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The simultaneous separation and determination of six flavonoids and troxerutin in rat urine and chicken plasma by reversed-phase high-performance liquid chromatography with ultraviolet–visible detection

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Abstract

The method of high-performance liquid chromatography (HPLC) with UV–vis detection was used and validated for the simultaneous determination of six flavonoids (puerarin, rutin, morin, luteolin, quercetin, kaempferol) and troxerutin in *rat urine* and chicken plasma. Chromatographic separation was performed using a VP-ODS column (150 mm × 4.6 mm, 5.0 μm) maintained at 35.0 °C. The mobile phase was a mixture of water, methanol and acetic acid (57:43:1, v/v/v, pH 3.0) at the flow rate of 0.8 mL/min. Six flavonoids and troxerutin were analyzed simultaneously with good separation. On optimum conditions, calibration curves were found to be linear with the ranges of 0.10–70.00 μg/mL (puerarin, rutin, morin, luteolin, quercetin, kaempferol) and 0.50–350.00 μg/mL (troxerutin). The detection limits were 0.010–0.050 μg/mL. The method was validated for accuracy and precision, and it was successfully applied to determine drug concentrations in rat urine and chicken plasma samples from rat and chicken that had been orally administered with six flavonoids and troxerutin.

Keywords: Flavonoids; Troxerutin; Reversed-phase high-performance liquid chromatography; Rat urine; Chicken plasma

1. Introduction

Flavonoids are a large family of over 4000 ubiquitous secondary plant metabolites, which can be further divided into five subclasses including flavonols, flavones, anthocyanins, catechins and flavonones [1]. Flavonoids have anti-inflammatory, anti-carcinogenic and other beneficial properties and are widely distributed in medicinal plants, fruit juices, teas and health beverage resulting in high human consumption [2,3]. Now a large number of literatures have been devoted to studies describing the potential anticancer activities of flavonoids. In many instances, these effects can be attributed to plausible biochemical mechanisms including enhanced apoptosis, growth arrest at one or more points in the cell cycle, inhibition of DNA synthesis, and modulation of signal transduction pathways by altered expression of key enzymes such as cyclooxygenases and protein kinases. Many experimental approaches have been used in these investigations, including use of cell lines, whole animals and, in a few instances, human cancer patients [4]. For example, in Turkey, the resin which was collected from branches of *P. terebinthus* ssp. *terebinthus* was used as an antiseptic for bronchitis and other respiratory and urinary system diseases because of a lot of flavonoids in resin [5]. Troxerutin shows a marked affinity for the venous wall, it may act to improve capillary function, reduce capillary fragility and reduce abnormal leakage. Applications also exist for reducing the occurrence of night cramps and other circulatory problems. Its common usage is mainly in the treatment of varicose veins and haemorrhoids [6].

Up to now, analysis of flavonoids has been accomplished by thin-layer chromatography [7–9], gas chromatography [10,11], capillary electrophoresis [12–17], electrochemical

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mensuration [18–20], high-performance liquid chromatography (HPLC) [1,2,5,6,21–30]. Especially, HPLC was widely used to separate and analyse flavonoids. For example, Wang et al. [26] reported the determination of flavonols (such as: myricetin, quercetin, kaempferol) in green and black tea leaves and green tea infusions by following HPLC method with diode array detection, and the mobile phase consisted of 30% acetonitrile in 0.025 M KH_2PO_4 buffer solution (v/v), which was adjusted to pH 2.5 by 6.0 M HCl. Fang et al. [27] adopted the elution program to determine quercetin, kaempferol, myricetin, rhamnetin, isorhamnetin, quercetrin, rutin, morin, galangin, fisetin, apigenin and luteolin in red wine by reversed-phase high-performance liquid chromatography (RP-HPLC) with UV–visible detector. However, to the best of our knowledge, there were few literatures to report the separation of troxerutin and puerarin or quercetin by RP-HPLC in previous studies. Especially, there was no way to simultaneously separate six flavonoids and troxerutin in rat urine and chicken plasma.

In this paper, the main objective of this study was to develop a reversed-phase high-performance liquid chromatographic method for the simultaneous separation and determination of six flavonoids and troxerutin in rat urine and chicken plasma after the oral administration. The proposed HPLC method was accurate, simple and efficient for simultaneous separation and determination of six flavonoids and troxerutin, and it can be applied to determine their content in rat urine and chicken plasma samples with satisfactory results.

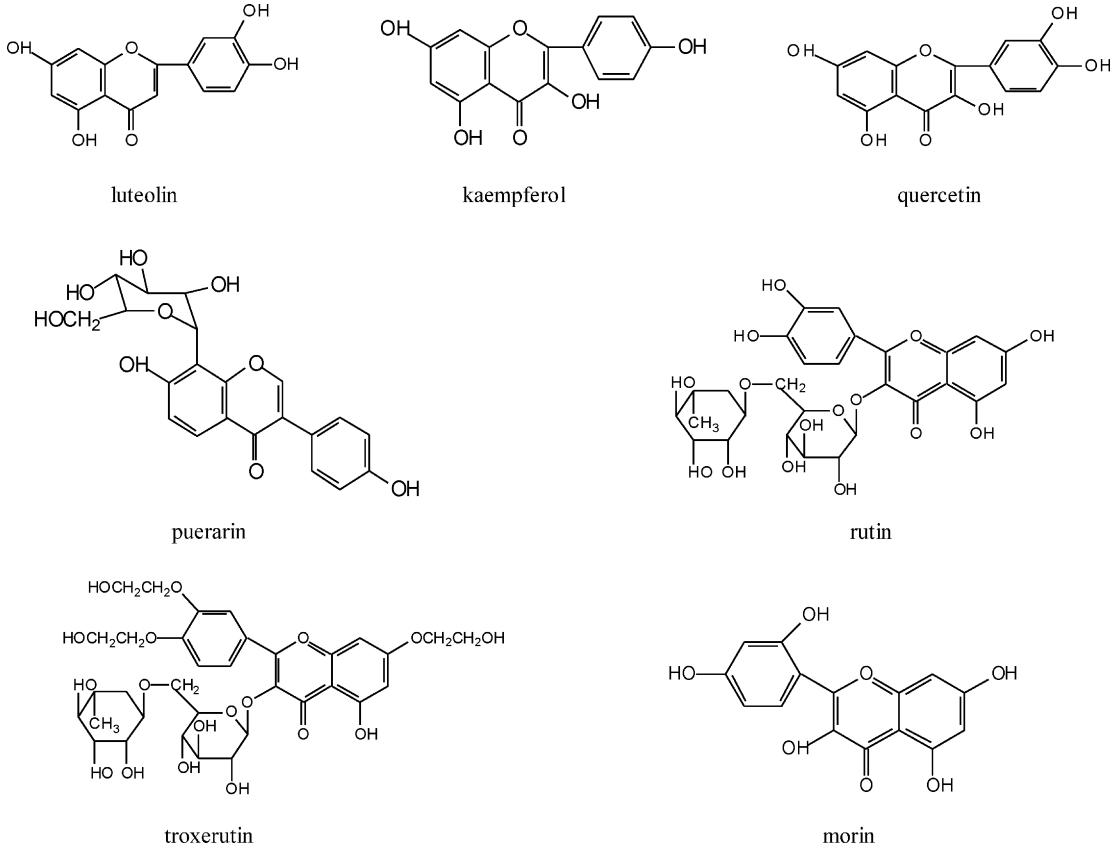
2. Experimental

2.1. Chemical and reagents

Flavonoid standards (see Scheme 1), including rutin, morin, luteolin, quercetin and kaempferol, which were purchased from Sigma–Aldrich. Puerarin and troxerutin (see Scheme 1) was kindly gifted by Yangzhou Institute of Drug Control. Ultrapure water at 18.3 MΩ resistance, used for HPLC mobile phase, was prepared using a Nanopure (New Haven, CT, USA) filtration system. HPLC-grade methanol was purchased from Fisher (Nepean, ON, Canada). All other chemicals were of analytical grade.

2.2. Chromatography

The HPLC (Shimadzu, Kyoto, Japan) instrument was equipped with a model series LC-10 ADVP pump, DGU-12 A degasser, SCL-10 AVP system controller, Rheodyne 7725 injector with a 20 μL loop and a SPD-10AVP UV–Vis detector. Separation and determination have been done on a VP-ODS column (5 μm particle size, 150 mm × 4.6 mm i.d. Shimadzu, Kyoto, Japan). Data acquisition was performed on class-VP software. The column was thermostatically controlled at 35 °C. The mobile phase was prepared by mixing water, methanol and acetic acid in a ratio of 57:43:1 (v/v/v, pH 3.0) at a flow rate of 0.8 mL/min. The mobile phase was filtered using 0.22 μm



Scheme 1. Molecular structures of the analytes.

membrane filter (Millipore, Milford, MA) and degassed by vacuum prior to use. The detection was set at a wavelength of 254 nm. An aliquot of 10 μL injections were made for each concentration under the specified chromatographic conditions.

2.3. Preparation of reference standard solutions

Individual standard stock solution of six flaovonids and troxerutin was prepared at a concentration of 1.0 mg/mL in methanol. Standard solutions were diluted to obtain three different concentrations of six flavonoids (1.0, 5.0, and 20.0 μg/mL) and troxerutin (5.0, 25.0, and 100.0 μg/mL), and were stored at −20 °C. And then the concentrations were determined for 7, 18 and 30 days. Data were compared with results obtained from freshly prepared standard solution. The change of troxerutin concentration was less than 2.3% after 7 days, 4.0% after 18 days, and the change of six flavonoids concentration was less than 5% for 30 days.

2.4. Sample preparation

2.4.1. Sample preparation – rat urine

Six female Wistar rats (weighing 350 ± 10 g, Experimental Animal Research Center of Medicine College, Yangzhou University, China) were housed in metabolic cages for collection of urine. The rats were provided standard laboratory food and water. Before being administered 200 mg/kg of six flaovonids and troxerutin, the rats were fasted for 24 h but with access to water. Urine samples were collected for a period of 8 h. All samples were stored at −20 °C prior to the analysis.

The blank rat urine, calibrator stock solutions, quality control stock solutions, and unknown samples were thawed and vortexed for 30 s. Calibrator and quality control solutions were freshly prepared by adding 50 μL of each stock solution to 450 μL of blank mice urine, then 1.0 mL of methanol was added. The mixture was vortexed for 1.0 min, and centrifugated at 12000 rpm for 10.0 min. Five-hundred microliters of each ultrafiltrate was transferred and 10 μL of them was injected for analysis.

2.4.2. Sample preparation – chicken plasma assay

Healthy female chickens with an average weight of 1.5 kg were provided by College of animal science and technology, Yangzhou University (Yangzhou, China). Animals were housed in a room with controlled temperature and humidity, and allowed free access to food and water. They were raised in the cage overnight before the experiments. The chickens were administered 30 capsules which contained 20 mg of each of the six flavonoids and troxerutin per capsule (made in our laboratory). Approximately 1.5 mL blood samples were collected from each chicken using a cannula inserted into the median cubital vein after drug administration of 90 min. Blood samples were centrifuged immediately, and plasma samples were frozen at −20 °C until required for LC analysis.

Venous blood samples from chickens were collected into heparinized plastic tubes. After immediate chilling, the plasma was separated by centrifugation for 15 min. The samples were stored at −20 °C until the assay. The blank chicken plasma, calibrator stock solutions, quality control stock solutions, and unknown samples were thawed and vortexed for 30 s. Calibrator and quality control solutions were freshly prepared by adding 50 μL of each stock solution to 450 μL of blank chicken plasma, then 1.0 mL of methanol was added. The mixture was vortexed for 1.0 min, and centrifugated at 12000 rpm for 10.0 min. Five-hundred microliters of each ultrafiltrate was transferred and 10 μL of them was injected for analysis.

2.5. Calibration

Calibration curves were produced by injecting prepared rat urine samples and prepared chicken plasma samples that were spiked with six flavonoids concentrations (0.10, 0.50, 1.00, 5.00, 10.00, 30.00, 50.00, 70.00 μg/mL) and troxerutin concentrations (0.50, 2.50, 5.00, 25.00, 50.00, 150.00, 250.00, 350.00 μg/mL). An aliquot of 10 μL of the resulting solution was injected into HPLC system, and each concentration was analyzed for five times. After determining the peak area, calibration lines of peak area versus analyte concentrations were plotted.

2.6. Recovery

The relative recovery in rat urine and chicken plasma samples were evaluated at three concentrations 1.00, 5.00 and 20.00 μg/mL for six flavonoids and 5.00, 25.00 and 100.00 μg/mL for troxerutin. The rat urine and chicken plasma were processed as described under preparation of sample solution before adding of the reference standards solution. An aliquot of 10 μL of the resulting solution was injected into the HPLC system. The peak area of six flavonoids and troxerutin were compared with those obtained by calibration curves. The variability of recovery results was determined.

2.7. Reproducibility

Spiked rat urine and chicken plasma samples with different concentrations of puerarin, rutin, morin, luteolin, quercetin, kaempferol (1.00, 5.00 and 20.00 μg/mL) and troxerutin (5.00, 25.00 and 100.00 μg/mL) were used for precision studies. The intra-day precision was calculated by analyzing five times on the same day. The inter-day variability was estimated by analyzing samples on 5 separate days. The relative standard deviation (RSD) was used to estimate the precision.

2.8. Detection limits

The spiked rat urine and chicken plasma solutions, which were diluted step by step, were injected into the HPLC equipment in quintuplicate. The limit of detection (LOD) was defined as the concentration for which a signal-to-noise ratio of 3.

2.9. Stability

Stability of six flavonoids and troxerutin in rat urine and chicken plasma was examined at room temperature and at −20.0 °C. Low, middle and high concentrations of six flavonoids

(1.00, 5.00 and 20.00 μg/mL) and troxerutin (5.00, 25.00 and 100.00 μg/mL) were spiked to matrix samples. And then they were left at room temperature for 7 days. The long-term storage stability of six flavonoids and troxerutin was also determined in rat urine and in chicken plasma for 7 and 18 days at −20.0 °C.

3. Results and discussion

3.1. High-performance liquid chromatography

3.1.1. Detection wavelength

The maximal absorbance wavelengths (λ_{max}) of six flaovonids and troxerutin were analyzed by scanning between 200 and 500 nm on the Shimadu UV-2501 in order to obtain the optimal detection wavelengths for the chromatography separation. The results of UV spectra showed that the absorbance peaks of six flavonoids and troxerutin were different, but they all had the best or better absorbance peaks at 254 nm. Therefore, the UV–vis detector was set at the wavelength of 254 nm in this method.

3.1.2. Optimization of mobile phase

Optimizing the HPLC separation procedure for quantitative analysis was a difficult process due to the highly complex chromatographic profile, which showed six flavonoids and troxerutin peaks. Different mobile phase conditions (organic solvent, acid percentage) were tested.

Methanol was the most widespread chromatographic mobile phase in the investigations of flavonoids at present. In this study, binary and ternary solvent (water; methanol; acetic acid) mixtures were tested as the mobile phase. When the proportion of acetic acid was invariable, the different volume ratio of methanol to water was investigated (shown in Fig. 1). It can be seen that the standard peaks appeared ahead of time by increasing the proportion of methanol in the mobile phase. When the volume ratio of water to methanol was below 50/50, rutin and troxerutin could not be separated. At the 57/43 (v/v, water/methanol), the six flavonoids and troxerutin, especially

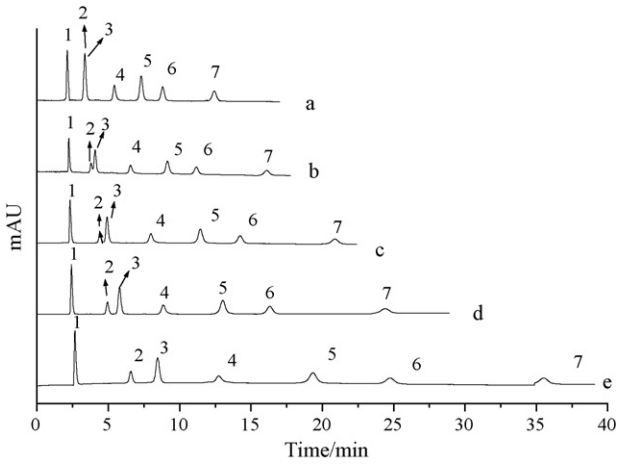


Fig. 1. Effect of water/methanol/acetic acid (v/v/v, pH 3.0) on chromatogram of six flavonoids and troxerutin Curve a: 50/50/1; curve b: 53/47/1; curve c: 55/45/1; curve d: 57/43/1; and curve e: 60/40/1. (1) Puerarin; (2) rutin; (3) troxerutin; (4) morin; (5) luteolin; (6) quercetin; and (7) kaempferol.

rutin and troxerutin, can be completely separated. And when volume ratio of water to methanol was beyond 60/40, the retention time delayed. So the volume ratio of water to methanol of 57/43 was selected. Due to easily ionizing ability of the phenolic hydroxyl group making the tailing phenomenon, the different pH of mobile phase was tested in the experiments by adding acetic acid. And the experimental results that the aqueous at pH of 3.0 was controlled (volume ratio of water, methanol to acetic acid for 57/43/1), which helped in separating all the standard peaks successfully. In addition, the other mobile phases, such as water/methanol (57:43, v/v), water/methanol/phosphoric acid (57:43:1, v/v/v), were also tested, and the better results were obtained by mobile phase of water/methanol/acetic acid (57:43:1, pH 3.0). So it was selected as mobile phase in this work.

3.1.3. Establishment of the calibration curves

The calibration curve of each compound was established by injecting eight different concentrations consisting of six

Table 1
Validation data from calibration curves and retention times of six flavonoids and troxerutin by RP-HPLC ($n = 5$)

Samples	Compounds	Retention time (min)	LOD (μg/mL)	Linear range (μg/mL)	Regression equation	Correlation coefficient
Spiked rat urine	Puerarin	2.42	0.02	0.10–70.00	$y = 18375.28 + 3676.01x$	0.9988
	Rutin	4.82	0.03	0.10–70.00	$y = 17632.27 + 937.06x$	0.9982
	Troxeyutin	5.69	0.05	0.50–350.00	$y = 5328.31 + 2603.21x$	0.9990
	Morin	8.92	0.04	0.10–70.00	$y = 27124.78 + 7038.54x$	0.9989
	Quercetin	13.06	0.02	0.10–70.00	$y = 8935.72 + 5042.61x$	0.9996
	Luteolin	16.34	0.02	0.10–70.00	$y = -15684.38 + 6021.36x$	0.9992
	Kaempoferol	24.43	0.03	0.10–70.00	$y = 10121.87 + 5615.35x$	0.9989
Spiked chicken plasma	Puerarin	2.42	0.020	0.10–70.00	$y = 13857.28 + 4666.01x$	0.9991
	Rutin	4.82	0.030	0.10–70.00	$y = 1698.27 + 621.60x$	0.9890
	Troxeyutin	5.69	0.050	0.50–350.00	$y = 5832.31 + 2702.12x$	0.9989
	Morin	8.92	0.040	0.10–70.00	$y = 21824.90 + 8028.33x$	0.9991
	Quercetin	13.06	0.020	0.10–70.00	$y = 8023.72 + 6062.61x$	0.9995
	Luteolin	16.34	0.020	0.10–70.00	$y = -14567.83 + 6027.59x$	0.9987
	Kaempoferol	24.43	0.030	0.10–70.00	$y = 9174.78 + 5767.72x$	0.9993

Table 2
Reproducibility of the studied flavonoids and troxerutin in *rat urine* and chicken plasma by RP-HPLC ($n = 5$)*

Samples	Compounds	RSD (%)*											
		Retention time						Integrated area					
		Run-to-run			Day-to-day			Run-to-run			Day-to-day		
		A	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*
Spiked rat urine	Puerarin	0.11	0.09	0.11	0.10	0.15	0.18	2.52	2.78	3.42	3.23	3.45	3.69
	Rutin	0.10	0.09	0.13	0.09	0.13	0.15	4.46	4.65	3.97	4.05	3.75	3.98
	Troxerutin	0.13	0.11	0.10	0.09	0.21	0.15	3.42	2.72	2.91	3.98	3.24	3.95
	Morin	0.15	0.13	0.14	0.12	0.13	0.17	2.87	3.04	2.87	3.71	3.61	4.29
	Quercetin	0.12	0.12	0.14	0.11	0.12	0.11	3.12	2.48	2.65	2.64	4.48	4.18
	Luteolin	0.13	0.11	0.13	0.17	0.13	0.14	2.17	2.19	3.75	2.83	3.59	3.64
	Kaempoferol	0.12	0.13	0.12	0.25	0.19	0.15	4.67	3.33	4.12	3.79	4.65	2.98
Spiked chicken plasma	Puerarin	0.19	0.15	0.13	0.25	0.21	0.15	3.24	3.01	2.89	3.36	3.13	2.92
	Rutin	0.13	0.10	0.10	0.21	0.20	0.15	4.85	4.36	4.20	4.97	4.77	3.89
	Troxerutin	0.15	0.12	0.10	0.16	0.14	0.13	3.68	3.51	3.39	4.78	4.32	3.78
	Morin	0.16	0.15	0.12	0.18	0.15	0.12	3.98	3.72	2.85	4.51	4.12	3.89
	Quercetin	0.14	0.13	0.11	0.19	0.16	0.14	5.36	4.62	3.92	5.82	5.45	5.13
	Luteolin	0.18	0.16	0.14	0.23	0.21	0.18	3.81	3.14	2.87	4.88	4.51	4.16
	Kaempoferol	0.21	0.15	0.11	0.24	0.21	0.17	3.15	3.01	2.75	4.73	4.15	3.91

* The result was obtained from 1.0 μg/mL (A), 5.0 μg/mL (B) and 20.0 μg/mL (C) of six flavonoids and 5.0 μg/mL (A), 25.0 μg/mL (B) and 100.0 μg/mL (C) troxerutin.

flavonoids and troxerutin, a good correlation was found between the peak area (y) and the concentrations (x). The results of each compound, such as linearity, linear range, limit of detection (LOD), retention time and correlation coefficient (R), were summarized in Table 1. The experimental results showed that the LODs of each flavonoid and troxerutin were very low (0.010–0.050 μg/mL), which indicated that this method had a high degree of sensitivity.

3.1.4. Reproducibility of HPLC analysis

The reproducibility of the HPLC analysis was carried out in two ways, i.e., retention times and peak areas. In this method, the relative standard deviation (RSD) for the reproducibility of the HPLC analysis was obtained through five injections of the spiked matrix standard solution with different concentrations, and the results were listed in Table 2. It showed that intra-day and inter-day relative standard deviations for retention time and for peak area were quite low, which indicated that this method possess good reproducibility.

3.1.5. Storage stability

The stability experiments were aimed at testing all possible conditions that the samples might experience after collection and prior the analysis. So the stability of six flavonoids and troxerutin in rat urine and chicken plasma samples were investigated. The results of storage stability were summarized as follows. The concentration of six flavonoids and troxerutin in rat urine and chicken plasma matrixes were determined for about 92% and 93% of initial amount remaining in rat urine and chicken plasma for 7 days at room temperature (stability defined as ≥90% of initial amount remaining [25]), respectively. And when they were stored at −20.0 °C, six flavonoids and troxerutin in sample matrixes were found to be stable for 18 days (about for 92% for rat urine and about 91% for chicken plasma) and 7 days (about 92% for rat urine and about 93% for chicken plasma), respectively.

3.2. Analysis of biological samples

3.2.1. Quantitative of flavonoids in rat urine and chicken plasma

In order to test the applicability of the present method for the analysis of six flavonoids and troxerutin, the rat urine and chicken plasma samples obtained upon oral administration of six flavonoids and troxerutin to rat and chicken were analyzed. The samples were treated according to the procedure described

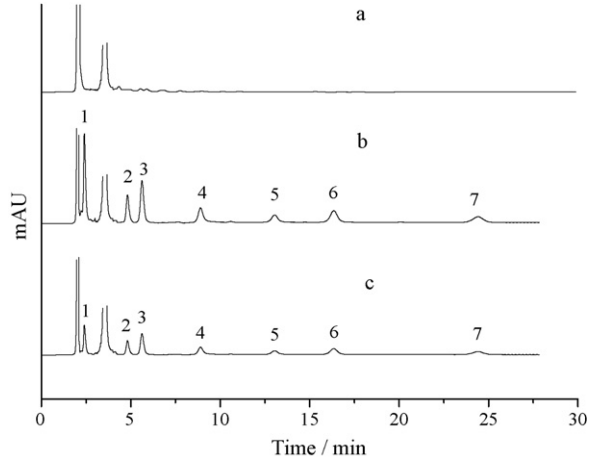


Fig. 2. Chromatograms of (a) blank rat urine; (b) six flavonoids and troxerutin in spiked rat urine: 5.0 μg/mL of six flavonoids and 25.0 μg/mL of troxerutin; (c) six flavonoids and troxerutin in urine obtained from one rat after the oral administration of six flavonoids and troxerutin in 8 h. (1) Puerarin; (2) rutin; (3) troxerutin; (4) morin; (5) quercetin; (6) luteolin; and (7) kaempoferol.

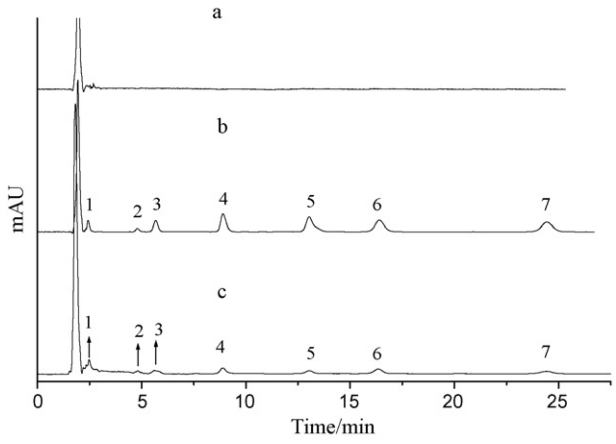


Fig. 3. Chromatograms of (a) blank chicken plasma; (b) six flavonoids and troxerutin in spiked chicken plasma: 5.0 μg/mL of six flavonoids and 25.0 μg/mL of troxerutin; (c) six flavonoids and troxerutin in chicken plasma obtained from one chicken after the oral administration of six flavonoids and troxerutin in 90 min. (1) Puerarin; (2) rutin; (3) troxerutin; (4) morin; (5) quercetin; (6) luteolin; and (7) kaempoferol.

in Section 2.4. And then the volume of 10 μL of the above solution was injected under the optimum conditions mentioned earlier. The chromatograms generated from the above rat urine and chicken plasma were shown in Figs. 2 and 3, respectively. It showed that there was no obvious interference on the analysis of six flavonoids and troxerutin in rat urine and chicken plasma matrixes. Based on the curve c of Figs. 2 and 3, the concentration of six flavonoids and troxerutin were calculated according to the previously established linear regression equations in Table 1,

Table 3
The concentration of six flavonoids and troxerutin in rat ruine and chicken plasma samples ($n = 5$)

Compounds	Rat urine		Chicken plasma	
	Concentration (μg/mL)	RSD (%)	Concentration (μg/mL)	RSD (%)
Puerarin	2.13	3.43	1.06	2.89
Rutin	2.54	2.98	1.04	3.21
Troxerutin	3.65	3.11	1.39	4.01
Morin	2.34	2.87	1.03	3.08
Quercetin	2.15	3.05	1.20	3.67
Luteolin	2.43	3.43	1.14	3.72
Kaempoferol	2.04	3.78	1.39	4.21

and the results were obtained (shown in Table 3). The experimental results showed that it would provide the possibility for the assessment of flavonoids and troxerutin concentrations in patients treated with these drugs.

3.2.2. Accuracy

The accuracy of the method, in terms of recovery efficiency, is a measure of the response of the analytical method to the entire quantity of the analyte contained in a sample. The recovery of the six flavonoids and troxerutin in rat urine and chicken plasma samples were calculated and the results were shown in Table 4 with the mean values of five replicate injections. The recoveries for the six flavonoids and troxerutin were between 97.32% and 103.15% in rat urine sample and between 97.14% and 103.61% in chicken plasma (see Table 4). From the results of recoveries, it can be seen that the present method for the analysis

Table 4
Recoveries of six flavonoids and troxerutin in *rat urine* and chicken plasma ($n = 5$)

Compounds	Amount added (μg/mL)	Recovery (%)		RSD (%)	
		Rat urine	Chicken plasma	*Rat urine*	Chicken plasma
Puerarin	1.0	101.43	101.59	3.12	3.14
	5.0	98.86	97.45	2.78	2.89
	20.0	101.63	103.61	2.85	2.71
Rutin	1.0	100.87	99.04	4.41	4.56
	5.0	98.68	101.68	3.85	4.15
	20.0	99.41	99.87	4.16	4.01
Troxerutin	5.0	97.32	98.52	3.43	3.34
	25.0	98.35	97.14	2.45	3.24
	100.0	100.21	101.59	2.39	2.92
Morin	1.0	103.15	98.32	4.95	3.14
	5.0	101.12	101.44	3.77	2.97
	20.0	99.61	99.11	2.86	2.48
Quercetin	1.0	97.71	98.78	2.97	3.49
	5.0	100.98	101.10	2.42	3.16
	20.0	99.08	101.08	2.31	2.45
Luteolin	1.0	98.47	98.72	5.12	3.38
	5.0	100.22	99.15	4.81	2.91
	20.0	99.58	101.58	3.71	2.67
Kaempoferol	1.0	102.08	101.13	6.65	4.61
	5.0	98.62	97.54	6.03	4.53
	20.0	99.12	98.23	4.99	3.98

Table 5
Comparison of detection limit for flavonoids between the present method and the portions of already available methods

Methods	Linear ranges	Detection limits
Determination of flavonoids by HPLC and capillary electrophoresis [1]	0.10–0.80 μg/mL	0.08–0.21 μg/mL
Determination of flavonoids in cultivated sugarcane leaves, bagasse, juice and in transgenic sugarcane by LC-UV detection [17]	5–350 μg/mL	0.40 mg/L
Determination of flavonoids in Semen Cuscutae by RP-HPLC [29]	0.64–96.00 μg/mL	64–103 ng/mL
Simultaneous determination of eight active components in Chinese medicine 'YIQING' capsule using HPLC [30]	0.50–576.00 mg/L	0.09–0.72 mg/L
The simultaneous separation and determination of six flavonoids and troxerutin in human urine by RP-HPLC with ultraviolet–visible detection [This work]	0.10–70.00 μg/mL for six flavonoids and 0.50–350.0 μg/mL for troxertuin	0.010–0.050 μg/mL

of six flavonoids and troxerutin in urine and plasma has good accuracy.

4. Conclusion

In summary, the RP-HPLC method mentioned here represented an excellent technique for firstly simultaneous separation and determination six flavonoids and troxerutin in rat urine and chicken plasma after the oral administration with good sensitivity, precision and reproducibility. The results of detection limit between present analytical method and the portions of already available methods can be summarized as Table 5. It showed the present method has lower detection limit. And the method employed simple sample preparation for rat urine and chicken plasma samples, and it is easily adaptable in many laboratories using commonly available HPLC equipment. Especially, it was successfully applied to determine drug concentrations in rat urine and chicken plasma samples that had been orally administered with six flavonoids and troxerutin, and it will play a reference role on the determination of flavonoids in other medicinal plants, pharmaceutical preparations or clinic analysis.

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