northwestern regions of China and usually recommended for the treatments of fever, enteritis, pregnant vomiting, carbuncle, mastitis, and swelling (Li et al., 1992, 1997). Modern research found that the major constituents of *S. austriaca* include flavonoids, sesquiterpenes, triterpenes, and steroids (Li et al., 2004; Zhu et al., 2009; Wu et al., 2011). Flavonoids are generally regarded as the major bioactive components, and total flavonoids from *S. austriaca* elicit significant anti-HBV and hepatoprotective effects (Xie et al., 2015; Zhang et al., 2015). Based on the previous pharmacological researches, flavonoids (vitexin (**1**), orientin (**2**), isoorientin (**3**), 2″-*O*-β-D-xylopyranosyl isoorientin (**4**), 2″-*O*-β-D-xylopyranosyl isovitexin (**5**), or 6-*C*-L-α-arabipyranosyl vitexin (**6**)) manifest a variety of pharmacological properties, including anti-inflammatory, anti-tumor, antiplatelet, antithrombotic, neuroprotective, and cardioprotective effects (Wang et al., 2015; Lee and Bae, 2015; Liu et al., 2016).

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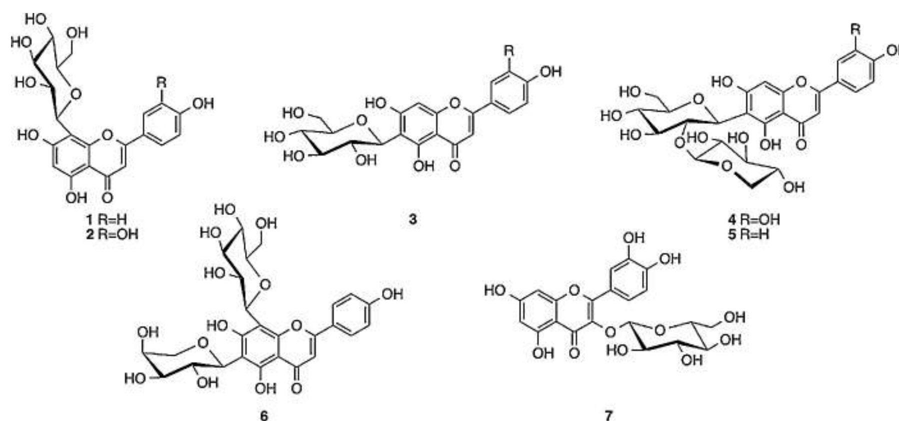

Until now, there are several studies were reported the determination of vitexin (**1**), orientin (**2**), isoorientin (**3**), 2″-*O*-β-D-xylopyranosyl isoorientin (**4**), 2″-*O*-β-D-xylopyranosyl isovitexin (**5**), or 6-*C*-L-α-arabipyranosyl vitexin (**6**) separately in biological samples with the method of HPLC, HPLC-MS/MS, and UPLC-MS/MS (Li et al., 2008a,b; Yan et al., 2013; Huang et al., 2012). In our previous investigations, we investigated the pharmacokinetic of these six flavonoid glycosides in rat plasma (Zhang et al., 2017). However, simultaneous determination of tissue distributions of these six flavonoid glycosides *in vivo* has not been reported. Here, a rapid liquid chromatography-tandem mass spectrometry (LC-MS/MS) method has been developed for the determination of **1**, **2**, **3**, **4**, **5**, or **6** in rat tissues after intravenous administration of total flavonoids of *S. austriaca*.

Materials and methods

Chemicals and reagents

Standard substances of **1**, **2**, **3**, **4**, **5**, and **6** were previously isolated from *Scorzonera austriaca* Wild., Asteraceae, in our laboratory, and identified by MS and NMR spectra. Total flavonoids of *S. austriaca* were prepared as previously described (Zhang et al., 2017), and the contents of the six components in the total flavonoids of *S. austriaca* were assayed by LC-MS/MS as described in Zhang et al. The internal standard (IS) isoquercitrin (**7**) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile and methanol of HPLC grade were purchased from Tedia company (Fairfield, OH, USA). HPLC grade formic acid was obtained from Tianjin Kemiou Chemical Reagent Co., Ltd. (Tianjin, China). Deionized water was purchased from Wahaha Co., Ltd. (Hangzhou, China).

LC-MS/MS conditions

Chromatographic separation was conducted on a Diamonsil® C_{18} column (4.6 × 250 mm, 5.0 μm ID) preceded by a Thermo Universal Filter 4.6/4 mm HPLC precolumn. The gradient elution consisted of acetonitrile (mobile phase A) and 0.1% aqueous formic acid (mobile phase B) was set as follows: starting at 5% A–95%B, increasing A to 20% in 5 min, maintaining 20% A for 9.5 min; then decreasing A to 5% in 0.5 min, keeping constant for 5 min. The flow rate was 0.80 ml/min with a split ratio of 1:1 and the sample injection volume was 10 μl. The total runtime was 20 min for each sample run.

MS detection was performed on a TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Scientific, San Jose, CA, USA) equipped with an ESI source in the negative ionization mode. The $[M-H]^-$ ions were used as the precursor for quantification in selected reaction monitoring (SRM) mode. The optimized tube lens and collision energy for six analytes and IS are shown in Table 1. Other parameters of MS analysis were as follows: spray voltage 3.0 kV, vaporizer temperature 300 °C, sheath gas (nitrogen) pressure, 35 AU; auxiliary gas (nitrogen) pressure, 10 AU; and capillary temperature, 270 °C. Xcalibur™ 2.2 Software was used for acquiring and processing data.

Preparation of standard and quality control (QC) samples

Mixed stock solution containing 200 μg/ml of **1** and **2**, 80 μg/ml of **3**, 220 μg/ml of **4**, **5**, and **6** was prepared in methanol. The stock solution of the six analytes was diluted with methanol to make a series of composited standard solutions. The stock solution of the IS was diluted to a concentration of 200 ng/ml with methanol as working solution. The samples of standard calibration curves were prepared by spiking 10 μl of the above working solutions into 190 μl of blank tissue homogenate. The concentrations were in the range of 2–2000 ng/ml for **1** and **2**, 0.8–800 ng/ml for **3**, 2.2–2200 ng/ml for **4**, **5**, and **6**, respectively. QC samples at low, middle and high concentrations (4, 50 and 1600 ng/ml for **1** and **2**; 1.6, 20 and 640 ng/ml for **3**; 4.4, 55 and 1760 ng/ml for **4**, **5**, and **6**) were also prepared by the same operation listed above. All standards were stored at −20 °C before analysis.

Sample preparation

The tissue homogenate sample (50 μl) was mixed with 150 μl acetonitrile containing 200 ng/ml isoquercitrin (**7**). The mixture was then vortex-mixed for 40 s and centrifugation at 14,850 × *g* for 10 min at 8 °C. The supernatant was transferred into another clean eppendorf tube and evaporated to dryness under a nitrogen stream in a water bath at 45 °C. Finally, the residue was dissolved

Table 1
MS/MS parameters for quantifications of six analytes and Internal Standard.

Analyte	Precursor to product ion (*m*/*z*) transition	Tube lens (V)	Collision energy (eV)
Vitexin	431 → 311	88	25
Orientin or isoorientin	447 → 327	80	25
2″-*O*-β-D-xylopyranosyl isoorientin	579 → 459	102	50
2″-*O*-β-D-xylopyranosyl isovitexin	563 → 293	89	38
6-*C*-L-α-arabipyranosyl vitexin	563 → 353	109	38
Internal standard	463 → 300	80	30

in 80 μl of acetonitrile-water (20:80, v/v) with vortex-mixing for 20 s, and centrifugation process was repeated. An aliquot of 10 μl was injected into the LC-MS/MS system for analysis.

Method validation

According to the FDA bioanalytical method validation guide (U.S. Food and Drug Administration, 2001), the LC-MS/MS method was validated in terms of selectivity, sensitivity, linearity, precision and accuracy, matrix effect and extraction recovery, carryover and stability. The selectivity was evaluated by analyzing blank tissue homogenates (liver was chosen as representative tissue) to check for the presence of any interfering peaks at the elution regions of analytes and IS. The linearity of each calibration curve was determined by plotting the peak area ratio (Y) of analytes to IS *versus* the nominal concentration (X) of analytes with weighted ($1/X^2$) least square linear regression. The lower limits of quantification (LLOQ) for the analytes were the lowest concentrations with signal-to-noise ≥10 that could be quantitatively determined with precision and accuracy ≤±20%. Intra- and inter-day accuracy and precision were evaluated by analyzing QC samples (five samples for each) at low, middle and high concentrations on the same day and on three consecutive days, respectively. Extraction recovery was assessed by comparing the responses of extracted QC samples with those of post-extraction blank tissue homogenates spiked with equivalent concentrations using six replicates. The matrix effect was calculated by comparing the peak areas obtained from samples where the extracted matrix was spiked with standard solutions to those obtained from the pure reference standard solutions at the same concentration. Stability studies in different tissue homogenate samples were performed at three QC levels under different storage conditions: Short-term temperature stability was determined by analyzing QC samples left at room temperature for at least 6 h that was longer enough to prepare samples. Long-term stability was studied by analyzing QC samples after storage at −20 °C for 80 days. Freeze and thaw stability was evaluated after three freeze (−20 °C) – thaw (room temperature) cycles. Post-preparative stability was investigated by analyzing the extracted QC samples kept in autosampler at 10 °C for at least 12 h.

Application to a tissue distribution study in rats

The animal study was approved by the Institutional Animal Ethical Committee of the First Hospital of Jilin University (ethic

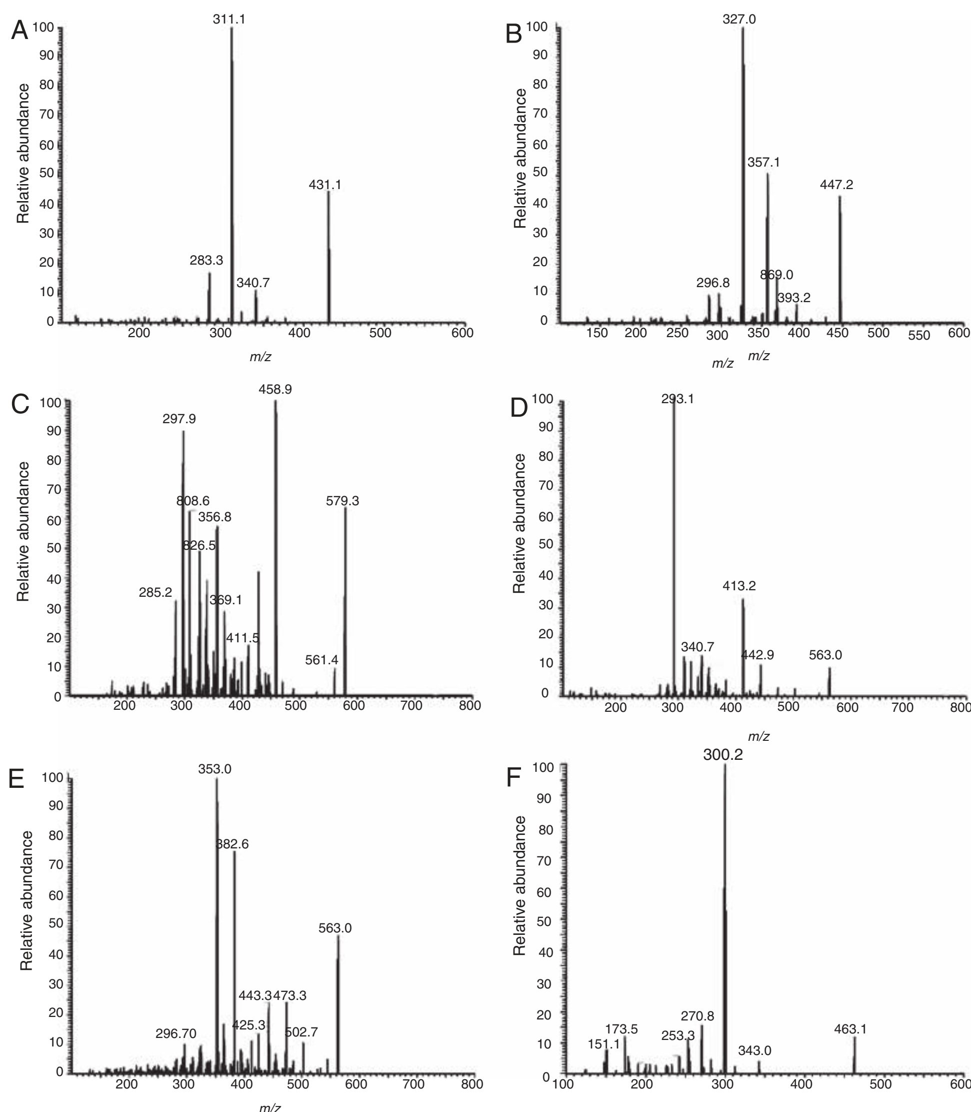

Fig. 1. The product ions scan spectra of the analytes and IS. (A) vitexin (**1**), (B) orientin (**2**) or isoorientin (**3**), (C) 2″-*O*-β-D-xylopyranosyl isoorientin (**4**), (D) 2″-*O*-β-D-xylopyranosyl isovitexin (**5**), (E) 6-*C*-L-α-arabipyranosyl vitexin (**6**), and (F) Internal Standard, isoquercetrin (**7**).

approval: 2016–523). Thirty male Wistar rats (200 ± 10 g) were fasted overnight but allowed access to water before drug administration. Six groups of rats (five rats per group) were intravenously administered a single dose of 8 mg/kg total flavonoids of *S. austriaca*, which was prepared in saline to make a solution of 1.63 mg/ml. These six groups of rats were euthanized by decapitation at 5 min, 30 min, 1 h, 3 h, 5 h and 7 h after dosing, respectively. Tissues including heart, liver, spleen, lung, kidney, stomach and brain were dissected and washed with saline, then were blotted by filter paper and weighed accurately. The weighed tissue samples were homogenized in ice-cold pure water (1:10, w/v for heart, liver, spleen, lung, kidney, stomach and brain tissue samples). These tissue homogenates were then stored at −20 °C until LC-MS/MS analysis.

Data analysis

Statistical analysis was performed using Origin85 (OriginLab Co., Northampton, MA, USA) software. Data were expressed as mean ± SD.

Results and discussion

Mass spectrometric and chromatographic conditions

In our study, various mobile phase compositions were examined to obtain optimal responses, good separation, and suitable retention times for the analytes. Acetonitrile, methanol, formic acid and ammonium acetate were tested as potential mobile phases. Finally a gradient elution with acetonitrile and 0.1% aqueous formic acid was chosen to obtain satisfactory separation and good peak shapes. For the MS condition, both negative and positive scan modes were tuned. The negative mode was selected for vitexin (**1**), orientin (**2**), isoorientin (**3**), 2″-*O*-β-D-xylopyranosyl isoorientin (**4**), 2″-*O*-β-D-xylopyranosyl isovitexin (**5**), and 6-*C*-L-α-arabipyranosyl vitexin (**6**) and IS. SRM mode was chosen to monitor both precursor and product ions (Fig. 1), which made the method more specific.

Method validation

Specificity and carryover

Representative LC-MS/MS chromatograms obtained from liver tissue samples are shown in Fig. 2, respectively. No significant interference was observed at the retention regions of the analytes and the IS. Furthermore, no quantifiable carryover effect was obtained in this study after the injection of an extracted upper limit of quantitation (ULOQ) sample.

Linearity and sensitivity

The standard curve of each analyte was constructed using the $1/X^2$ weighted linear least-squares regression model. Calibration curves, correlation coefficients, and linear ranges of six analytes in tissue samples are listed in Table 2, respectively.

Accuracy and precision

Intra-day and inter-day precision and accuracy of **1**, **2**, **3**, **4**, **5**, and **6** in liver tissue homogenates at three levels of QC samples ($n = 5$) are presented in Table 3. Intra- and inter-day precision of six analytes in liver tissue homogenates ranged from 1.46% to 12.35% (RSD). Accuracy of six analytes in liver tissue homogenates ranged from −10.35% to 15.00% (RE). All the assay values of tissues were within acceptable limits.

Extraction recovery and matrix effect

Extraction recovery and matrix effect of **1**, **2**, **3**, **4**, **5**, and **6** at three QC concentrations in liver tissue homogenates are presented in Table 4. Mean extraction recovery of six analytes and IS was 85.74–97.22% and matrix effect of six analytes and IS was from 85.53% to 98.73%, respectively. The results indicated that no obvious matrix effect for six analytes occurred in current LC-MS/MS determination.

Stability

The stability of **1**, **2**, **3**, **4**, **5**, and **6** in rat tissue homogenates under different storage conditions was evaluated and the results were presented in Table 5. The results of the stability tests for the six analytes showed that they were stable in tissue homogenate samples after storage at −20 °C for 80 days, after three freeze (−20 °C) – thaw (room temperature) cycles and being left at room temperature for at least 6 h. The processed samples that kept in autosampler at 10 °C for at least 12 h also remain stable.

Application to a tissue distribution study in rats

The distribution of **1**, **2**, **3**, **4**, **5**, and **6** was investigated in rats following a single intravenous administration of total flavonoids of *S. austriaca* (8 mg/kg) by collecting tissues including the heart, liver, spleen, lung, kidney, stomach and brain (Figs. 3–8). The results of the present study indicated that all analytes underwent a wide and rapid distribution into tissues within the time course examined, and no long-term accumulation of the compounds in tissues was observed. From the results, the highest levels of six analytes were observed at 5 min or 30 min after administration in the kidneys. These flavonoid glycosides were mainly distributed in blood-abundant tissues such as kidney, lung, stomach and liver, which implied that the distribution of the compounds depended on the blood flow or perfusion rate of the organ. The highest distribution in kidney suggested that kidney tissue might be the primary excretion organ of these flavonoid glycosides. Additionally, the concentrations of six analytes were lowest in the brain, indicating that it was difficult for the flavonoid glycosides to cross the blood–brain barrier of normal rats.

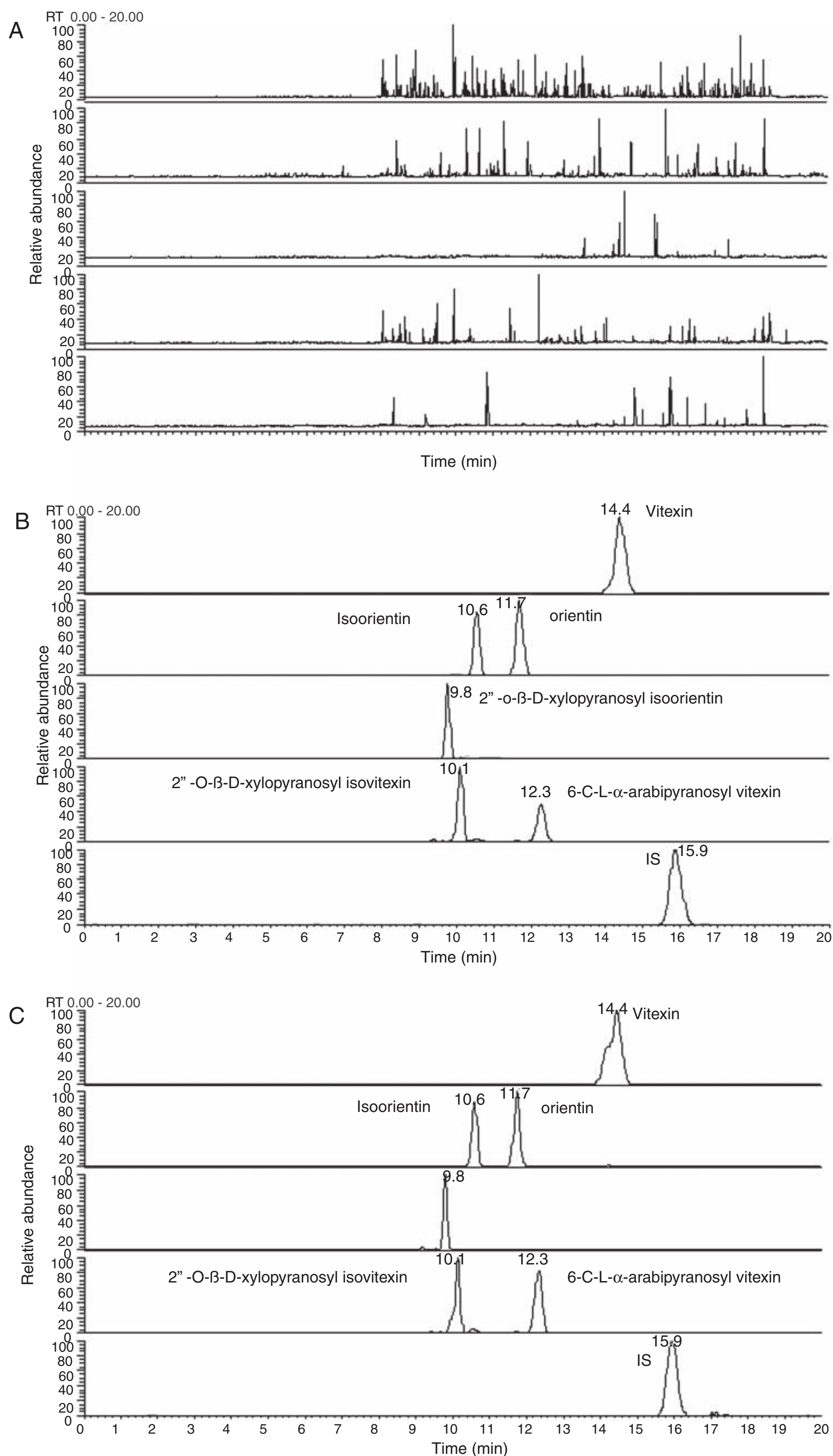

Fig. 2. Typical chromatograms of six compounds in rat plasma: (A) blank liver tissue sample; (B) blank liver tissue sample spiked with the six analytes and Internal Standard; (C) 1.0 h liver tissue after an intravenous administration of total flavonoids of *Scorzonera austriaca* (8 mg/kg).

Table 2
Calibration curves and linearity for six analytes in all tissue samples.

Analytes	Calibration curve							Linear range (ng/ml)
	Heart	Liver	Spleen	Lung	Kidney	Stomach	Brain	
Vitexin	$Y=0.00329X-0.00015$, $r^2=0.9920$	$Y=0.00551X-0.00095$, $r^2=0.9956$	$Y=0.00172X+0.00049$, $r^2=0.9995$	$Y=0.00592X+0.00198$, $r^2=0.9950$	$Y=0.00402X-0.00319$, $r^2=0.9994$	$Y=0.00204X+0.00101$, $r^2=0.9947$	$Y=0.00741X-0.00305$, $r^2=0.9915$	2.0–2000
Orientin	$Y=0.00741X-0.00032$, $r^2=0.9961$	$Y=0.00314X-0.00056$, $r^2=0.9988$	$Y=0.00180X+0.00089$, $r^2=0.9940$	$Y=0.00905X-0.00225$, $r^2=0.9932$	$Y=0.00319X-0.00315$, $r^2=0.9992$	$Y=0.00326X-0.00069$, $r^2=0.9982$	$Y=0.00154X-0.00128$, $r^2=0.9960$	2.0–2000
Isoorientin	$Y=0.00952X+0.00412$, $r^2=0.9929$	$Y=0.01882X-0.00865$, $r^2=0.9902$	$Y=0.00113X+0.00022$, $r^2=0.9969$	$Y=0.00927X+0.00061$, $r^2=0.9987$	$Y=0.00485X-0.00097$, $r^2=0.9926$	$Y=0.01847X-0.00514$, $r^2=0.9940$	$Y=0.03624X-0.00103$, $r^2=0.9958$	0.8–800
2″-*O*-β-D-xylopyranosyl isoorientin	$Y=0.00026X+0.00021$, $r^2=0.9951$	$Y=0.00089X-0.00016$, $r^2=0.9905$	$Y=0.00079X+0.00037$, $r^2=0.9912$	$Y=0.00014X-0.00011$, $r^2=0.9950$	$Y=0.00092X-0.00017$, $r^2=0.9990$	$Y=0.00120X+0.00095$, $r^2=0.9966$	$Y=0.00058X+0.00030$, $r^2=0.9914$	2.2–2200
2″-*O*-β-D-xylopyranosyl isovitexin	$Y=0.00625X-0.00201$, $r^2=0.9907$	$Y=0.00166X-0.00026$, $r^2=0.9924$	$Y=0.00708X+0.00501$, $r^2=0.9903$	$Y=0.00599X-0.00156$, $r^2=0.9902$	$Y=0.00511X+0.00107$, $r^2=0.9988$	$Y=0.00194X-0.00117$, $r^2=0.9939$	$Y=0.00315X+0.00241$, $r^2=0.9973$	2.2–2200
6-*C*-L-α-arabipyranosyl vitexin	$Y=0.00400X-0.00095$, $r^2=0.9914$	$Y=0.00105X-0.00150$, $r^2=0.9932$	$Y=0.00080X+0.00051$, $r^2=0.9942$	$Y=0.00532X+0.00008$, $r^2=0.9996$	$Y=0.00200X+0.00017$, $r^2=0.9959$	$Y=0.00162X-0.00028$, $r^2=0.9915$	$Y=0.00148X-0.00198$, $r^2=0.9992$	2.2–2200

Table 3
Accuracy and precision of six analytes in liver tissue samples ($n = 5$).

Sample	Compound	QC (ng/ml)	Intra-day(%)		Inter-day (%)	
			Precision (RSD)	Accuracy (RE)	Precision (RSD)	Accuracy (RE)
Liver	vitexin	2.00	8.62	13.88	8.47	9.71
		4.00	7.82	15.00	6.42	15.45
		50.0	2.80	−2.80	3.75	−2.25
		1600	1.46	0.26	3.09	−0.74
	orientin	2.00	2.20	13.21	9.76	2.72
		4.00	4.40	9.43	10.02	−0.32
		50.0	3.39	−0.39	5.27	−0.88
		1600	2.10	3.00	6.99	1.16
	isoorientin	0.8	8.02	−2.26	9.87	−1.78
		1.60	6.30	4.49	8.67	1.91
		20.0	4.30	−1.22	6.17	0.44
		640	1.79	−10.35	6.80	−8.65
	2″-O-β-D-	2.20	1.82	16.39	11.29	2.52
	xylopyranosyl	4.40	3.64	7.69	8.98	−0.40
	isoorientin	55.0	5.51	−7.56	4.32	−7.74
		1760	2.51	0.56	3.42	−2.07
	2″-O-β-D-	2.20	5.02	13.28	9.25	5.92
	xylopyranosyl	4.40	8.66	5.77	7.58	4.84
	isovitexin	55.0	4.86	−1.85	6.16	2.55
		1760	1.51	1.51	4.28	3.33
	6-C-L-α-	2.20	8.14	13.02	12.35	1.42
	arabipyranosyl	4.40	3.60	0.40	9.77	0.36
	vitexin	55.0	4.00	−3.37	7.53	−0.19
		1760	1.85	−1.13	5.87	0.93

Table 4
The recovery and matrix effect of six analytes and internal standard in rat plasma ($n = 5$).

Sample	Compound	QC (ng/ml)	Extraction recovery (%)	Matrix effect (%)
Liver	vitexin	4.00	85.74 ± 4.70	87.54 ± 7.24
		50.0	88.40 ± 3.52	93.19 ± 5.27
		1600	90.43 ± 2.84	94.14 ± 7.75
	orientin	4.00	96.79 ± 8.69	85.84 ± 9.19
		50.0	89.13 ± 3.55	89.93 ± 6.13
		1600	88.18 ± 5.05	91.10 ± 8.68
	isoorientin	1.60	92.14 ± 7.72	86.05 ± 8.51
		20.0	97.22 ± 7.60	95.64 ± 6.24
		640	93.81 ± 6.03	91.68 ± 4.91
	2″-O-β-D-	4.40	92.17 ± 7.43	98.73 ± 8.13
	xylopyranosyl	55.0	91.88 ± 3.40	85.53 ± 5.97
	isoorientin	1760	95.00 ± 3.03	86.71 ± 7.43
	2″-O-β-D-	4.40	91.90 ± 6.52	89.14 ± 6.30
	xylopyranosyl	55.0	86.21 ± 3.44	95.08 ± 6.04
	isovitexin	1760	91.32 ± 4.63	88.73 ± 3.41
	6-C-L-α-	4.40	86.12 ± 1.22	93.96 ± 7.02
	arabipyranosyl	55.0	86.96 ± 3.46	91.16 ± 8.45
	vitexin	1760	90.84 ± 6.73	97.07 ± 4.04

Table 5
Stability results of six analytes in liver tissue samples ($n = 3$).

Sample	Compound	QC (ng/ml)	Short-term temperature stability		Post-preparative stability		Freeze and thaw stability		Long-term stability	
			RSD%	RE%	RSD%	RE%	RSD%	RE%	RSD%	RE%
Liver	vitexin	4.00	8.54	9.89	9.08	1.62	4.84	14.86	2.36	9.08
		50.0	2.60	1.71	11.02	8.71	10.22	10.07	3.11	−3.19
		1600	3.49	−2.83	14.10	8.55	6.43	4.01	4.52	2.77
	orientin	4.00	12.58	−0.93	2.88	−2.85	3.64	0.87	3.98	5.89
		50.0	3.84	9.76	8.15	7.85	4.35	7.53	4.14	11.49
		1600	1.96	11.52	7.41	4.43	2.71	4.22	5.15	8.46
	isoorientin	1.60	10.09	−6.24	11.32	0.65	2.66	−3.73	10.09	−6.24
		20.0	3.89	−5.95	6.73	3.76	5.55	1.48	3.89	−5.95
		640	6.15	−6.63	7.39	−5.19	3.30	−11.25	9.67	−0.54
	2″-O-β-D-	4.40	3.67	−2.58	2.14	−1.70	7.82	−2.18	10.93	1.21
	xylopyranosyl	55.0	4.68	−8.13	8.05	−7.66	10.53	−6.85	1.20	−12.48
	isoorientin	1760	5.36	−7.04	10.78	−2.75	10.83	−3.60	4.10	−10.24
	2″-O-β-D-	4.40	10.91	−0.09	3.05	−7.11	6.96	6.17	10.15	−5.89
	xylopyranosyl	55.0	1.27	5.60	4.17	6.87	3.29	11.70	4.09	2.46
	isovitexin	1760	0.46	9.30	1.49	6.29	1.77	6.52	6.13	4.60
	6-C-L-α-	4.40	2.63	1.94	9.34	4.41	1.90	−2.55	9.08	3.96
	arabipyranosy	55.0	2.02	6.64	6.44	6.49	8.89	−0.26	4.34	2.85
	vitexin	1760	4.71	7.61	6.02	4.48	5.78	8.70	4.59	7.22

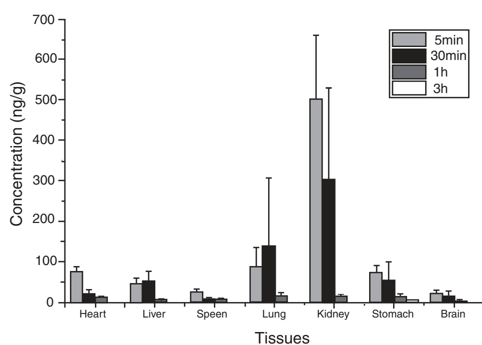

Fig. 3. Concentrations of isoorientin (**3**) in rat tissues at 5 min, 30 min, 1 h and 3 h after intravenous administration of total flavonoids of *Scorzonera austriaca* (8 mg/kg) in rats. Data are means ± SD.

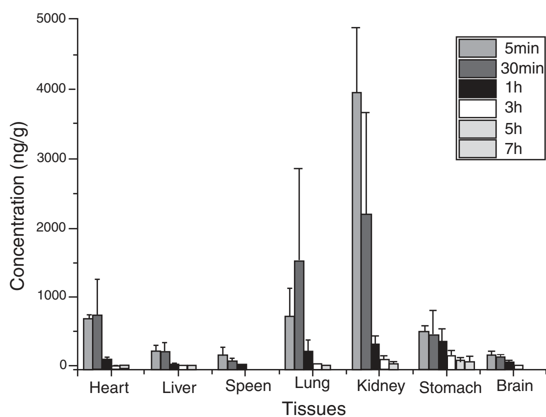

Fig. 4. Concentrations of 2″-*O*-β-D-xylopyranosyl isoorientin (**4**) in rat tissues at 5 min, 30 min, 1 h, 3 h, 5 h and 7 h after intravenous administration of total flavonoids of *Scorzonera austriaca* (8 mg/kg) in rats. Data are means ± SD.

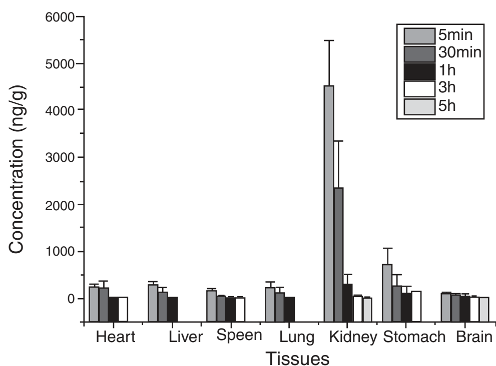

Fig. 5. Concentrations of orientin (**2**) in rat tissues at 5 min, 30 min, 1 h, 3 h and 5 h after intravenous administration of total flavonoids of *Scorzonera austriaca* (8 mg/kg) in rats. Data are means ± SD.

Conclusions

The present study established an LC–MS/MS method for the simultaneous determination of vitexin (**1**), orientin (**2**), isoorientin (**3**), 2″-*O*-β-D-xylopyranosyl isoorientin (**4**), 2″-*O*-β-D-xylopyranosyl isovitexin (**5**), and 6-*C*-L-α-arabipyranosyl vitexin (**6**). This method was successfully applied to a tissue distribution study of the six components in rat tissues after intravenous administration of total flavonoids of *S. austriaca*. The tissue distribution results might be useful for further study of the bioactive mechanism of *S. austriaca*.

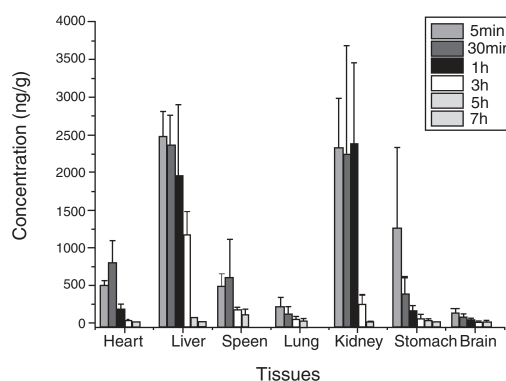

Fig. 6. Concentrations of 2″-*O*-β-D-xylopyranosyl isovitexin (**5**) in rat tissues at 5 min, 30 min, 1 h, 3 h, 5 h and 7 h after intravenous administration of total flavonoids of *Scorzonera austriaca* (8 mg/kg) in rats. Data are means ± SD.

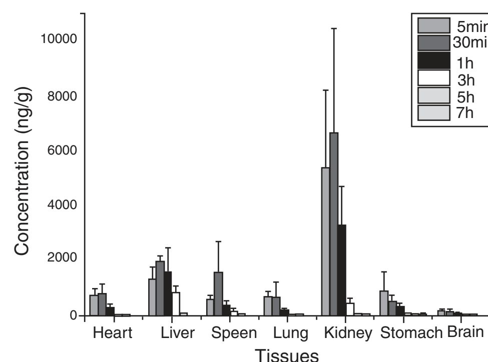

Fig. 7. Concentrations of 6-*C*-L-α-arabipyranosyl vitexin (**6**) in rat tissues at 5 min, 30 min, 1 h, 3 h, 5 h and 7 h after intravenous administration of total flavonoids of *Scorzonera austriaca* (8 mg/kg) in rats. Data are means ± SD.

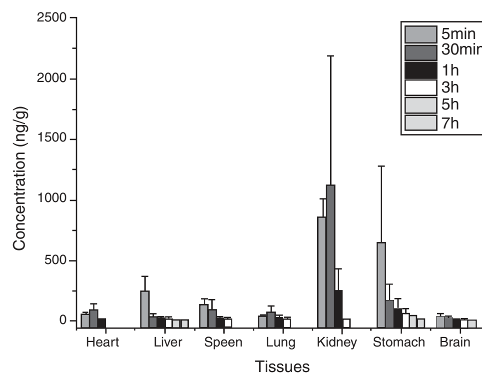

Fig. 8. Concentrations of vitexin (1) in rat tissues at 5 min, 30 min, 1 h, 3 h, 5 h and 7 h after intravenous administration of total flavonoids of *Scorzonera austriaca* (8 mg/kg) in rats. Data are means ± SD.

Ethical statement

The animal study was approved by the Institutional Animal Ethical Committee of Jilin University, 1163 Xinmin Rd, Changchun 130021, PR China.

Authors' contributions

SZ carried out the data analyses and wrote the manuscript. YX and GW designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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References

Huang, Y., He, F., Zheng, L., Zhang, Z., Lan, Y., Wang, Y., 2012. Simultaneous determination of isoorientin, scutellarin and cynaroside in rat plasma and pharmacokinetics by UPLC-MS/MS. Zhongguo Zhong Yao Za Zhi 37, 529–532.

Li, P.Z., Wu, H.J., Wu, W.Q., 1992. Effect on enteritis of *Scorzonera austriaca*. J. Chin. Pharm. Univ. 23, 357–360.

Li, Y.J., Shao, M.X., Zhao, C.C., 1997. 88 case treatments of hyperemesis gravidarumby of *Scorzonera austriaca*. Chin. Naturop. 4, 13.

Li, J., Wu, Q.X., Shi, Y.P., Zhu, Y., 2004. A new sesquiterpene lactone from *Scorzonera austriaca*. Chin. Chem. Lett. 15, 1309–1310.

Li, D., Wang, Q., Xu, L., Li, M., Jing, X., Zhang, L., 2008a. Pharmacokinetic study of three active flavonoid glycosides in rat after intravenous administration of *Trollius ledebourii* extract by liquid chromatography Biomed. Chromatography 22, 1130–1136.

Li, D., Wang, Q., Yuan, Z.F., Zhang, L., Xu, L., Cui, Y., Duan, K., 2008b. Pharmacokinetics and tissue distribution study of orientin in rat by liquid chromatography. J. Pharm. Biomed. Anal. 47, 429–434.

Lee, W., Bae, J.S., 2015. Antithrombotic and antiplatelet activities of orientin *in vitro* and *in vivo*. J. Funct. Foods 17, 388–398.

Liu, L., Wu, Y., Huang, X., 2016. Orientin protects myocardial cells against hypoxia-reoxygenation injury through induction of autophagy. Eur. J. Pharmacol. 776, 90–98.

U.S. Food and Drug Administration, 2001. Guidance for Industry: Bioanalytical Method Validation. U.S. Department of Health and Human Services, FDA Rockville.

Wang, Y., Zhen, Y., Wu, X., Jiang, Q., Li, X., Chen, Z., Zhang, G., Dong, L., 2015. Vitexin protects brain against ischemia/reperfusion injury via modulating mitogen-activated protein kinase and apoptosis signaling in mice. Phytomedicine 22, 379–384.

Wu, Q.X., Su, Y.B., Zhu, Y., 2011. Triterpenes and steroids from the roots of *Scorzonera austriaca*. Fitoterapia 82, 493–496.

Xie, Y., Wang, J., Geng, Y., Qu, Y., Zhang, Z., Wang, G., 2015. Experimental studies on inhibitory effects of total flavonoids in *Scorzonera austriaca* Wild on hepatitis B virus *in vitro*. Zhongguo Sheng Hua Yao Wu Za Zhi 35, 41–47.

Yan, C., Liu, H., Lin, L., 2013. Simultaneous determination of vitexin and isovitexin in rat plasma after oral administration of Santalum album L. leaves extract by liquid chromatography tandem mass spectrometry. Biomed. Chromatogr. 27, 228–232.

Zhu, Y., Wu, Q.X., Hu, P.Z., Wu, W.S., 2009. Biguaiascorzolides A and B: Two novel dimeric guaianolides with a rare skeleton, from *Scorzonera austriaca*. Food Chem. 114, 1316–1320.

Zhang, T., Xie, Y., Zhang, Z., Wang, G., 2015. Study on hepatoprotective effects of total flavonoids in *Scorzonera austriaca* Wild *in vivo* and *in vitro*. Zhongguo Sheng Hua Yao Wu Za Zhi 35, 6–9.

Zhang, S., Xie, Y., Wang, J., Geng, Y., Zhou, Y., Sun, C., Wang, G., 2017. Development of an LC-MS/MS method for quantification of two pairs of isomeric flavonoid glycosides and other ones in rat plasma: application to pharmacokinetic studies. Biomed. Chromatogr., http://dx.doi.org/10.1002/bmc.3972.