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Short communication

A rapid LC/MS/MS quantitation assay for naringin and its two metabolites in rats plasma

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Abstract

Naringin is a flavonoid that exists in many plants and traditional Chinese medicines. In this study, a highly sensitive and specific electrospray ionization (ESI) liquid chromatography–tandem mass spectrometry (LC/MS/MS) method was developed for quantification of naringin and its two metabolites, naringenin and naringenin glucuronide. Naringin and naringenin were extracted from rat plasma with ethyl acetate, using hesperidin as an internal standard. Components in the extract were separated on a 100 mm × 2.0 mm Betabasic 5 μm C_{18} ODS column by isocratic elution with 70% methanol. The components were analyzed in the multiple-reaction-monitoring (MRM) mode in the precursor/product ion pair of *m*/*z* 581.3/273.4 for naringin, *m*/*z* 273.4/153.1 for naringenin and *m*/*z* 611.5/303.4 for hesperidin, respectively. Linear calibration curves were obtained in the range of 5–1000 ng/ml, using 0.1 ml rat plasma. The within-day coefficients of variation (CVs) were 3.1, 1.8 and 2.2% for naringin, 3.0, 3.3, 3.1% for naringenin at 5, 50 and 500 ng/ml ($n = 5$). The between-day CVs were 3.4, 1.7 and 4.9% for naringin and 4.0, 3.0, 4.6% for naringenin ($n = 5$) at 5, 50 and 500 ng/ml respectively. A formulation based on PEG400 was used and orally administered to Sprague-Dawley male rats. Plasma drug concentrations were measured by this method and the pharmacokinetics was analyzed by WinNonlin computer software. Plasma concentration–time profiles of naringin were found to increase quickly and decline rapidly within 2 h and could not be detected after 24 h. Naringenin and naringenin glucuronide occurred slower and the T_{max} were about 9 and 7.5 h later, respectively.

Keywords: Pharmacokinetics; Naringin; LC/MS/MS; Rats

1. Introduction

Naringin (4′,5′,7-trihydroxy flavanone-7-rhamnonglucoside) is a flavonoid present in many *Citrus* fruits and traditional Chinese medicines [1,2]. Like most flavonoids, naringin has anti-inflammatory [3], anti-ulcer [4], superoxide, and antioxidation [5] activities. Early research has shown that orally administered naringin can be metabolized into naringenin and naringenin glucuronide [6–8]. Naringenin, the aglycone of naringin, has also been found to exhibit anti-ulcer [4], antioxidant [5], and inhibition of breast cancer proliferation [9]. Several liquid chromatographic methods with UV-detection for detecting naringin in rabbit plasma [6], human plasma [7], human urine [8], and grapefruit juice have been developed. Ishii et al., reported the limits of detection by an assay in human urine was approximately 5 ng for naringin and 1 ng for naringenin [8], which is not sensitive enough to assay the plasma concentration of naringin due to the low oral dosage. To this day, research using the HPLC method with UV-detection to assay the naringin metabolite are unable to assay the naringin and its metabolite naringenin concurrently. Lack of specificity and low sensitivity still limit the effective detection of naringin and its metabolites.

The LC/MS/MS is a method that provides superior selectivity, sensitivity and a rapid rate of analysis. The method is accurate and highly sensitive for detecting plasma concentration in pharmacokinetics which accounts for its increasing

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popularity [10–16]. Our research established this new method of using LC/MS/MS for the determination of naringin and its metabolite naringenin concurrently in the plasma of Sprague-Dawley male rats.

2. Experimental

2.1. Materials and reagents

Naringin and naringenin were purchased from Sigma (St. Louis, MO, purity >95%), while the internal standard (IS) hesperidin, was purchased from the National Institute for the control of pharmaceutical and biological products (HPLC grade). Stock solutions of naringin, naringenin and hesperidin were prepared by dissolving them in methanol. All other chemicals and solvents were of analytical-reagent grade and were used without further purification.

2.2. Instrumentation

Analyses were performed using a SHIMADZU HPLC system (SHIMADZU, Columbia, MD), consisting of an SCL-10A system controller, an LC-10AD HPLC pump, a DGU 12A-degasser, and a manual injector. The mass spectrometric was made using an Applied Biosystems MDS Sciex API3000 Triple Quadrapole mass spectrometer (MS/MS) equipped with a heated nebulizer interface (500 °C). Data was collected and processed using Sciex Analyst 1.1 on a DELL computer.

2.3. Chromatographic conditions

The HPLC column (100 mm × 2.0 mm) was a 5 μm BetaBasic-C_{18} ODS column (KEYSTONE Bellefonte, PA). Column temperature was maintained at room temperature. The mobile phase consisted of methanol (70%) and water (30%), which was filtered through a 0.2 μm nylon filter before use. The flow rate was 0.4 ml/min and the total run time was 3.0 min for each injection.

2.4. MS/MS detection

Precursor ions for analytes and internal standard were determined from mass spectra obtained during infusion of the standard solution into the API3000 mass spectrometer. The mass spectrometer chose the positive ionization mode as the detection mode with the collision gas off. At the same time the mass spectrometer temperature was set at 370 °C. Under these conditions, the analytes yielded protonated molecules at *m*/*z* 581.3 for naringin, *m*/*z* 273.4 for naringenin and *m*/*z* 611.5 for hesperidin. Each of the protonated molecules was subjected to collision-inducted dissociation to determine the product ions. The product ions for naringin, naringenin, and hesperidin are shown in Fig. 1A–C. The primary naringin fragments lost glucoside and turned to naringenin, whose protonated molecular was *m*/*z* 273.4. Others are shown in Fig. 1B and (C). A positive ions multiple reaction monitoring (MRM) was used as the detecting mode. From the product ions results, we chose the protonated molecular pairs for MS/MS detection, which were *m*/*z* 581.3/273.4, *m*/*z* 273.4/151.3 and *m*/*z* 611.5/303.4 for naringin, naringenin, and hesperidin, respectively.

The mass spectrometer was operated using electrospray ionization (ESI) with an ion spray voltage of +4500 V, and using nitrogen as the collision gas, nebulizer gas and curtain gas. The curtain gas was set at 0.8 l/min. The standard solution of naringin, naringenin and hesperidin (20 μl injections of each 100 ng/ml solution) was used to optimize the detecting condition in the presence of LC mobile phase. After the parameter was optimized, others were shown as follows: declustering potential 62; focusing potential 350; entrance potential 11.0; collision cell exit potential 23.0; nebulizer gas 3.0; collision gas 7.0; temperature 370 °C. The rate of heated gas (gas2) was set at 6 l/min. The dwelling time was 100 ms.

2.5. Preparation of standards and quality control (QC) samples

Stock solutions of naringin and naringenin were prepared with methanol at a concentration of 100 μg/ml, followed by dilution with methanol to 2 μg/ml. The working standard rat's plasma solutions were serially diluted to a concentration of 5, 10, 50, 100, 500, 1000 ng/ml of naringin and naringenin with blank rat plasma. An internal standard was prepared in methanol at a concentration of 1000 ng/ml. Naringin and naringenin QC samples were prepared separately at the concentration of 5, 50, 500 ng/ml, the same as the plasma standard.

2.6. Dilution of test samples

Dilution samples were prepared by using the blank rat plasma to dilute the plasma samples into the working calibration curve.

2.7. Sample preparation

A 0.1 ml aliquot sample plasma was transferred to a clean glass tube. Ten microliters of methanol with 1000 ng/ml internal standard was added and vortexed. Then 400 μl ethyl acetate (LC grade) was added to the tube and vortexed again for 2 min. After the sample was centrifuged at 6000 × *g* for 10 min, the upper organic layer was transferred into a 5 ml glass tube. 200 μl ethyl acetate was added to the deposition again and then the deposition was extracted as those mentioned above again. The organic layer was mixed together and dried with nitrogen at room temperature. The dried residue was dissolved in 100 μl of the mobile phase. A 20 μl aliquot of the sample was injected into the LC/MS/MS.

The assay of naringenin with its glucuronide was the same as that reported earlier by others [6–8]. For determination of

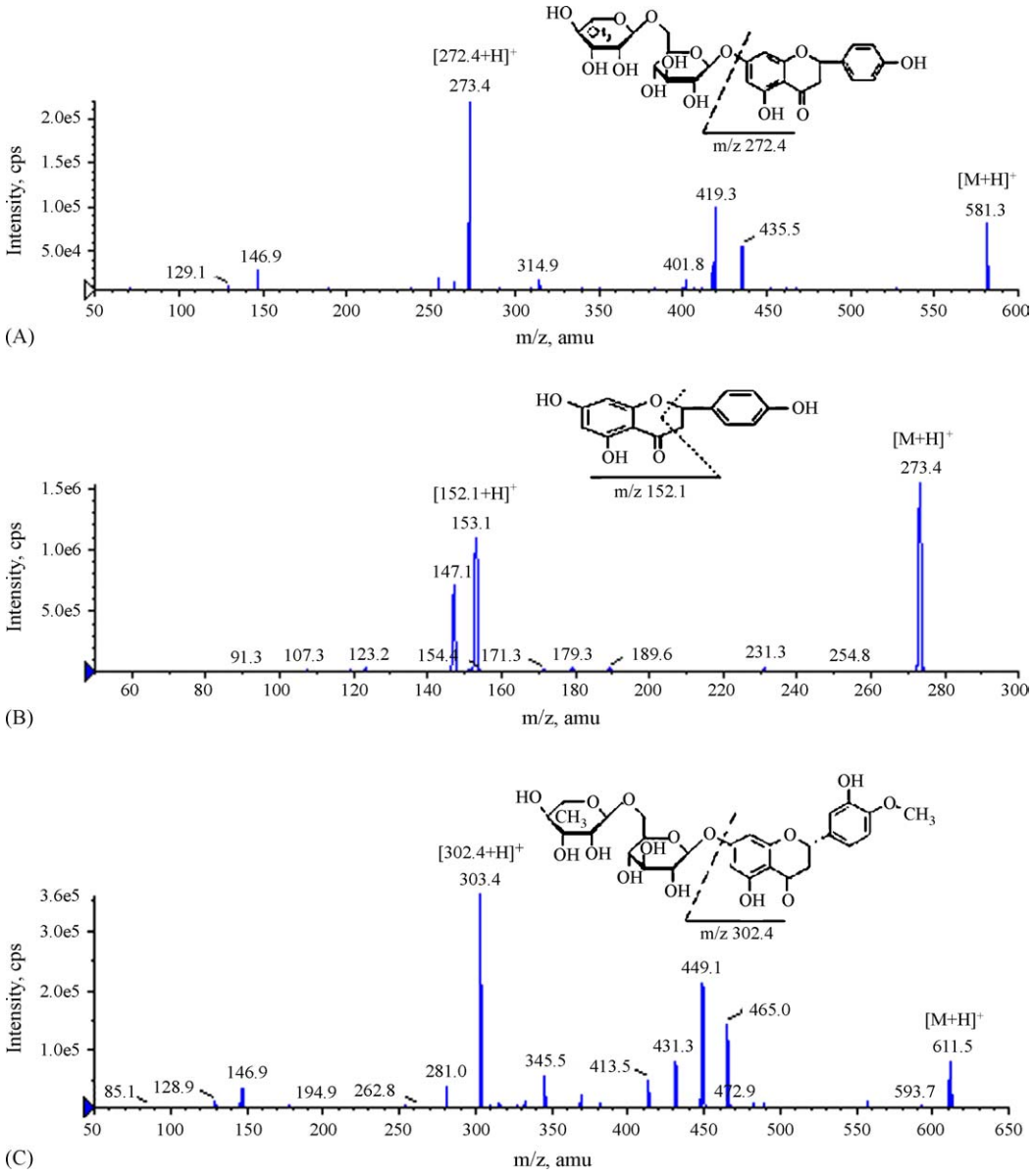

Fig. 1. Product ion mass spectra of naringin (A), naringenin (B) and hesperidin (C) (internal standard).

total naringenin (including naringenin and its glucuronide), plasma (100 μl) was incubated with 10 μl enzyme (glucuronidase 500 unit/ml) at 37 °C for 2 h. After adding 10 μl 0.5 N HCl, 10 μl methanol containing 1000 ng/ml hesperidin as internal standard was used to partition with the plasma sample and vortexed for 2 min, the next extracting steps were the same as above.

2.8. Assay validation

Plasma samples for the standard curve were prepared by spiking 0.1 ml of rat plasma, with various amounts of naringin, naringenin, and a constant amount of hesperidin. The linearity was evaluated in the concentration range of 5–1000 ng/ml of rat plasma. The within-day precision values were determined in five replicates at concentration of 5, 50, 500 ng/ml of naringin and naringenin in rat plasma, and these replicates were processed independently. The between-day precision was determined by three different concentrations in 5 days, and calculated the mean concentrations and coefficient of variation. The accuracy of the assay was determined by comparing the nominal concentrations with the corresponding calculated concentrations via linear regression. The specificity of the assay was prepared by one major product ion from the molecular of naringin and naringenin in blank plasma. No disturbance was found at the same retention time of naringin and naringenin. The recovery of naringin and naringenin was estimated by comparing the peak area of the extract of naringin and naringenin with that of the unextracted naringin, naringenin at three concentrations of

5, 50, 500 ng/ml. The accuracy and precision of LOQ were determined by detecting the same sample five times and determined in five separate samples.

2.9. Pharmacokinetics of naringin in the rat

Six male Sprague-Dawley rats (body weight 250–300 g) were purchased from the College of Military Medicine Animal Research Center (Guangzhou, China). Naringin was dissolved into 127.9 mg/ml with 50% PEG400. The rats were dosed via oral gavage with 746.7 mg/kg naringin. Approximately 0.5 ml blood was withdrawn from each rat according to a schedule of 0 (predose), 5, 15, 30, 45, 60, 120, 240, 360, 480, 720 and 1440 min after dosing. The blood samples were centrifuged at 6000 × *g* for 10 min and the supernatants of each were collected and frozen at −20 °C for analysis.

Plasma concentration–time profiles were analyzed by WinNonlin computer software, Version 4.0 (Pharsight Corporation, Mountain View, CA), using a noncompartmental method.

3. Results and discussion

3.1. LC/MS/MS assay

Under ESI conditions, naringin gave MH^+ at *m/z* 581.3 as the base ion, naringenin and hesperidin gave MH^+ at *m/z* 273.4 and *m/z* 611.5 as the base ion. The ions *m/z* 581.3, 273.4 and 611.5 were selected for collision-induced dissociation (CID) experiments. Naringin generated one major product ion at *m/z* 273.4 (Fig. 1A), naringenin generated two product ions at *m/z* 147.1 and *m/z* 153.1 (Fig. 1B), and hesperidin generated two product ions at *m/z* 449.1 and 303.4 (Fig. 1C).

The precursor/product ion pairs at *m/z* 581.3/273.4, 273.4/153.1 were selected in the MRM mode for quantitation of naringin and naringenin. The precursor/product ion pair at *m/z* 611.5/303.4 of internal standard hesperidin was selected for the assay.

3.2. Assay validations

The assay was validated in rat plasma. The MRM chromatograms of naringin and naringenin are shown in Fig. 2. There was no detectable disturbance in blank plasma. As shown, naringin and naringenin were separated; likewise, naringenin and hesperidin were also separated. The retention time was 0.5, 0.5, and 1.07 min for naringin, hesperidin and naringenin, respectively. The absence of a signal at the retention time of naringin, naringenin and hesperidin in the blank plasma established the specificity of the assay. The limit of quantitation (LOQ) was set at 0.5 ng/ml in rat plasma for naringin and 0.2 ng/ml for the naringenin on the base of a signal-to-noise level above 10:1, the accuracy values of the assay varied from 93.02 to 96.37% for naringin and 94.89 to 96.34% for naringenin. The within-day precision values

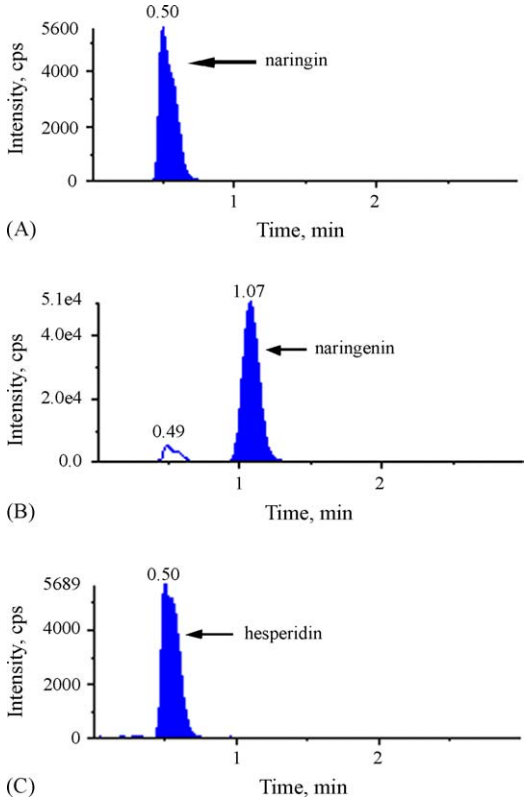

Fig. 2. The positive ions multiple reaction monitoring (MRM) chromatograms of rat plasma at *m/z* 581.3 → 273.4, *m/z* 273.4 → 153.1 and *m/z* 611.5 → 303.4 obtained from (A) blank rat plasma containing 100 ng/ml naringin; (B) blank rat plasma containing 100 ng/ml naringenin; (C) blank rat plasma containing 100 ng/ml IS. (The arrow indicates the position of naringin, naringenin or IS peak.)

ranged from 3.9 to 5.3% for naringin and 4.5 to 5.7% for naringenin.

The assay was linear from 5 to 1000 ng/ml, using 0.1 ml rat plasma, with regression coefficients (r^2) >0.9996 for naringin and (r^2) >0.9956 for naringenin. The validation results for quality control samples of naringin and naringenin at low (5 ng/ml), medium (50 ng/ml), and high (500 ng/ml) are shown at Table 1, which reflected the within-day precision and between-day precision expressed as %CV. As shown, the values ranged from 1.8 to 3.1% for naringin and 3.0 to 3.3% for naringenin. The between-day precision values ranged from 1.7 to 4.9% for naringin and 3.0 to 4.6% for naringenin. The accuracy values of the assay varied from 100.5 to 103.4% for naringin and 98.4 to 101.5% for naringenin. The mean recovery values for the entire procedure were found to be 90.58, 91.64, 94.15% for naringin and 93.17, 91.25, 89.78% for naringenin at 500, 50, 5 ng/ml in rat plasma ($n = 5$).

3.3. Application

The pharmacokinetics of naringin after oral administration in Sprague-Dawley male rats was studied. Pharmacokinetics was assessed using the noncompartmental method. The

Table 1
Assay validation characteristics of naringin and naringenin in rat plasma by ESI LC/MS/MS

Concentration (ng/ml plasma)	Within-day calculated					Average ± S.D.	CV (%)	Accuracy (%)
Naringin								
5	5.06	5.13	5.04	5.43	5.21	5.17 ± 0.16	3.1	103.4
50	51.8	51.0	49.8	52.0	52.1	51.34 ± 0.96	1.8	102.7
500	504.0	510.0	498.0	514.0	486.0	502.4 ± 10.99	2.2	100.5
Naringenin								
5	4.68	4.87	5.02	4.98	5.04	4.918 ± 0.148	3.0	98.4
50	51.4	48.6	50.8	47.3	49.5	49.52 ± 1.654	3.3	99.0
500	512.0	527.0	503.0	484.0	512.0	507.6 ± 15.758	3.1	101.5

	Between-day calculated					Average ± S.D.	CV (%)
Naringin							
5	4.98	5.03	5.16	4.72	4.86	4.95 ± 0.17	3.4
50	50.4	52.3	51.2	50.1	50.6	50.92 ± 0.87	1.7
500	484.0	493.0	517.0	532.0	472.0	499.60 ± 24.50	4.9
Naringenin							
5	4.62	4.83	4.91	5.04	5.12	4.90 ± 0.19	4.0
50	51.8	50.4	49.5	48.1	51.4	50.24 ± 1.49	3.0
500	507.0	521.0	493.0	517.0	509.0	503.4 ± 23.30	4.6

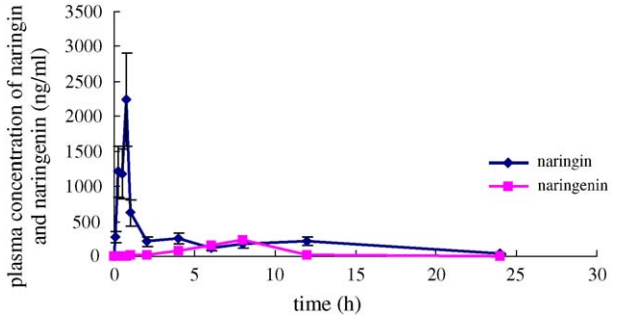

Fig. 3. Mean plasma concentration–time plots of naringin and naringenin after a single oral dose of naringin (746.7 mg/kg) to six Sprague-Dawley male rats. Each point represents mean ± S.D.

mean plasma concentration–time profiles of naringin, naringenin, and its glucuronide are shown in Figs. 3 and 4, and the pharmacokinetics parameters are shown in Table 2. The concentration of naringin in plasma reached nearly 2300 ng/ml in 45 min and declined rapidly within 2 h (Fig. 3). Naringin cannot be detected after 24 h. The concentration of naringenin (Fig. 3) and naringenin glucuronide (Fig. 4) in plasma increased more slowly after oral administration of naringin. The T_{max} of naringenin and naringenin glucuronide occurred at 9 and 7.5 h.

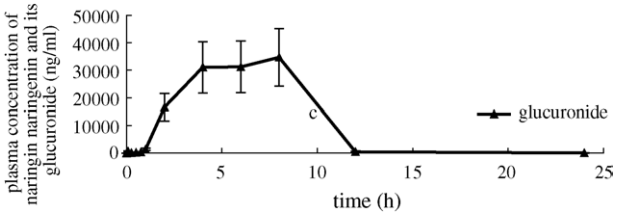

Fig. 4. Mean plasma concentration–time plots of naringenin glucuronide after a single oral dose of naringin (746.7 mg/kg) to six Sprague-Dawley male rats. Each point represents mean ± S.D.

Table 2
Relevant pharmacokinetic parameters of naringin, naringenin, and naringenin glucuronide in Sprague-Dawley rats following oral administration at 746.7 mg/kg using noncompartmental method except as indicated

Parameters	Average ± S.D. (n = 6)
Naringin	
T_{max} (h)	0.75 ± 0.25
C_{max} (ng/ml)	3782.50 ± 986.82
$AUC_{0–24}$ (ng/ml h)	6026.32 ± 1562.63
MRT (h)	6.47 ± 1.18
CL (ml/h)	0.028 ± 0.008
HL-λz (h)	5.34 ± 1.07
Naringenin	
T_{max} (h)	9.00 ± 1.50
C_{max} (ng/ml)	227.05 ± 88.41
$AUC_{0–24}$ (ng/ml h)	1252.24 ± 461.66
MRT (h)	8.16 ± 1.59
CL (ml/h)	0.30 ± 0.070
HL-λz (h)	2.98 ± 0.65
Naringenin glucuronide	
T_{max} (h)	7.50 ± 1.00
C_{max} (ng/ml)	43575.00 ± 8409.00
$AUC_{0–24}$ (ng/ml h)	238269.00 ± 53253.10
MRT (h)	6.37 ± 0.60
CL (ml/h)	0.0004 ± 0.00019
HL-λz (h)	1.84 ± 0.16

4. Discussion

Naringin considered the most potent okadaic acid-antagonistic flavonoid, has been shown to exhibit bioactivity in a number of different ways such as anti-inflammatory [3], anti-ulcer [4], superoxide, and antioxidation [5]. Previous methods were used HPLC-UV to detect the naringin and its metabolites. Compared with the HPLC method, the lower limits of this method for naringin was 0.5 ng/ml, approximately 100 times higher than that of HPLC. Compared

with LC/MS/MS, the HPLC method cannot detect naringin, naringenin, and naringenin glucuronide at the same time. Additionally, two internal standards must be applied with a retention time of more than 10 min. In our study, the retention time was 0.5 min for naringin, 0.5 min for hesperidin, and 1.07 min for naringenin, which obviously reduced the detection time when compared with the HPLC method. Utilization of one internal standard in this method can efficaciously simplify the detecting procedure better than the HPLC method. As previously noted, the LC/MS/MS is a highly rapid, sensitive, and specific method for the quantitation assay of naringin and its two metabolites in rat plasma. It has also been successfully applied to investigate the pharmacokinetics of naringin.

In our results, naringin was quickly absorbed into body and could be detected 5 min after oral administration. The T_{max} was 45 min. The plasma concentration of naringin also declined quickly. Its metabolites, naringenin and naringenin glucuronide, were produced slowly after oral administration and T_{max} for them were 9 and 7.5 h, respectively. For naringin, naringenin, and naringenin glucuronide, the C_{max} was 3782.50 ± 986.82, 227.05 ± 88.41, 43575.00 ± 8409.00 ng/ml and the AUC_{0-24} was 6026.32 ± 1562.63, 1252.24 ± 461.66, 238269.00 ± 53253.10 ng/ml h, respectively. The AUC_{0-24} of naringenin glucuronide was higher than naringin and naringenin, indicating that naringenin glucuronide is the main existent form in rat plasma after oral administration.

5. Conclusion

A highly sensitive and specific LC/MS/MS method for the quantification of naringin and its two metabolites was developed and successfully applied to investigate the pharmacokinetics of naringin following oral administration in Sprague-Dawley male rats.

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