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SPE-HPLC method for the determination of four flavonols in rat plasma and urine after oral administration of *Abelmoschus manihot* extract

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Abstract

A SPE-HPLC method was developed and validated for the simultaneous determination of flavonols, isoquercitrin (**1**), hibifolin (**2**), myricetin (**3**), quercetin-3′-*O*-D-glucoside (**4**) and quercetin (**5**) in rat plasma and urine after oral administration of the total flavonoids from *Abelmoschus manihot* (TFA). The astragalin (**6**) and kaempferol (**7**) were used as internal standards (IS). Plasma and urine samples were pretreated by solid-phase extraction using Winchem^{TM} C_{18} reversed-phase cartridges. Analysis of the plasma and urinary extract was performed on YMC-Pack ODS-A C_{18} and Thermo ODS-2HYEPRSIL C_{18} reversed-phase column, respectively and a mobile phase of acetonitrile–0.1% phosphoric acid was employed. HPLC analysis was conducted with different elution gradients. The flow rate was 1.0 mL/min and the detection wavelength was set at 370 nm. Calibration ranges in plasma for flavonols **2**–**5** were at 0.011–2.220, 0.014–2.856, 0.022–4.320, and 0.028–5.600 μg/mL, respectively. In urine calibration ranges for flavonols **1**, **2**, **4** and **5** were at 2.00–16.00, 8.56–102.72, 2.70–21.60, and 3.00–24.00 μg/mL, respectively. The RSD of intra- and inter-day was less than 5.40% and 4.89% in plasma, and less than 3.96% and 6.85% in urine for all the analyses. A preliminary experiment to investigate the plasma concentration and urinary excretion of the flavonols after oral administration of TFA to rats demonstrated that the present method was suitable for determining the flavonols in rat plasma and urine.

Keywords: *Abelmoschus manihot*; Flavonols; Plasma; Urine; SPE-HPLC

1. Introduction

The flower of *Abelmoschus manihot* (Linn.) Medicus, a traditional Chinese medicine, was found to have anti-inflammatory, antibacterial and anticoagulant effects [1,2] and used for the treatment of chronic renal disease, mouth ulcers and burn [1,3]. Because the decoction of the entire flower extract was taken orally, the pharmacological activity observed in human is attributed to the entire flower extract, which included flavonoid, pigment, resin, polysaccharide, etc. But the main biological active constituents of the flower are the flavonoids [4,5]. The total flavonoids from the flowers of *A. manihot* (TFA), which are the purified flowers extract, have been shown to possess protective effects against myocardial and cerebral anoxia and ischemia [6–8]. The phytochemical analysis on the flavonoids of *A. manibot* in our lab indicated that isoquercitrin (**1**), hibifolin (**2**), myricetin (**3**), quercetin-3′-*O*-glucoside (**4**) and quercetin (**5**) are the principal five flavonols in the flowers of the title plant (Fig. 1). Although some determination methods were developed for the analysis of isoquercitrin (**1**), myricetin (**3**) and quercetin (**5**) in plasma and urine [9–14], no methods have been reported for analysis of hibifolin (**2**) and quercetin-3′-*O*-glucoside (**4**) in plasma or urine. Also no report was found on the simultaneous determination of TFA's main flavonols in biological fluids after oral administration of TFA.

In the present study, we developed and validated a simple and reliable method for the simultaneous determination of flavonols **1**–**5** in rat plasma and urine using a solid-phase extraction (SPE) technique and HPLC with photodiode-array (PDA) ultraviolet detection. And the method was applied for the pharmacokinetic

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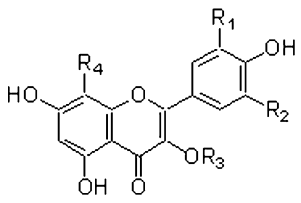

	R_1	R_2	R_3	R_4
1	OH	H	glc	H
2	OH	H	H	O-glu A
3	OH	OH	H	H
4	O-glc	H	H	H
5	OH	H	H	H
6	H	H	glc	H
7	H	H	H	H

Fig. 1. The structures of **1**–**5** from *A. manihot* and **6** and **7** (internal standard).

studies of compounds **1**–**5** in rat plasma and urine after oral administration of TFA.

2. Experimental

2.1. Chemicals and reagents

The reference standards of flavonols **1**–**5** were isolated from the flowers of *A. manihot* by the author. The structures of **1**–**5** were identified on the basis of their spectral data (^{1}H NMR, ^{13}C NMR and UV) and by comparing with literature values. Astragalin (**6**) and kaempferol (**7**) (provided by Dr. Jun Cheng, School of Pharmaceutical Sciences, Peking University) were selected as an internal standard (IS) in plasma and urine, respectively (Fig. 1). Purity analysis suggested that their purities were all above 99% by HPLC method. The total flavonoids (TFA) from the ethanol extraction of the flowers of *A. manihot* were obtained by macroporous resin and polyamide chromatography.

HPLC grade acetonitrile was purchased from Honeywell International Inc. (Burdick & Jackson, Muskegon, MI, USA); analytical grade methanol was purchased from Beijing Reagent Co. Ltd. (Beijing, PR China) and Winchem^{TM} C_{18} (SPE) cartridge column was purchased from Winchem Technology Co. Ltd. (Beijing, PR China). Distilled and deionised water was used for the preparation of all solutions.

2.2. Animals

Male Sprague-Dawley rats (190–230 g) were obtained from the Laboratory Animal Centre of Peking University Health Science Centre (Beijing, PR China). Rats used in pharmacokinetic studies were housed in an animal room for three days before the test and fed with food and water *ad arbitrium*. In urine pharmacokinetic experiment, each rat was held in stainless steel metabolism cage. The animals were fasted 24 h before the experiment. A 35 mg/mL TFA (the total flavonoids extract from the flowers of *A. manihot*) aqueous solution was administered orally at a dose of 350 mg/kg, which contained 20.74, 72.17, 7.89, 11.90 and 1.20 mg/kg of compounds **1**–**5**, respectively.

2.3. Instrumentation and conditions

A JASCO HPLC system (JASCO, Tokyo, Japan) consisting of a solvent gradient delivery pump, and a photodiode-array detector was used. An YMC-Pack ODS-A C_{18} reversed-phase column (5 μm, 250 × 4.6 mm) and a Thermo ODS-2HYEPRSIL C_{18} reversed-phase column (5 μm, 250 × 4.6 mm) with a Phenomenex® C_{18} (ODS, Octadecyl) guard column (5 μm, 4.0 × 3.0 mm) were used in separation and quantitation of the flavonols in plasma and urine, respectively.

The elution gradient for HPLC analysis was conducted using acetonitrile (A) and 0.1% phosphoric acid solution (B). The UV detection wavelength was set at 370 nm.

For plasma sample, the initial elution condition was A–B (16:84, v/v), linearly changed to A–B (20:80, v/v) at 30 min, and then linearly changed to A–B (24:76, v/v) at 45 min. The percentage of mobile phase A increased linearly to 36% at 55 min and held for 5 min. The analysis was performed at room temperature and took 60 min. The flow rate was 1.0 mL/min and injection volume was 20 μL.

For urine sample, initial condition was A–B (15:85, v/v), linearly changed to A–B (16:84, v/v) at 18 min, linearly changed to A–B (20:80, v/v) at 22 min, linearly changed to A–B (22:78, v/v) at 28 min, and then held A–B (22:78, v/v) until 36 min. The percentage of mobile phase A increased linearly to 40% at 46 min and held for 4 min. The analysis took 50 min and the column temperature was maintained at 25 °C. The flow rate was 1.0 mL/min and injection volume was 40 μL.

2.4. Preparation of standard solutions

For plasma sample, pure solutions of compounds **2**–**5** were prepared separately with methanol at concentrations of 0.444, 0.408, 0.432 and 0.400 mg/mL, respectively. A stock solution containing compounds **2**–**5** was prepared by mixing and diluting the pure solutions with methanol to yield concentrations of 44.4, 57.1, 86.4 and 112.0 μg/mL, respectively. The astragalin (**6**, **IS**) solution at a final concentration of 41.6 μg/mL was prepared with the same solvent. All solutions were stored at 4 °C and were determined to be stable for at least 1 month. Working solutions (for preparing plasma standards at known concentrations) were produced by diluting the stock solution in methanol at ratios of 1:0, 1:2, 1:10, 1:20, 1:40, 1:100 and 1:200, respectively.

For urine sample, pure solutions of compounds **1**, **2**, **4** and **5** were prepared separately with methanol at concentrations of 0.400, 0.856, 0.360 and 0.200 mg/mL, respectively. A stock solution containing compounds **1**, **2**, **4** and **5** was prepared by mixing and diluting the pure solutions with methanol to yield concentrations of 40.0, 171.2, 54.0 and 60.0 μg/mL, respectively. The kaempferol (**7**, **IS**) solution at a final concentration of 208.0 μg/mL was prepared with the same solvent. Working solutions were just processed by adding the stock solution 300, 200, 100, 75, 50, 25 μL to 500 μL urine, respectively.

2.5. Calibration

Standard samples were prepared by adding 20 μL of working solutions for plasma sample to 400 μL blank plasma, and different amount stock solution for urine sample to 500 μL blank urine. Then plasma samples were added 10 μL of **6** (41.6 μg/mL) and 40 μL 20% phosphoric acid solution, and urine samples were spiked with 15 μL of **7** (208.0 μg/mL). Extraction proceeded as described in sample preparation section.

2.6. Sample preparation

A 400 μL plasma sample was added to a 5 mL tube and spiked with 10 μL of astragalin as IS (41.6 μg/mL) and 40 μL 20% phosphoric acid solution to adjust the plasma for pH 2.5. The treated plasma sample was added and flowed through the SPE cartridge (3 mL, packed with 200 mg of 40 μm octadecyl silica) with gravity. The solid-phase cartridge was washed with 5 mL deionised water, then the compounds were eluted with 3 mL 60% methanol. The methanolic eluate was evaporated to dryness under a stream of nitrogen at 35 °C. The residue was reconstituted in 100 μL of 50% acetonitrile in 1% phosphoric acid solution (pH 3.0). A 20 μL of the sample was injected into the HPLC system for analysis.

A 500 μL urine sample was added to a 5 mL tube and spiked with 15 μL of kaempferol as IS (208.0 μg/mL). The mixture was vortex-mixed for 1 min and applied to Winchem^{TM} C_{18} (SPE) cartridge column. The treated urine sample was also prepared by SPE as above, and the compounds were eluted with 2 mL 60% methanol. The eluate was collected and vortex-mixed for 1 min. A 40 μL of the sample was injected into the HPLC system for analysis.

2.7. Precision, accuracy and recovery

For plasma sample, precision, accuracy and recovery experiments for the flavonols were performed by using 20 μL of high, middle and low concentrations of working standard solutions. The solutions were added to 400 μL blank plasma, respectively. Then 10 μL internal standard (41.60 μg/mL) and 40 μL 20% phosphoric acid solution were added to the plasma successively.

For urine sample, high, middle and low amounts of working standard solutions were spiked to 500 μL blank urine, respectively. Then 15 μL of internal standard (208.0 μg/mL) were added to the urine.

The plasma or urine sample was processed according to the standard SPE method.

The intra-day precision and accuracy were calculated by determining six and five replicates for plasma and urine sample, respectively. The inter-day precision was assessed by replicate analysis of the same samples on five and three continual days for plasma and urine sample, respectively.

Percent recovery was determined by comparison of the measured concentration to the theoretical concentration. A mean of six and four replicates was used for the determination of plasma and urine recovery, respectively.

2.8. Pharmacokinetic studies

Blood samples were collected from the retrobulbar capillary plexus at 5, 10, 15, 20, 30, 40, 60 and 120 min after administration. Because of limited volume, plasma specimens were collected from the first rat for the first 20 min following TFA administration. A second rat was utilized for collection of specimens from 30 min to 120 min. Five rats were utilized for each of the time period. Plasma was separated by centrifugation at 8000 rpm for 10 min, and then stored at −20 °C until analyses.

Urine samples were collected at 2, 5, 8, 12, 24, 36, 48 and 60 h after administration. The urine were centrifuged at 3000 rpm for

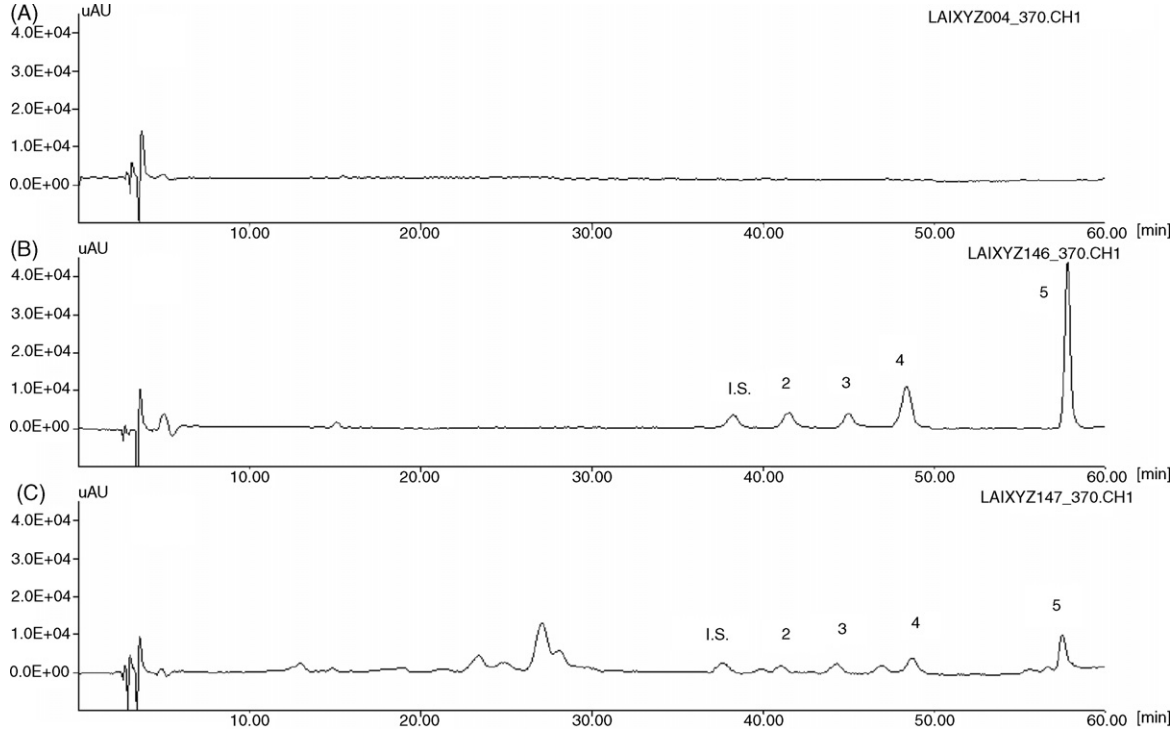

Fig. 2. Typical chromatograms for the determination of flavonols **2**–**5** in plasma samples: (A) a blank plasma sample; (B) the plasma sample spiked with flavonols **2**–**5** and astragalin (**IS**); (C) the extract of plasma sample from a rat after 20 min of oral administration of TFA.

15 min. Then the urine samples were stored at −20 °C until analyses.

3. Results and discussion

Optimization of extraction methods for the flavonol compounds in plasma was conducted by comparison of liquid–liquid extraction (LLE) to solid-phase extraction (SPE). SPE was found to produce cleaner extract and eliminate emulsification problems encountered with LLE. SPE conditions were further optimized by evaluation of washing and eluting solvents. It was determined that washing SPE column with deionised water following addition of the biological specimens could remove proteins and other interfering substances. Following the water washing, flavonols could be eluted quantitatively with 3 mL of 60% methanol.

For urine sample, the use of SPE column could effectively eliminate the interfering material and was found to be satisfactory.

Fig. 2 showed the typical HPLC chromatograms of extracts derived a blank plasma sample, a plasma sample spiked with standard solution and a rat plasma sample collected at 20 min after oral administration TFA. Fig. 3 showed the typical HPLC chromatograms of extracts derived a blank urine sample, a urine sample spiked with standard solution, and a rat urine sample collected at 5 h after oral administration TFA.

The flavonols and IS were identified by comparison of their retention times with those of the reference standards. Peak identification was further verified by analysis of the UV spectrum.

Table 1
The t_R(min) of compound **1–7** in HPLC

Compounds	t_R (min)	
	In plasma	In urine
Isoquercitrin (**1**)		21.40
Hibifolin (**2**)	41.45	28.93
Myricetin (**3**)	45.00	
Quercetin-3′-*O*-D-glucoside (**4**)	48.39	33.12
Quercetin (**5**)	57.77	43.65
Astragalin (**6**) (**IS**)	38.24	
Kaempferol (**7**) (**IS**)		48.97

Their representative retention times in plasma and urine were listed in Table 1.

In this method, calibration curves of the test compounds in plasma and urine were linear, which are listed in Tables 2 and 3, respectively.

A series of different diluted plasma and urine standard samples was utilized to determine the limit of detection (LOD) with signal/noise (S/N) ≥ 3:1 (Tables 2 and 3).

To determine the limit of quantification (LOQ) with signal/noise (S/N) ratio of 10:1 (Table 2) for plasma sample, the lowest concentration of the linear range of calibration curve was tested with five replicates as the limit of quantification (LOQ) (Table 3) for urine sample, a series of different diluted urine standard samples were investigated.

Three concentrations of flavanols were used to analyze precision and accuracy in plasma and urine samples. Analytical precision and accuracy data are shown in Tables 4 and 5,

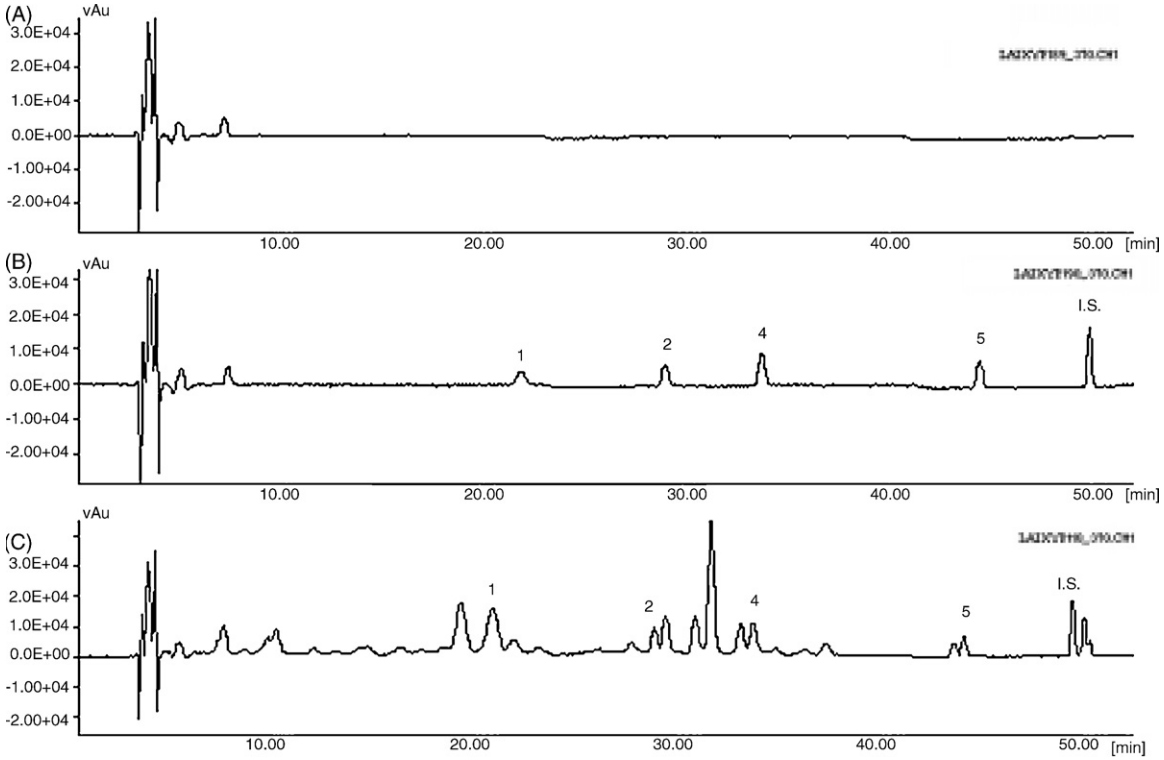

Fig. 3. Representative chromatograms of the four flavonols (**1**, **2**, **4** and **5**) in urine samples: (A) a blank urine sample; (B) a urine sample spiked with flavonols (**1**, **2**, **4** and **5**) and kaempferol (**IS**); (C) the extract of urine sample obtained after 5 h of oral administration of TFA.

Table 2
Calibration curves in plasma for flavonols (**2–5**) (μg/mL)

Compound	Standard curves	r^2	Test range	LOQ	LOD
Hibifolin (**2**)	$Y = 1.1213X - 0.0028$	0.9981	0.011–2.220	0.055	0.022
Myricetin (**3**)	$Y = 0.8320X - 0.0261$	0.9945	0.014–2.856	0.071	0.029
Quercetin-3′-*O*-glucoside (**4**)	$Y = 1.3949X + 0.0216$	0.9967	0.022–4.320	0.108	0.043
Quercetin (**5**)	$Y = 2.3334X + 0.0025$	0.9963	0.028–5.600	0.028	0.014

Y, peak area ratio (analyte/internal standard); *X*, concentration of compound in rat plasma (μg/mL); LOQ, the limit of quantification (signal/noise ratio of 10:1); LOD, the limit of detection (signal/noise ratio of 3:1).

Table 3
Calibration curves in urine for flavonols (**1**, **2**, **4**, **5**) (μg/mL)

Compound	Standard curves	r^2	Test range	LOQ	LOD
Isoquercitrin (**1**)	$Y = 0.0758X + 0.0300$	0.9974	2.00–16.00	2.00	1.00
Hibifolin (**2**)	$Y = 0.0144X + 0.0220$	0.9934	8.56–102.72	8.56	4.28
Quercetin-3′-*O*-glucoside (**4**)	$Y = 0.0868X + 0.0456$	0.9961	2.70–21.60	2.70	1.35
Quercetin (**5**)	$Y = 0.1338X - 0.3579$	0.9920	3.00–24.00	3.00	1.50

Y, peak area ratio (analyte/internal standard); *X*, concentration of compound in rat urine (μg/mL); LOQ, the limit of quantification (the lowest concentration of the linear range of calibration curve); LOD, the limit of detection (signal/noise ratio of 3:1).

respectively, which were expressed as the mean detected concentration and relative standard deviation (RSD). The RSD of intra- and inter-day was less than 7% for all the analytes, indicating that the precision was acceptable for all the compounds.

Plasma and urine samples containing three different concentrations of the analytes (low, medium and high working standard solutions) were used for the determination of percent recoveries of the flavonols. The flavonols in plasma sample had recoveries between 75.85% and 111.40% with the relative standard deviation (RSD) was no more than 8.97% and the recoveries in urine sample were between 92.07% and 103.66% with the relative standard deviation (RSD) was less than 11.00%. Results were shown in Tables 4 and 5, respectively.

The concentration–time profiles of the four flavonols (**2–5**) in rat plasma following oral administration of TFA were shown in Fig. 4. The data represented the mean from five rats. The time variations of urinary excretions of the four flavonols (**1**, **2**, **4** and **5**) following oral administration of TFA were shown in Fig. 5. Compound **1** could not be detected in plasma samples, while it could be found in urine. And in urine samples, compound **3** was absent, indicating that **3** might be metabolized into other compounds. The cumulative urinary excretions of the four flavonols (**1**, **2**, **4** and **5**) within 60 h were 0.115, 0.491, 0.630 and 4.663% of the dose, respectively. The urinary excretion–time profiles of the four flavonols (**1**, **2**, **4** and **5**) were shown in Fig. 6. The maximum excretions of isoquercitrin (**1**) and quercetin-3′-*O*-glucoside (**4**) were observed

Table 4
Intra- and inter-day accuracy and precision and recovery for the flavonols (**2–5**) in rat plasma

Spiked concentration (μg/mL)	Intra-day ($n = 6$)		Inter-day ($n = 3$)		Recovery (%) ($n = 6$)	RSD (%)
	Measured concentration (μg/mL)	RSD (%)	Measured concentration (μg/mL)	RSD (%)		
Hibifolin (**2**)						
1.110	1.040 ± 0.015	1.45	1.046 ± 0.010	1.01	99.17 ± 5.34	5.39
0.222	0.254 ± 0.008	3.04	0.248 ± 0.007	2.96	107.70 ± 3.39	3.15
0.111	0.124 ± 0.006	4.82	0.127 ± 0.003	2.42	103.07 ± 3.65	3.54
Myricetin (**3**)						
1.428	1.320 ± 0.040	3.03	1.315 ± 0.012	0.91	94.26 ± 8.46	8.97
0.285	0.321 ± 0.006	2.04	0.315 ± 0.010	3.24	104.51 ± 5.97	5.71
0.143	0.160 ± 0.005	3.04	0.160 ± 0.005	3.26	109.30 ± 2.96	2.71
Quercetin-3′-*O*-glucoside (**4**)						
2.160	2.361 ± 0.025	1.04	2.363 ± 0.028	1.19	108.34 ± 5.78	5.34
0.432	0.412 ± 0.014	3.41	0.393 ± 0.019	4.89	92.07 ± 4.03	4.38
0.216	0.212 ± 0.011	5.40	0.206 ± 0.007	3.21	95.97 ± 5.18	5.39
Quercetin (**5**)						
2.800	3.097 ± 0.065	2.09	3.049 ± 0.052	1.71	111.40 ± 7.57	6.80
0.560	0.451 ± 0.019	4.27	0.450 ± 0.014	3.17	75.85 ± 3.99	5.27
0.280	0.238 ± 0.005	1.92	0.241 ± 0.005	2.03	83.06 ± 3.10	3.74

Table 5
Intra- and inter-day accuracy and precision and recovery for the flavonols (**1**, **2**, **4**, **5**) in rat urine

Spiked concentration (μg/mL)	Intra-day ($n=5$)		Inter-day ($n=3$)		Recovery (%)	RSD (%)
	Measured concentration (μg/mL)	RSD (%)	Measured concentration (μg/mL)	RSD (%)		
Isoquercitrin (**1**)						
8.000	7.680 ± 0.096	1.25	7.633 ± 0.023	0.30	94.96 ± 6.12	6.45
6.000	6.167 ± 0.163	2.64	6.388 ± 0.310	4.86	103.15 ± 4.76	4.62
4.000	3.913 ± 0.139	3.56	4.176 ± 0.204	4.89	103.66 ± 4.27	4.12
Hibifolin (**2**)						
34.240	36.331 ± 1.110	3.06	37.325 ± 2.267	6.07	103.29 ± 7.94	7.69
25.680	25.786 ± 0.788	3.06	28.227 ± 1.933	6.85	101.47 ± 11.16	11.00
17.120	19.224 ± 0.417	2.17	19.427 ± 0.393	2.02	103.36 ± 10.05	9.72
Quercetin-3′-*O*-glucoside (**4**)						
10.800	8.725 ± 0.358	4.10	9.210 ± 0.623	6.77	97.57 ± 7.38	7.57
8.100	6.567 ± 0.254	3.87	7.117 ± 0.420	5.91	92.07 ± 7.29	7.92
5.400	4.326 ± 0.171	3.96	4.627 ± 0.051	1.10	92.11 ± 6.16	6.68
Quercetin (**5**)						
12.000	10.594 ± 0.289	2.72	11.023 ± 0.340	3.09	100.55 ± 10.95	10.89
9.000	7.822 ± 0.137	1.76	7.992 ± 0.326	4.08	97.58 ± 4.16	4.27
6.000	5.604 ± 0.084	1.50	5.737 ± 0.111	1.94	92.90 ± 7.36	7.92

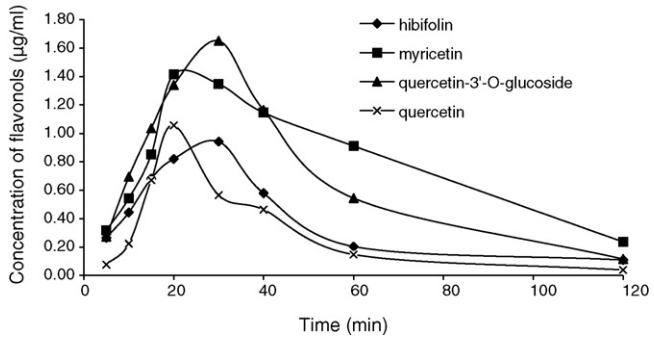

Fig. 4. Mean ($n=5$) concentration–time profile for hibifolin, myricetin, quercetin-3′-*O*-glucoside and quercetin in rat plasma after oral administration of TFA.

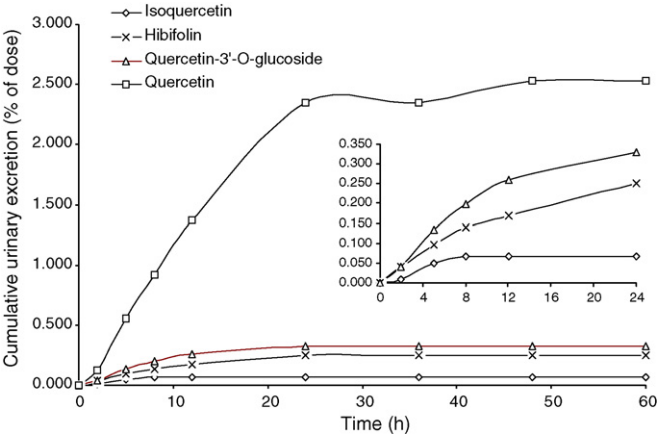

Fig. 5. Cumulative excretion of isoquercitrin, hibifolin, quercetin-3′-*O*-glucoside and quercetin into urine after oral administration of TFA to rats. Each point represents mean ($n=3$) as the percentage of the administered dose of TFA.

between 2 h and 5 h. The maximum excretions of hibifolin (**2**) and quercetin (**5**) were observed between 12 h and 24 h. Neither the four flavonols nor their metabolites could be observed after 24 h.

Hibifolin (**2**) was the most abundant constituent in TFA and was reported to possess anti-inflammatory activity through lipoxygenase inhibition [15]. In this paper, we established a method to determine hibifolin (**2**) in plasma and urine for the first time. The results showed that it was rapidly absorbed, reached the peak concentration at 30 min, and was quickly excreted into urine, then disappeared from the urine after 24 h. Quercetin-3′-*O*-glucoside (**4**) was another main constituent in TFA and its pharmacokinetics was never reported before. The results showed that it had similar absorption and excretion as hibifolin (**2**) in the plasma and urine. The pharmacokinetic study on the flavonols after oral administration of TFA indicated that the flavonols were rapidly absorbed, quickly excreted into urine and disappeared

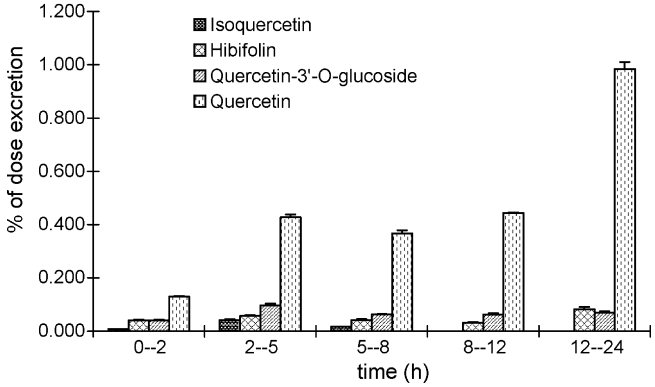

Fig. 6. Urinary excretion-time profile of isoquercitrin, hibifolin, quercetin-3′-*O*-glucoside and quercetin after oral administration of TFA to rats. Urinary excretion (%dose of recovered) = amount of the drug excreted to urine/amount of the drug administered to rats.

from the urine after 24 h. This result agreed with the acquirement that it should be taken commonly three times per day [16].

4. Conclusions

In the present study we developed a simple, reliable and validated SPE-HPLC method for the simultaneous determination of flavonols **1**–**5** in rat plasma and urine after oral administration of TFA. The method was utilized for the simultaneous determination of flavonols concentration in plasma and used to describe urinary excretion–time profile and cumulative excretion of the flavonols after oral administration of TFA. This is the first pharmacokinetic study on the flavonols after oral administration of TFA.

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