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Article

Simultaneous Analysis of Quercetin and Naringenin in Rat Plasma by Liquid Chromatography–Tandem Mass Spectrometry: Application to a Pharmacokinetic Study After Oral Administration

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Abstract

A rapid and specific LC–MS-MS method has been developed for simultaneous analysis of quercetin and naringenin in rat plasma. The method was applied to the pharmacokinetics studies of quercetin and naringenin after oral administration of Pollen Typhae extract. The samples were prepared by the protein precipitation method. The analysis was carried out on an ACQUITY UPLC™ BEH C18 column with gradient elution using mobile phase, which included acetonitrile and water (containing 0.1% formic acid). The flow rate was 0.4 mL/min. All analytes including internal standard (IS) were monitored by selected reaction monitoring with an electrospray ionization source. Linear responses were obtained for quercetin ranging from 0.5 to 100 ng/mL and naringenin ranging from 5 to 1000 ng/mL. The intra- and interday precisions (RSD) were less than 10.78 and 11.20%. The extraction recovery of the analytes was acceptable. Stability studies showed that quercetin and naringenin were stable in the preparation and analytical process. The validated method was successfully used to determine the concentration–time profiles of quercetin and naringenin.

Introduction

Pollen Typhae (named Puhuang in Chinese) is a well-known traditional Chinese medicine (TCM), which plays an important role in the treatment of stranguria, hematuria, dysmenorrheal, metrorrhagia and injuries from falls (1). It was shown that Pollen Typhae possessed various pharmacological activities, such as improving the microcirculation, immunosuppression, raising cAMP levels, lowering cholesterol and anticoagulation (2–4). The major active ingredients of Pollen Typhae were flavonoids, which were responsible for antioxidant, anti-inflammatory, antigenotoxic and antiprotozoal activities (5–8).

The extract of Pollen Typhae had an immunosuppressive activity on the immune responses in mice and could be deserved further research studies as an immunosuppressant (5). These might explain why Pollen Typhae could attract more and more interests in the world. Quercetin and naringenin are two major flavonoids in the extract of Pollen Typhae (9). The recent study showed that quercetin and naringenin were considered antioxidant compounds with promising activity against oxidative damage in human cells (10). Quercetin has been associated with a reduced incidence of heart disease and cancer (11–14), hypothesized to be due to their antioxidant properties. Naringenin potentiated intracellular signaling responses to low insulin doses, suggesting

that naringenin sensitizes hepatocytes to insulin (15). Quercetin and naringenin were the two representative flavonoid aglycones.

Earlier publications have described some methods for the determination of naringenin and quercetin utilizing LC–MS/MS (16–17). In contrast, the method in our article is fast, more suitable and lower limit of quantification (LLOQ) for the determination of these flavonoids. The total run time was 4 min per sample. The method was fully validated and applied to the pharmacokinetic study after oral administration of Pollen Typhae extract to rats. It was expected that the results of this study would provide some references for the clinical application of Pollen Typhae.

Experimental

Chemicals and reagents
Quercetin (98%, purity, Figure 1A), naringenin (98%, purity, Figure 1B) and luteoline (98%, purity, IS, Figure 1C) were purchased from the National Institute for Food and Drug Control (Beijing, China). Acetonitrile (HPLC/MS-grade) and methanol (HPLC/MS-grade) were purchased from Fisher (USA). HPLC-quality water was obtained using a Cascada™ IX-water Purification System (Pall Co., USA). Other reagents were all of analytical grade.

Instrumentation and chromatography
Liquid chromatography
Identification and quantification of analytes were carried out using an ACQUITY™ UPLC™ system (Waters Corp., Milford, MA, USA). An ACQUITY UPLC™ BEH C18 column (100 × 2.1 mm, 1.7 μm) was used for LC separation. The column temperature was maintained at 40°C, and an autosampler was set at 4°C. The gradient elution was employed with 0.1% formic acid (HOOCH) in water as solvent A and acetonitrile as solvent B. The gradient program was as follows: 0–1.7 min 80% A to 68% A, 1.7–3.2 min 68% A to 30% A and 3.2–4.0 min 30–20% A. The flow rate was set at 0.4 mL/min, and the injection volume was 10 μL. The total run time was 4 min for each sample.

Mass spectrometer conditions
The detection was performed on an Xevo TQ-S mass spectrometer (Waters Corp., Milford, MA, USA) equipped with an ESI interface,

operated in negative ionization mode. The capillary voltage was 2500 V, and the cone voltage was 30 V. The desolvation temperature was held at 400°C, and the source temperature was 150°C. Nitrogen was used as desolvation and cone gas with the flow rate of 800 and 150 L/h, respectively. Other parameters were also optimized for maximum sensitivity (Table I).

Preparation of calibration standards and quality control samples
Primary stock solutions of quercetin, naringenin and IS for preparation of standards and quality control (QC) samples were prepared from separate weightings. The primary stock solutions of the analytes and IS were prepared in methanol at a concentration of 0.02 mg/mL and stored at −20°C. When the primary stock solutions were saved for more than 1 week, they needed to be re-prepared. A series of working standard solutions of the analytes ranging from 0.5 to 100 ng/mL for quercetin, 5 to 1,000 ng/mL for naringenin and IS solution at 100 ng/mL were prepared by diluting the stock solutions with methanol. All solutions were stored at −20°C for the assay within 1 week. Calibration standards were prepared using blank rat plasma spiked with different concentrations of working solutions of quercetin and naringenin to yield the concentrations of 0.5–100 and 5–1000 ng/mL, respectively.

QC samples were prepared in the same way as the calibration samples, representing three level concentrations of quercetin in plasma at 1, 10 and 80 ng/mL and naringenin at 10, 100 and 800 ng/mL.

Sample preparation
IS (20 μL) was added to blank plastic tube and evaporated to dryness at 60°C in advance. Then, an aliquot of 100 μL rat plasma was transferred to the plastic tube. For the method validation, 10 μL of working standards and 10 μL of 30% formic acid were added to blank plasma. For the blood samples of the pharmacokinetic study, only 10 μL of 30% formic acid was added. After vortexing for 30 s, 300 μL of acetonitrile was added and the mixture was vortexed for 1 min followed by centrifugation at 12,000 rpm for 10 min at 4°C. The supernatant was transferred into a clean plastic tube and evaporated to dryness under a gentle nitrogen stream. The residue was reconstituted in 100 μL of methanol solution, vortexed for 1 min, centrifuged at
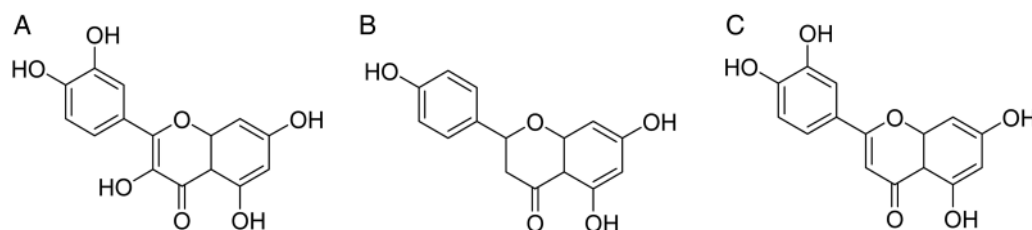

Figure 1. Chemical structures of quercetin (**A**), naringenin (**B**) and luteoline (**C**, IS).

Table I. Optimized Selected Reaction Monitoring Parameters for the Detection of Analytes and Internal Standards

Compound	Formula/mass	Parent m/z	Cone votage	Daughters	Collision energy	Iron mode
Quercetin	302.04	301.07	54	150.93	18	ES⁻
Naringenin	272.07	271.04	50	150.89	16	ES⁻
Luteoline	286	284.97	46	132.9	34	ES⁻

12,000 rpm for 10 min. After centrifuging, a 3.0 μL aliquot of the solution was injected into the UPLC–MS-MS system.

Method validation

This method was validated according to FDA guidelines (US Food and Drug Administration, 2001) with respect to specificity, linearity, LLOQ, accuracy, precision, extract recovery, matrix effect and stability.

Specificity

The specificity study is to investigate whether endogenous constituents and other substances existing in samples will interfere with the detection of the analytes and IS. The specificity of this method was established by comparing the blank plasma from six different sources of rats and those containing analytes and IS, particularly to find out interference from endogenous components (18).

Linearity and LLOQ

The linearity of each calibration curve was determined by the observed peak area ratios of analytes to IS (Y) versus the spiked concentrations of analytes (X) at least six-point calibration curves. The acceptance criterion for a calibration curve was a correlation coefficient (*r*) of 0.99 or better. The LLOQ, which was defined as the lowest concentration in the calibration curve with acceptable the relative standard deviation (RSD), was within ±20% and accuracy within 100 ± 20%. The deviation criteria of these back-calculated concentrations from the spiked concentrations were set within ±15%, except for the LLOQ.

Precision and accuracy

The precision and accuracy assays were determined by analyzing six QC replicates at three levels on the same day and three batches on three consecutive validation days. The assay accuracy was expressed as (observed concentration/nominal concentration) × 100%. The intra- and interday precision was expressed as RSD, and the accuracy was defined as the RE (18). The precision and the accuracy should not exceed 15%.

Extraction recovery and matrix effect

The extraction recoveries of quercetin, naringenin and IS were determined by comparing the peak areas from blank plasma samples spiked with QC working solutions and IS before extraction with those from blank plasma samples spiked after extraction. Matrix effects were evaluated by comparing the peak areas from blank plasma samples spiked with QC working solutions and IS after extraction with the areas obtained by direct injection of corresponding standard solutions. When the peak area ratios of the analytes and the IS solution were all between 85 and 115%, the matrix effect may be considered negligible (19).

Stability

The stability of the analytes in plasma was investigated under the following conditions: 24 h at room temperature, three freeze (−20°C) and thaw (room temperature) cycles, stored at −20°C for 30 days. They were investigated by assaying QC plasma samples of the three concentration levels and considered stable when 85–115% of the initial concentrations were got.

Pharmacokinetic study

Animals

Six male Sprague Dawley rats (270 ± 20 g) were used, which were supplied by Beijing Vital River Laboratory Animal Technology (Beijing, China), and were fasted overnight before administration while water was taken *ad libitum*. The animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publications No. 85–23, revised 1996) and with approval from the Animal Care Committee of Beijing University of Chinese Medicine (no. SYXK 2011-0024). The permission date is 2011-06-28–2016-06-28, and the location is no. 11, North Third Ring Road, Chaoyang District, Beijing 100029, P.R. China.

Dosing and sampling

Pollen Typhae extract freshly prepared in 0.5% CMC-Na solution was given to rats at intragastric dose (quercetin was 2.05 mg/kg and naringenin was 1.82 mg/kg). Blood samples (0.5 mL) were collected from the ocular fundus veins of rats before administration and after 0.25, 0.5, 1, 3, 4, 6, 8, 10, 12 and 24 h. The blood samples were immediately centrifuged to separate out plasma and stored at −20°C until analysis.

Data analysis

The pharmacokinetic parameters of quercetin and naringenin were calculated by Kinetica 4.4 software (Thermo Scientific, USA). Noncompartmental analysis was used to determine standard pharmacokinetic parameters of the analytes. All the results were expressed as means ± standard deviation (SD) of six replicates.

Results

Method development

Mass spectrometry

To optimize the precursor/product ions of the analytes and IS for MRM mode analysis, both positive/negative ESI modes were finely tuned. Due to the baseline noise was high under the positive conditions, and the response values of the analytes and IS were high under the negative conditions, the negative ESI mode was selected. The full-scan mass spectrum of quercetin, naringenin and IS revealed [M–H]⁻ ions at *m/z* 301.07, 271.04 and 284.97, respectively. The fragment ions at 150.93, 150.89 and 132.9 *m/z* were used as the prominent product ions of quercetin, naringenin and IS, respectively. The detailed parameters of the analytes and IS are shown in Table I.

Optimization of chromatographic conditions

To separate the analytes and IS, different compositions of mobile phase and elution modes were tried. To improve peak shapes and obtain good sensitivity and selectivity, a gradient elution program with a mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile (B) was applied, which had a satisfactory separation.

Optimization of sample preparation conditions

Initially, we used pure acetonitrile as the precipitant, but it brought about matrix effect, which improved the mass spectrometer signal toward the analytes and IS. The mean peak area of QC samples at low concentration was above 120% of equivalent drug acetonitrile solution samples. To solve this problem, we add 10 μL of 30% formic acid or 60% formic acid to samples before precipitating with acetonitrile, and the 30% formic acid with acetonitrile worked well, making
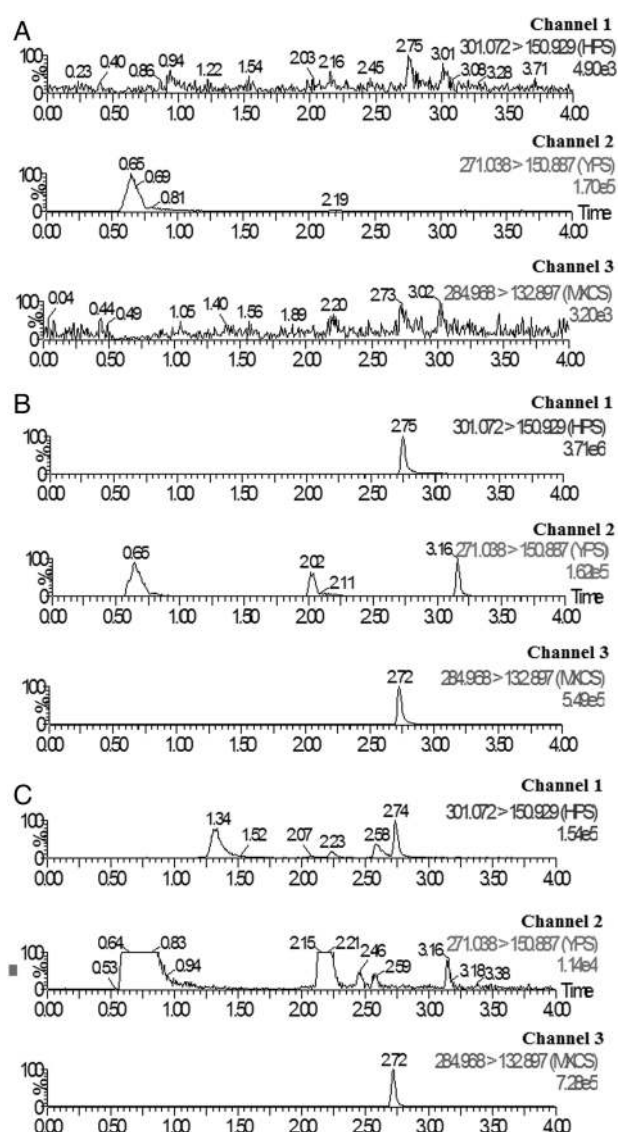
Figure 2. Chromatograms for quercetin (Channel 1), naringenin (Channel 2) and IS (Channel 3) in rat plasma samples: (**A**) a blank plasma sample; (**B**) a blank plasma sample spiked with analytes; (**C**) a plasma sample from a rat at 4 h after oral administration of Pollen Typhae extract.

the improved signal back to normal. Therefore, we chose 10 µL of 30% formic acid with 300 µL of acetonitrile.

Method validation

Specificity

Figure 2 shows the chromatographic profiles of blank plasma, blank plasma spiked with quercetin and naringenin and IS and plasma obtained 4 h after oral administration of Pollen Typhae extract. From Figure 2B, we can know that the retention times for quercetin, naringenin and IS were 2.75, 3.16 and 2.72 min, respectively. From the figure of blank plasma sample (Figure 2A), no significant interferences from endogenous substances were observed at the retention times of naringenin and IS. Although there was a peak at the retention time of quercetin, the value was very low and we can ignore it. Figure 2C demonstrated chromatographic profiles of plasma obtained 4 h after oral administration of Pollen Typhae extract. As a result, studies showed that there were no endogenous interferences in blank tissues.

Linearity and LLOQ

The excellent linear relationships are shown in Table II, where Y represents the peak area ratio of analytes to IS and X is the plasma concentration of analytes. Two calibration curves were established within the range of 0.5–100 and 5–1,000 ng/mL and each range has at least six concentrations. Representative regression equations for the calibration curves are $Y = 0.4217X - 4.0378$, $Y = 1.22256 \times 10^{-3}X + 0.0155$ for quercetin and naringenin. The weighting factor was $1/X$. The LLOQ for quercetin were 0.5 ng/mL, and naringenin was 5 ng/mL, which are sensitive enough for the pharmacokinetic studies of these compounds in rats.

Precision and accuracy

The intra- and interday precision and accuracy are demonstrated in Table III and investigated by analyzing QC samples. Intra-day RSD was below 10.78% and inter-day RSD was below 11.2%. The RE was −2.39–4.11% for quercetin, and −0.05–5.63% for naringenin over the three concentration levels, indicating a highly accurate and precise method.

### Extraction recovery and matrix effect

The absolute extraction recoveries and matrix effect ($n = 6$) of quercetin, naringenin and IS are shown in Table IV. Recoveries of the compounds were >75.82 ± 2.13%, and the recovery of the

Table II. Linearity and LLOQ for the Analysis of the Studied Compounds Under Standard Solutions

Compound	Linearity (ng/mL)	Calibration curve	Correlation coefficient (r)	LLOQ (ng/mL)
Quercetin	0.504–100.8	$Y = 0.4217X - 4.0378$	0.9958	0.504
Naringenin	5.04–1008	$Y = 1.2256 \times 10^{-3}X + 0.0155$	0.9996	5.04

Table III. The Intra- and Interrun Precisions and Accuracies of the Analytes in Rat Plasma ($n = 6$)

Analytes	Spiked concentration (ng/mL)	Intrarun			Interrun		
		Measured concentration (ng/mL)	Precision (%, RSD)	Accuracy (%, RE)	Measured concentration (ng/mL)	Precision (%, RSD)	Accuracy (%, RE)
Quercetin	1	1.06 ± 0.11	10.78	−0.38	1.06 ± 0.12	11.20	5.63
	10	10.15 ± 0.17	1.70	−0.58	10.09 ± 0.14	1.40	0.88
	80	80.27 ± 1.32	1.65	−0.16	80.12 ± 1.03	1.29	0.15
Naringenin	10	10.58 ± 0.78	7.40	4.11	10.17 ± 0.73	7.14	1.74
	100	98.63 ± 9.83	9.97	−2.39	99.95 ± 9.43	9.43	−0.05
	800	802.21 ± 25.91	3.23	−0.82	801.85 ± 27.38	3.42	0.18

Table IV. The Extraction Recoveries and Matrix Effects of the Analytes ($n = 6$)

Analytes	Spiked concentration (ng/mL)	Recovery (%)	RSD (%)	Matrix effect (%)
Quercetin	1	75.82 ± 2.13	2.74	112.33 ± 2.01
	10	76.47 ± 2.78	3.50	102.00 ± 3.70
	80	79.55 ± 2.96	3.68	96.62 ± 5.16
Naringenin	10	98.34 ± 9.06	9.19	89.58 ± 9.45
	100	101.13 ± 8.87	8.70	87.48 ± 3.47
	800	95.79 ± 5.47	5.64	86.58 ± 1.70
Luteoline	100	97.72 ± 2.65	2.62	105.57 ± 8.92

Table V. The Stabilities of the Analytes in Rat Plasma Sample and Plasma Extract at Different Conditions ($n = 6$)

Analytes	Spiked concentration (ng/mL)	Three freeze–thaw cycles		30 days' storage at −20°C		24 h at room temperature	
		Measured concentration (ng/mL)	RSD (%)	Measured concentration (ng/mL)	RSD (%)	Measured concentration (ng/mL)	RSD (%)
Quercetin	1	1.07 ± 0.10	9.25	1.04 ± 0.07	6.68	1.01 ± 0.10	9.64
	10	10.08 ± 0.64	6.39	10.28 ± 0.49	4.73	9.69 ± 0.60	6.22
	80	79.97 ± 2.12	2.65	80.62 ± 1.61	1.99	80.85 ± 1.53	1.90
Naringenin	10	9.58 ± 0.70	7.35	9.98 ± 0.36	3.62	10.19 ± 0.45	4.39
	100	97.94 ± 4.57	4.67	100.90 ± 2.28	2.26	101.10 ± 2.52	2.49
	800	798.94 ± 8.95	1.12	803.71 ± 9.49	1.18	798.97 ± 8.06	1.01
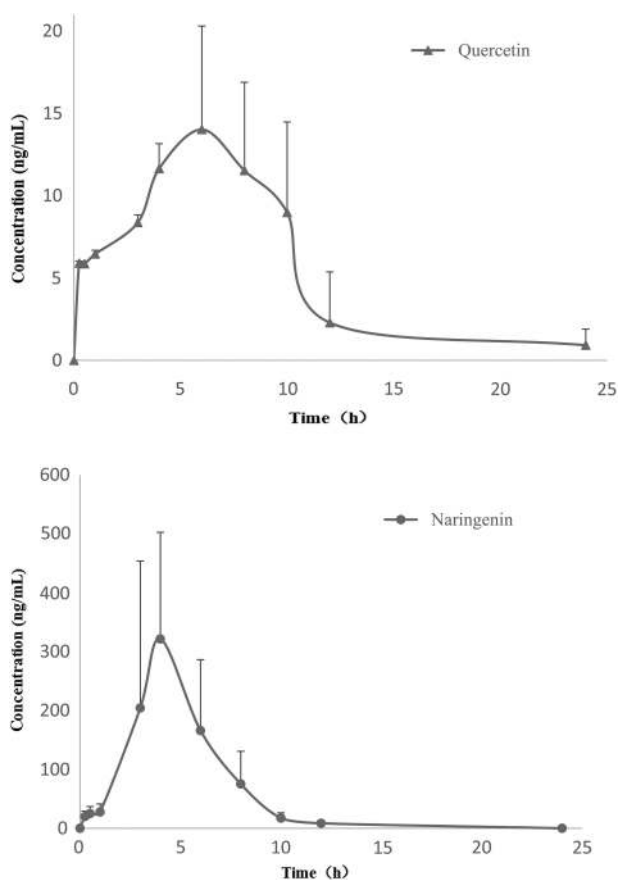
Figure 3. Mean (+SD) plasma concentration–time profiles of quercetin and naringenin after oral administration of Pollen Typhae extract to six rats.

IS was 97.72 ± 2.65%. Matrix effects of quercetin, naringenin and IS in rat plasma were between 85 and 115%, so there was no significant matrix effects on the analytes in this method.

Table VI. Pharmacokinetic Parameters of Quercetin and Naringenin After Oral Administration of Pollen Typhae Extract (Data are Mean ± SD, $n = 6$)

Parameters	Unit	Quercetin	Naringenin
T_{max}	h	6.1 ± 1.3	4.2 ± 1.0
C_{max}	ng/mL	16.7 ± 4.9	411.3 ± 193.0
AUC_{0-24}	ng h/mL	128.0 ± 21.8	1277.4 ± 567.8
$AUC_{0-\infty}$	ng h/mL	134.0 ± 22.2	1294.8 ± 565.0
$T_{1/2}$	h	5.0 ± 1.3	1.4 ± 0.2
MRT	h	8.6 ± 0.9	5.1 ± 0.5

Stability

Stability study showed that the concentrations of quercetin and naringenin were stable in plasma stored at 20°C for 24 h, −20°C for 30 days and after three freeze–thaw cycles at low, medium and high concentrations, respectively. All the values are shown in Table V.

Pharmacokinetic study

In this study, the method described above was applied to measure the plasma concentrations of quercetin and naringenin in rats administered orally with Pollen Typhae extract. The mean plasma concentration–time profiles are shown in Figure 3, and the main pharmacokinetic parameters are summarized in Table VI.

Quercetin plasma concentration reached a maximum at 6.1 h after administration with an average C_{max} of 16.7 ng/mL. The area under the curve ($AUC_{0-\infty}$) was 134 ng h/mL, and MRT was 8.6 h.

Naringenin plasma concentration reached a maximum at 4.2 h after administration with an average C_{max} of 411.3 ng/mL. The $AUC_{0-\infty}$ was 1294.8 ng h/mL and MRT was 5.1 h.

Discussion

Quercetin and naringenin are two major flavonoids in the extract of Pollen Typhae, which were considered antioxidant compounds with promising activity against oxidative damage in human cells. However,

little data were available regarding the pharmacokinetic characterization of quercetin and naringenin in rats after oral administration of Pollen Typhae extract. This is the first study of UPLC–MS-MS quantitative assay for investigating the pharmacokinetics of quercetin and naringenin in rats after oral administration of Pollen Typhae extract. There were no interferences from endogenous substances. Pharmacokinetic results showed that the two compounds reached a maximum at about 6.1 h and 4.2 h, respectively, indicated that they may be absorbed slowly after oral administration. Furthermore, the elimination of them may be also slow in rats because of the $T1/2$ was about 8.6 and 5.1h, respectively. These may be useful for the further clinical use and the pharmacological studies of Pollen Typhae.

Conclusion

A rapid and specific UPLC–ESI-MS/MS method was established for simultaneous determination of quercetin and naringenin in rat plasma, which was highly sensitive and accurate. The method has been successfully applied to pharmacokinetic studies of quercetin and naringenin in rats after oral administration of Pollen Typhae extract, and the pharmacokinetic parameters would be a suitable reference in clinical application.

Funding

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