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LC–MS/MS determination of naringin, hesperidin and neohesperidin in rat serum after orally administrating the decoction of *Bulpleurum falcatum* L. and *Fractus aurantii*

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Abstract

To identify and quantify biologically active components in rat serum after orally administrating the decoction of *Bulpleurum falcatum* L. and *Fractus aurantii*, one of traditional Chinese medicines (TCMs), high-performance liquid chromatography (HPLC)–tandem mass spectrometry method was developed and validated. The HPLC separation was carried out on a Waters Nova Pak C_{18} column using acetonitrile and water as mobile phase after the sample of rat serum was cleaned up with solid-phase extraction. Atmospheric pressure chemical ionization in the negative ion mode and selected reaction monitoring (SRM) method was developed to determine the active components. Three flavonoids of hesperidin, neohesperidin and naringin were identified in the serum by comparing their retention times and three independent SRM precursor/product ion transitions with those of corresponding reference standards. The concentrations of naringin, hesperidin and neohesperidin in rat serum determined by SRM measurement were 16.3, 11.9 and 14.3 ng/ml, respectively, after orally administrating the decoction of *B. falcatum* L. and *F. aurantii*. This method was validated in terms of recovery, linearity, accuracy and precision (intra- and interday variation). The recoveries from spiked control samples were 93.0, 89.3 and 91.2% for hesperidin, neohesperidin and naringin, respectively. Linearity in rat serum was observed over the range of 2.0–50.0 ng/ml. Percent bias (accuracy) and precision were well within the acceptable range and the relative standard deviation (R.S.D.) of the measured rat serum samples was less than 10% ($n = 5$).

Keywords: LC–MS/MS; Hesperidin; Naringin; Neohesperidin; Qualification; Quantification

1. Introduction

Modern drugs in the form of single chemical entities have been successful in the treatment of acute conditions, while traditional Chinese medicines (TCMs) are more appropriate to the treatment of chronic diseases [1]. However, the relationship between clinical effects and biologically active components of herbal medicines is still controversial. To resolve the problem, it is important to perform component analysis to define actually absorbed components when complex mixtures are given orally [2].

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Bulpleurum falcatum L. and *Fractus aurantii* are two component herbs of traditional Chinese formulae Xuefuzhuyutang (XFZY), which is a traditional Chinese formula and used to treat arteriosclerosis. Our pharmacological results in rats indicated that the decoction of *B. falcatum* L. and *F. aurantii* could markedly reduce the lipoprotein (α), low-density lipoprotein and triglyceride levels [3]. So the active components in rat serum are of interest. The main components in *F. aurantii* are flavonoids including naringin, hesperidin and neohesperidin and those of *B. falcatum* L. are saikosaponins [4,5].

It is known that saikosaponins are degraded in the gastrointestinal tract before absorption [6], so they were beyond the scope of detection. The flavonoids were the interested targets. Several liquid chromatographic methods with UV detection for measuring one or two components of naringin, hesperidin and neohesperidin in different matrix have been reported [7–12], but there are inherent limitations with high-performance liquid chromatography (HPLC)–UV methods—lower sensitivity and lack of specificity. The detection limit of hesperidin and naringin in processed foods by fast semi-micro-HPLC was 0.5 mg/g [9]. The determination of naringin and neohesperidin in orange juice by liquid chromatography make progress with detection limits of 10 and 2 μg/ml, respectively [11]. The lower limit of quantification was 25 ng/ml for naringin in human urine by HPLC [12]. Gradient elution liquid chromatography–selected ion monitoring mass spectrometry (SIM LC–MS) was used to study the contents of eleven flavonoids including naringin, hesperidin and neohesperidin in orange juice. Limits of detection for neohesperidin, naringin and hesperidin were 20 ng/ml [13], which were still not sensitive enough to determine naringin, hesperidin and neohesperidin in the biological samples.

Generally, identification and quantification of components in serum are required for investigation of their potential contribution to the activity of *B. falcatum* L. and *F. aurantii* examined. However, TCMs showed a daunting complexity of compounds and biological activities from each of the component herbs [14]. To date, no work has been reported on the screening of active multi-components in rat serum after administration of *B. falcatum* L. and *F. aurantii*. In the present study, qualitative and quantitative analyses of components in rat serum at picogram levels were carried out using liquid chromatography (LC)–atmospheric pressure chemical ionization (APCI)–tandem mass spectrometry (MS/MS). The validation of this method was performed using neoeriocitrin as the internal standard. This study indicates that the methods performed well in terms of validation criteria of selectivity, linearity, precision and accuracy.

2. Experimental

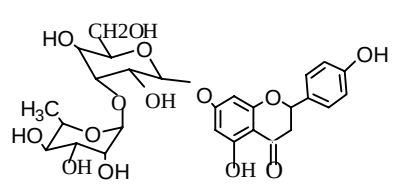
2.1. Materials

Naringin was purchased from Acoros (Geel, Belgium). Hesperidin, and neohesperidin were purchased from Sigma (St. Louis, MO). Neoeriocitrin, as internal standard (I.S.), was purchased from Fluke (Buchs, Switzerland). The chemical structures of the flavonoids used in this study are shown in Fig. 1. Acetonitrile was HPLC grade from Tedia Company Inc., USA. Water was purified with Milli-Q system (Millipore, Bedford, MA, USA). Methanol was HPLC grade from Yuwang Company Inc. (Shandong, China). *B. falcatum* L. and *F. aurantii* were provided by Xiyuan Hospital, Beijing, China.

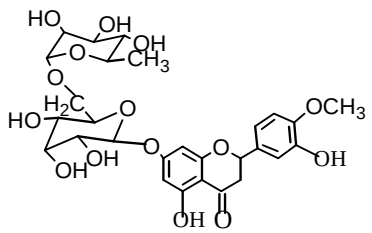
2.2. Preparation of decoction and collection of serum samples

B. falcatum L. (100 g) and *F. aurantii* (200 g) were mixed and extracted by refluxing with 3000 ml of 95% ethanol for 1 h and then filtered. This procedure was repeated. The combined ethanol extracts were concentrated with vacuum rotary evaporator to 80 g. The residue of the extracts was dissolved and diluted to concentration of 0.135 g herbs/ml with distilled water.

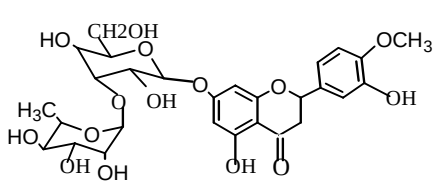
Wistar rats, weighing 200 ± 20 g, were fed with high-fat diet (2% cholesterol, 10% lard, 0.2% methylthiouracil, 0.5% cholate and 87.3% common feed) after having been fed with common feed for one week. Meanwhile the rats were given 1.35 g/kg per day (corresponding to the maximum clinical dose) the decoction of *B. falcatum* L. and *F. aurantii* orally for 7 days. After the last time of administration, the rats were abstained from food except water for 12 h. The blood was sampled from the aorta abdominal and



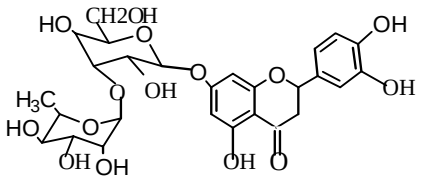
Naringin ($C_{27}H_{32}O_{14}$) Mr=580.5



Hesperidin ($C_{28}H_{34}O_{15}$) Mr=610.5



Neohesperidin ($C_{28}H_{34}O_{15}$) Mr=610.5



Neoeriotrin ($C_{27}H_{32}O_{15}$) Mr=596.5

Fig. 1. The chemical structures of naringin, hesperidin, neohesperidin and neoeriocitrin.

pooled. The serum was obtained by centrifuging the blood at 2000g for 20 min and stored at −78 °C till analysis.

2.3. Sample preparation and standard

2.3.1. Preparation of the samples

For quantitative analysis, an aliquot (0.2 ml) of rat serum was fortified with 20 ng of I.S. and the mixture was ultrasonated for 5 min. The sample was purified using Waters Oasis HLB solid-phase extraction (SPE) cartridge (30 mg packing, Waters, Milford, MA, USA). The cartridge was firstly conditioned with 1 ml methanol and then equilibrated with 1 ml water. After sample loading, the cartridge was washed with 1.0 ml of 5% methanol. Finally, the analyte was eluted with 1.0 ml of methanol. The methanol eluent was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 100 μl mobile phase and 25 μl of the solution was injected into the LC–MS/MS system.

For qualitative analysis, an aliquot (1.0 ml) of serum was extracted by SPE. After evaporation of solvent, the residue was dissolved in 100 μl mobile phase and 25 μl of the solution was injected into the LC–MS/MS system.

2.3.2. Preparation of samples for calibration curve and quality control

Calibration samples and quality control (QC) samples were prepared to cover the range of 2.0–50.0 ng/ml. Stock solutions of 0.2 mg/ml concentrations of naringin, hesperidin, neohesperidin and neoeriocitrin were prepared by separately dissolving an accurately weighed amount of these compounds in appropriate volumes of methanol.

A combined stock solution was prepared by combining 0.5 ml of each of the stock solutions of naringin, hesperidin, neohesperidin and 8.5 ml mobile phase in a volumetric flask. The working solutions were prepared by diluting the combined stock solution in series to the concentrations of 20.0, 50.0, 100.0, 200.0 and 500.0 ng/ml of each compound and spiked with I.S. at the concentration of 100 ng/ml. Calibration samples were prepared by spiking 20 μl of the working solutions to the blank serum. The concentrations of the calibration samples were 2.0, 5.0, 10.0, 20.0 and 50.0 ng/ml.

Quality control samples were prepared in the same way as the calibration samples at concentrations corresponding to 4.0, 24.0 and 48.0 ng/ml.

2.4. Instruments and conditions

The HPLC system included a Waters 2690 equipped with a gradient controller, an automatic sample injector and 996-photodiode array detector. Separation was performed on a Nova Pak C_{18} column (250 mm × 4.6 mm i.d., 4 μm) from Waters. The mobile phase was acetonitrile–water (25:75, v/v) and the flow rate was 0.8 ml/min. The column temperature was set at 40 °C. The injection volume was 25 μl.

MS experiments were performed on a Finnigan TSQ (San Jose, CA, USA) mass spectrometer equipped with an APCI interface. Mass spectrometric conditions were optimized in order to achieve maximum sensitivity. The APCI conditions were as follows: corona discharge voltage, 4.5 kV; heated capillary temperature, 280 °C; nebulization temperature, 450 °C; nitrogen was used both as sheath gas (70 psi; 1 psi = 6894.76 Pa) and auxiliary gas (25 a.u.). Argon was used as the collision gas with collision energy of 35 V.

3. Results and discussion

3.1. Qualitative analysis: screening the components in rat serum

One concern about the determination of the components was to ensure reliable characterization of the targeted compounds in rat serum. The preferred approach was to compare the mass spectra and retention times of target compounds with those of corresponding reference standards from LC–MS/MS. The criteria generally accepted include the agreement of the retention time of a tested component with that of the reference standard within 2% under the same conditions [15] and the MS/MS mass spectra data should agree with those of a reference standard analyzed under the same conditions. However, sometimes satisfactory full-scan LC–MS/MS mass spectra cannot be obtained due to the very low levels of the target compounds in biological samples. One strategy is to compare at least two and preferably three independent selected reaction monitoring (SRM) precursor/product ion transitions of target compounds with corresponding reference standards and the criteria was the absolute relative abundance of the precursor/product ion transitions agreed within ±10% [15]. The strategy in this work is to compare three independent SRM precursor/product ion transitions and the retention times of target compounds with corresponding reference standards under the same conditions.

Three independent SRM LC–MS analyses of each target compound in rat serum were carried out. The precursor/product ion transitions of naringin, hesperidin and neohesperidin are listed in Table 1. The results indicate that HPLC chromatographic retention times agree with those of the three standard flavonoids within 0.6% under the same conditions. Three independent SRM precursor/product ion transitions were compared for the target compounds in the serum extract with the reference standards. The identical chromatographic retention times observed for

Table 1
Reproducibility of retention time and relative abundance observed for precursor-product ion transition from SRM analyses of hesperidin, neohesperidin and naringin in rat serum extracts ($n = 5$)

Flavonoid	Retention time (min) from standards	R.S.D. from the rat serum extracts (%)	Precursor ion product ion transitions from standards	Abundance of product ion relative to the base peak from the rat serum extracts	R.S.D. from the rat serum extracts (%)
Naringin	5.7	0.2	579 → 271	100	0
			579 → 459	51	1.1
			579 → 151	17	2.3
Hesperidin	6.2	0.6	609 → 301	100	0
			609 → 325	5	5.0
			609 → 343	5	10.4
Neohesperidin	6.8	0.5	609 → 301	100	0
			609 → 343	6	5.0
			609 → 489	5	9.1

all of these transitions supported the common origin of all product ions. Also the absolute relative abundance of the precursor/product ion transitions agreed within 4% when the relative abundance of product ion were 5. These data provided the ultimate results in high-sensitivity and high-specificity qualitative determination of targeted compounds in the rat serum.

3.2. Optimization of experimental conditions

The chromatographic conditions were optimized to obtain good separation of the target compounds and avoid the interferences. The retention times of naringin, hesperidin and neohesperidin were 5.7, 6.2 and 6.8 min, respectively.

In the negative ion mode, the most abundant ions observed were singly charged pseudomolecular ions ($[M–H]^-$) for naringin, hesperidin and neohesperidin. Therefore, $[M–H]^-$ were chosen as precursor ions in the collision-induced dissociation (CID) MS/MS experiments. Product ion mass spectra were obtained at different collision energies to characterize each compound's fragmentation pattern and to select appropriate CID energy that produced useful abundance of fragment ions for each compound. Under the conditions of 35 V CID energy, naringin exhibited two predominant ions 271 and 459, while hesperidin and neohesperidin showed one main product ion 301, as shown in Fig. 2. The selected product ions for SRM transitions for quantification on the basis of best sensitivity and minimum interference were 579/271, 609/301 and 609/301 for naringin, hesperidin and neohesperidin, respectively.

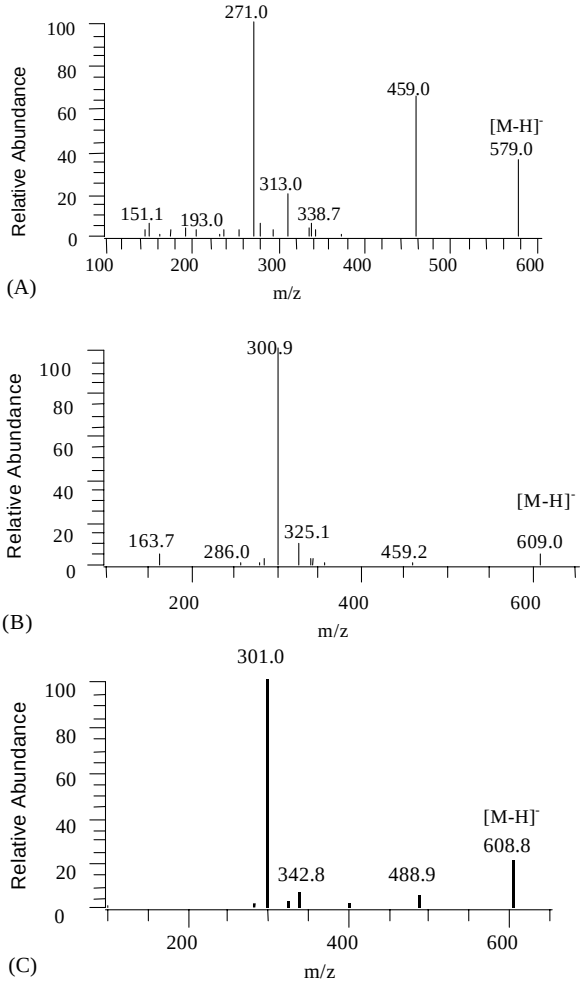


Fig. 2. The product ion mass spectra of naringin (A), hesperidin (B) and neohesperidin (C) in the negative ion mode. Conditions: APCI interface; corona discharge current, 4.5 μA; heated capillary temperature, 280 °C; nebulization temperature, 450 °C; sheath gas (N_2) pressure, 70 psi; auxiliary gas (N_2), 25 a.u. Argon was used as collision gas with collision energy of 35 V.

3.3. Quantitative analysis

The validation of the LC–MS/MS method included intra- and interday precision and accuracy studies on three days. The accuracy and precision were calculated from studies carried out in triplicate at three different concentrations of 4.0, 24.0 and 48.0 ng/ml in serum and analyzed in each day and three such batches were processed.

3.3.1. Specificity

The specificity was defined as non-interference with the endogenous substances in the regions of interest. LC–MS/MS analysis of the blank serum samples actually showed no endogenous peak interference present with the quantification of naringin, hesperidin and neohesperidin and their internal standard. Representative chromatograms of blank rat serum fortified with naringin, hesperidin and neohesperidin and extracted blank rat serum are shown in Fig. 3(A) and (B), respectively.

3.3.2. Recovery

Absolute recoveries of the analytes were studied in triplicate in blank serum spiked with analytes.

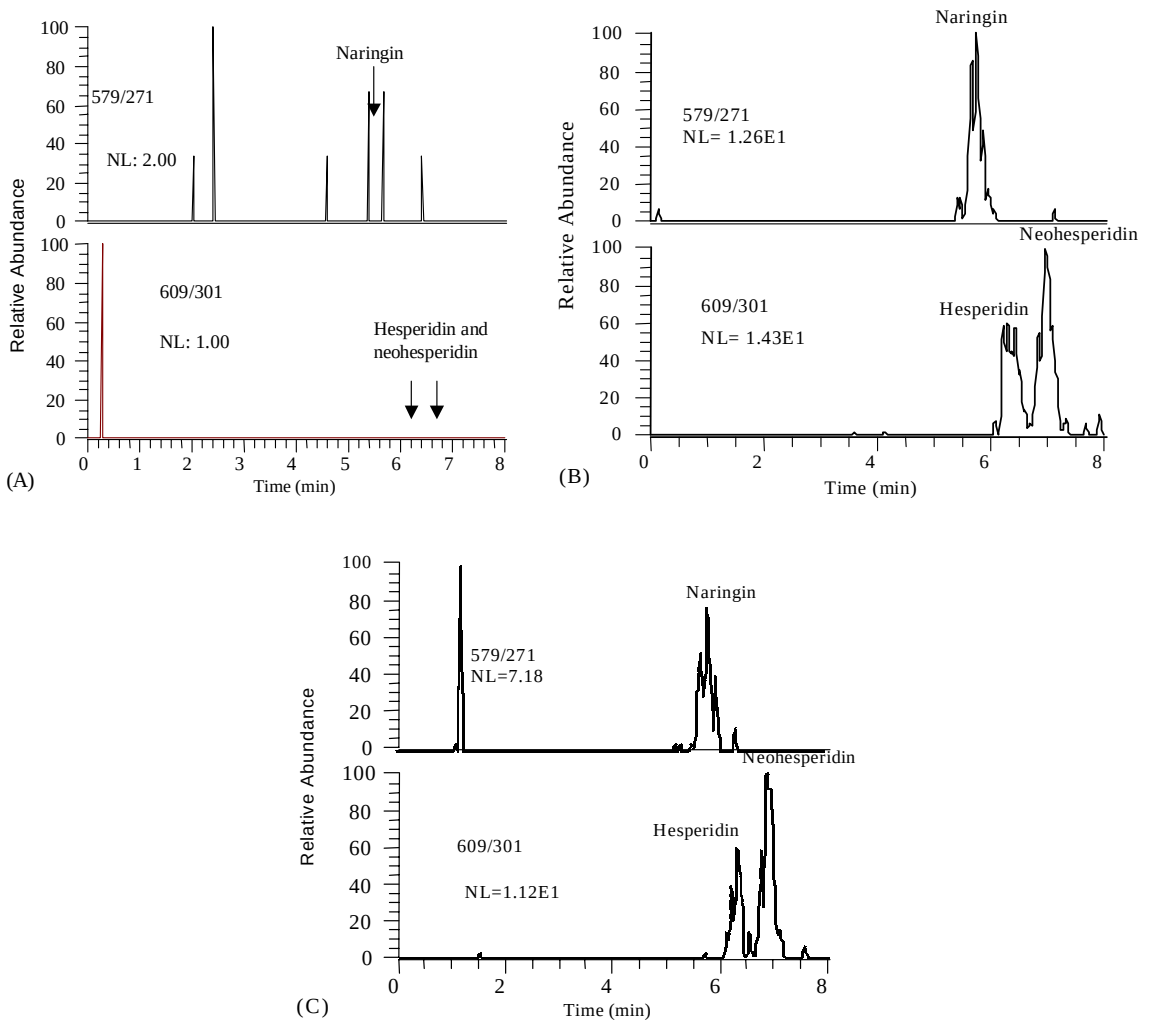


Fig. 3. Selected reaction monitoring of: (A) blank rat serum; (B) blank rat serum fortified with naringin, hesperidin and neohesperidin (20.0 ng/ml each); (C) naringin, hesperidin and neohesperidin in rat serum after administrating of the decoction. Conditions are the same as in Fig. 2.

Recoveries were determined by comparing the peak area from the extracted sample versus those for the reconstituted blank serum extracts spiked after extraction. The recoveries of hesperidin, neohesperidin and naringin were 93.0, 89.3 and 91.2%, respectively.

3.3.3. Calibration curve

Calibration standard at 2.0, 5.0, 10.0, 20.0 and 50.0 ng/ml in rat serum for the naringin, hesperidin and neohesperidin were used to construct the corresponding calibration curves by plotting peak area ratio of the analytes and the internal standard against the analytes concentrations. The weighed ($1/x^2$) least-squares regression line was fitted over the range of 2.0–50.0 ng/ml for naringin, hesperidin and neohesperidin and correlation coefficients were greater than 0.99 for all the three flavonoids. Data for calibration curves of naringin, hesperidin and neohesperidin are shown in Table 2.

Table 2
Data for calibration curve of naringin, hesperidin and neohesperidin

	Nominal concentration (ng/ml)	Calculated concentration (ng/ml, $n = 3$)		
		Naringin	Hesperidin	Neohesperidin
	2.0	1.82 (15.0)	1.75 (12.3)	1.86 (16.4)
	5.0	4.53 (10.2)	4.62 (8.1)	4.70 (7.5)
	10.0	9.52 (8.5)	9.33 (7.6)	9.56 (10.8)
	20.0	18.6 (10.5)	18.4 (9.2)	19.1 (8.4)
	50.0	47.9 (6.7)	47.3 (11.2)	48.1 (7.2)
Slope		0.0705	0.1468	0.0942
Intercept		0.0050	0.0014	−0.0020
R		0.995	0.992	0.996

The values given in parentheses indicate error (%, R.S.D.).

3.3.4. Precision and accuracy

Table 3 presents the accuracy and intra- and interday precision which was determined by analyzing triplicate of QC samples at three concentrations on three successive days. The results show that the analytical method was accurate and the bias was within the acceptable limits of 20% at all concentration levels studied. The intra- and interday precision was assessed by the analysis of variance of measured QC concentration in triplicate. The precision for naringin, hesperidin, and neohesperidin were found to be less than 20%. The results are listed in Table 3.

3.3.5. Lower limit of quantification

The lower limit of quantification (LLQ) was defined as the lowest concentration on calibration curve with acceptable precision (<20% relative standard deviation (R.S.D.)). The described assay had an LLQ of 2.0 ng/ml in rat serum for the three flavonoids based on 200 μl aliquots of rat serum. The precision and accuracy at the LOQ for this method was less than 20%. Limits of detection (S/N = 3) for naringin, hesperidin and neohesperidin were 0.7 ng/ml.

3.4. Analysis of serum samples

The method was applied to investigate the concentration of naringin, hesperidin and neohesperidin in rat serum after orally administrating the decoction of *B. falcatum* L. and *F. aurantii*. The serum was collected after 12 h post dose and stored at −78 °C until further analysis. Typical representative chromatograms of treated rat serum are given in Fig. 3(C). The average concentrations of naringin, hesperidin

Table 3
The recovery, precision and accuracy of the method

Analyte	Recovery		Concentration (ng/ml)	Precision (%, R.S.D.)		Bias (%)	
	Mean (%)	R.S.D. (%)		Intraday	Interday	Intraday	Interday
Hesperidin	93	7.9	2	18.9	15.7	15.1	19
			24	12.2	13.1	11.2	15.8
			48	7	10.2	9.1	10.9
Neohesperidin	89.3	8.9	2	13.3	20.3	21.5	19.2
			24	6.6	10.7	13.1	13.9
			48	6.1	12.8	9.8	7.9
Naringin	91.2	10.8	2	11.2	17	18.2	19.3
			24	4.4	13.7	6.7	12
			48	12.3	14.5	9.5	10.3

and neohesperidin were 16.3, 11.9 and 14.3 ng/ml with the R.S.D. less than 10% ($n = 5$).

4. Conclusions

Chemical identification and quantification of components in lower concentrations and micro quantities are the barriers in the studies of active components of TCMs in biological fluids. The identification of components in biological fluids by the only use of chromatography is unreliable. So, a highly selective and sensitive LC–MS/MS method was developed and validated for the simultaneous qualification and quantification of components in rat serum after oral administration of the decoction of *B. falcatum* L. and *F. aurantii*. The method is useful for screening potential drugs at low levels in biological fluids. As a result, naringin, hesperidin and neohesperidin were identified in rat serum. This method is highly suitable for carrying out preclinical or pharmacokinetic studies of TCMs.

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