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Simultaneous determination of vitexin-4″-O-glucoside, vitexin-2″-O-rhamnoside, rutin and vitexin from hawthorn leaves flavonoids in rat plasma by UPLC–ESI-MS/MS

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ABSTRACT

A sensitive and accurate ultra-performance liquid chromatography electrospray ionization tandem mass spectrometry (UPLC–ESI-MS/MS) method was developed and validated for the simultaneous determination of vitexin-4″-O-glucoside (VGL), vitexin-2″-O-rhamnoside (VRH), rutin (RUT) and vitexin (VIT) in rat plasma after intravenous administration of hawthorn leaves flavonoids (HLF). Following protein precipitation by methanol, the analytes were separated on an ACQUITY UPLC BEH C_{18} column packed with 1.7 μm particles by gradient elution using a mobile phase composed of acetonitrile and water (containing 0.1% formic acid) at a flow rate of 0.20 mL/min. The analytes and diphenhydramine (internal standard, IS) were detected in the multiple reaction monitoring (MRM) mode by means of an electrospray ionization (ESI) interface (*m*/*z* 292.96 for vitexin-4″-O-glucoside, *m*/*z* 293.10 for vitexin-2″-O-rhamnoside, *m*/*z* 299.92 for rutin, *m*/*z* 310.94 for vitexin and *m*/*z* 166.96 for IS). The calibration curve was linear over the range 10–40,000 ng/mL for vitexin-4″-O-glucoside, 10–50,000 ng/mL for vitexin-2″-O-rhamnoside, 8–1000 ng/mL for rutin and 16–2000 ng/mL for vitexin. The intra- and inter-run precisions (relative standard deviation, RSD) of these analytes were all within 15% and the accuracy (the relative error, RE) ranged from −10% to 10%. The stability experiment indicated that the four analytes in rat plasma samples and plasma extracts under anticipated conditions were stable. The developed method was applied for the first time to pharmacokinetic studies of the four bioactive compounds of hawthorn leaves flavonoids following a single intravenous administration of 20 mg/kg in rats.

1. Introduction

Hawthorn leaves flavonoids (HLF) are the major bioactive constituents of hawthorn leaves and are present in large amounts in standardized leaf extracts [1], with a content exceeding 80% (w/w). HLF include many kinds of flavanoids, e.g. vitexin-4″-O-glucoside (VGL), vitexin-2″-O-rhamnoside (VRH), vitexin (VIT), rutin (RUT), hyperoside, quercitrin, quercetin, etc. [2,3]. Today, HLF are known to exhibit a wide variety of pharmacological functions like cardiovascular regulation [4], hypolipidemic [5], antioxidative [5–7], anti-inflammatory and α-glucosidase inhibitor effects [8].

Till now, most studies have been based on the pharmacodynamics, pharmacology or constituents of HLF and very little attention has been paid to the pharmacokinetic study of the multiple components of HLF in vivo [9,10]. Due to the combinatorial multi-component therapies of traditional Chinese medicines (TCMs) [11], it is necessary to develop a more comprehensive and global assay to fully evaluate the pharmacokinetics of these active ingredients in TCMs [12–14]. Furthermore, a selective and sensitive analytical method for simultaneous quantification and pharmacokinetic studies of HLF in plasma was developed in order to gain a better understanding of the relationship between the pharmacokinetics, pharmacology and bioavailability of the multiple components, which influence the clinical effects of HLF and its rational dosage regiments. According to their contents and physiological activities [15,16], VGL, VRH, RUT and VIT, which are representative and specific ingredients of HLF, can be chosen as markers for the quantitation of HLF. The molecular structures of the four compounds were shown in Fig. 1. According to types of glycosidic bonds and glycosylation sites, VGL, VRH and VIT are flavone C-glycosides, and RUT (quercetin 3-O-glycoside) is a flavone O-glycoside. Several analytical methods, such as high-performance liquid chromatography with ultraviolet detection (HPLC-UV) [17–20] and capillary-zone electrophoresis with electrochemical detection (CE-ED) [21], have been used to determine several of the four ingredients mentioned above in vivo. However, these measurements were not sensitive enough to determine simultaneously the four analytes of HLF in rat plasma for a pharmacokinetic study after intravenous administra-

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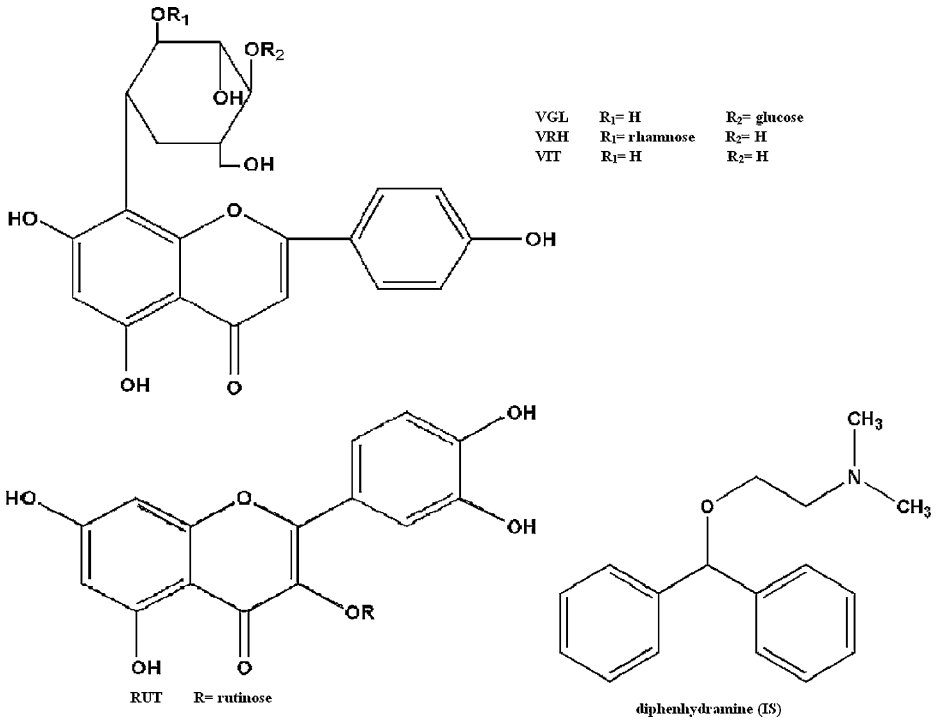

Fig. 1. Chemical structures of analytes and the IS compound.

tion. LC–MS has been demonstrated to be a powerful tool for the identification of natural products in biological matrices owing to their ionization and, in addition, it has a high sensitivity and specificity. Tandem mass spectrometry has the additional advantages of meeting the requirements of high-throughput determination of biosamples and ensuring unequivocal identification and quantification of target analytes as opposed to UV detection where identification is primarily by retention time of a marker compound. Ying et al. developed a UPLC–MS/MS to determine the main active monomer component of HLF (VRH) in biological samples [22]. However, the study did not fully reflect the pharmacokinetics of HLF owing to multiple components.

The purpose of this study is to develop a rapid, selective and specific ultra-performance liquid chromatography electrospray ionization tandem mass spectrometry (UPLC–ESI-MS/MS) method, which enables simultaneous determination of four flavonoids (VGL, VRH, RUT and VIT) in rat plasma. To our knowledge, this is the first report of the development, validation and application of a UPLC–ESI-MS/MS method for the simultaneous determination of four flavonoids in rat plasma and an investigation of their pharmacokinetics after a single intravenous administration of HLF.

2. Materials and methods

2.1. Materials

Vitexin-2″-O-rhamnoside, vitexin and rutin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (100%, Beijing, China). Vitexin-4″-O-glucoside was isolated from HLF in our laboratory and its purity was tested and found to be more than 99.1% by HPLC. HLF were provided by Zhongjin Medicine Co., Ltd. (total flavonoid content >90.3%, Jincheng, China). A quantity of HLF was weighed and dissolved in 0.9% sodium chloride solution containing 10% propylene glycol, agitated then passed through a 0.2 μm micropore filter. The contents of VGL, VRH, RUT and VIT in the resulting HLF solution were 8.65%, 13.14%, 0.24% and 0.31%, respectively. Diphenhydramine (IS) was a kind gift of the Department of Analytical Chemistry of Shenyang Pharmaceutical University (Shenyang, China). Acetonitrile and methanol of HPLC grade were purchased from Dikma Company, Inc. (Richmond Hill, NY, USA). Water was purified in a Barnstead EASYpure® II RF/UV ultrapure Water System (Dubuque, IA, USA) for all the experiments. All other chemicals were of analytical grade.

2.2. Chromatographic conditions and instrumentation

2.2.1. Ultra-performance liquid chromatography

UPLC analysis was performed using an ACQUITY™ UPLC system (Waters Corp., Milford, MA, USA) with conditioned autosampler at 4 °C. The separation was carried out using an ACQUITY UPLC BEH C18 column (50 mm × 2.1 mm, i.d., 1.7 μm; Waters Corp., Milford, MA, USA). The column temperature was set at 35 °C. The analysis was achieved with gradient elution using (A) acetonitrile and (B) water (containing 0.1% formic acid) as the mobile phase at a flow rate of 0.20 mL min^{-1}. Gradient condition of the mobile phase was as follows: A, 10–30%, 0–1.0 min; A, 30–80%, 1.0–1.1 min; an isocratic elution of A, 80%, 1.1–2.5 min; A, 80–10%, 2.5–2.6 min; and returned to the initial condition (acetonitrile–water, 10:90, v/v) for a 3.0 min re-equilibration.

2.2.2. Mass spectrometry

Mass spectrometric detection was carried out using a Waters ACQUITY™ TQD triple quadrupole tandem mass spectrometer (Waters Corp., Manchester, UK) with an ESI interface. The ESI source was operated in negative-ionization mode for the analytes, and in positive ionization mode for the IS. The optimal ESI source parameters for analytes were as follows: capillary 2.0 kV, extractor 3.0 V and RF 0.1 V. The ESI source parameters for IS were: capillary 3.9 kV, extractor 4.0 V and RF 0.2 V. The temperature of the source and desolvation were set at 100 and 400 °C, separately. Nitrogen was used as the desolvation gas (500 L/h) and cone gas (50 L/h). For collision-induced dissociation (CID), argon was used as the collision gas at a flow rate of 0.20 mL min^{-1} (approximately 2.81 × 10^{-3} mbar). The

Table 1
Transition reactions of the analytes and internal standards.

Analytes	Transition	Dwell (S)	Cone voltage (V)	Collision energy (eV)
VGL	592.89 → 292.96	0.2	55	40
VRH	577.00 → 293.10	0.2	65	35
RUT	609.03 → 299.92	0.2	75	40
VIT	431.20 → 311.94	0.2	55	25
IS	256.08 → 166.96	0.2	30	15

multiple reaction monitoring (MRM) mode was used for quantification. Transition reactions of the analytes and internal standards are given in Table 1. All data were acquired using Masslynx™ NT4.1 software (Waters Corp., Milford, MA, USA).

2.3. Applicability of the method to pharmacokinetic studies

2.3.1. Animals

Male Sprague–Dawley rats (weighing 250–300 g) were purchased from the Laboratory Animal Center of Shenyang Pharmaceutical University. The animals were kept in an environmentally controlled breeding room for 1 week before the experiments, and fed with standard laboratory food as well as water ad libitum. They were fasted overnight (16–20 h) with access to water before dosing. All animal experiments were performed in strict accordance with the protocol approved by the Institutional Animal Care and Use Committee of Shenyang Pharmaceutical University.

2.3.2. Plasma sample preparation

Aliquots of 300 μL methanol and 20 μL IS (20 ng/mL methanol solution) were added to plasma samples (100 μL) in a silylated centrifuge tube. The mixture was vortexed for 3 min and centrifuged at 12,000 × *g* for 10 min at 3 °C to separate the protein from the organic phase. Then, 200 μL of the supernatant was collected and transferred to a 1.5 mL tube and evaporated to dryness at 50 °C in a centrifugal concentrator (Labconco Corp., MO, USA). The residue was dissolved in 100 μL initial mobile phase, vortexed for 1 min and centrifuged at 12,000 × *g* for 5 min at 3 °C again. After centrifuging, a 5.0 μL aliquot of the solution was injected into the UPLC–ESI-MS/MS system for analysis.

2.3.3. Pharmacokinetics

20 mg/kg of HLF solution was administered to the rats (equivalent to 1.73 mg/kg of VGL, 2.63 mg/kg of VRH, 0.048 mg/kg of RUT and 0.062 mg/kg of VIT, respectively) intravenously via the femoral vein. Blood samples (0.3 mL) were collected in heparinized eppendorf tubes (1.5 mL) from the vena orbitalis at 0 (predose), 0.033, 0.083, 0.17, 0.25, 0.33, 0.50, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, and 12.0 h after administration. As soon as possible, the heparinized blood was centrifuged at 2000 × *g* for 10 min at room temperature. The plasma samples were stored at −20 °C until analysis. The data analysis of the plasma concentrations of the four analytes versus time and calculation of the pharmacokinetic parameters in rats were carried out using statistics software DAS 2.0 (Mathematical Pharmacology Professional Committee of China, Shanghai, China). The results are represented as the mean ± standard deviation of the mean and the main pharmacokinetic parameters of the HLF.

2.4. Preparation of calibration standards and quality control samples

Four standards (VGL, VRH, RUT and VIT) were accurately weighed separately (Sartorius AG electronic balance, CP 225D, *d* = 0.01 mg, Germany) and then dissolved in an appropriate volume of methanol to produce corresponding stock standard solutions. The stock solution containing four flavonoids (VGL, VRH, RUT and VIT) was prepared to contain concentrations of 4.0 mg/mL for VGL, 5.0 mg/mL for VRH, 0.1 mg/mL for RUT and 0.2 mg/mL for VIT, respectively. Working standard solutions were freshly prepared by mixing and diluting the above stock solution with methanol at appropriate ratios. For the validation of the method, three concentration levels of QC plasma samples were prepared with blank plasma containing VGL (20, 20,000, 32,000 ng/mL), VRH (20, 25,000, 40,000 ng/mL), RUT (8, 500, 800 ng/mL) and VIT (16, 1000, 1600 ng/mL). The internal standard (IS) working solution was similarly prepared to give a concentration of 20 ng/mL. All the stock and working standard solutions were stored at 4 °C and brought to room temperature before use.

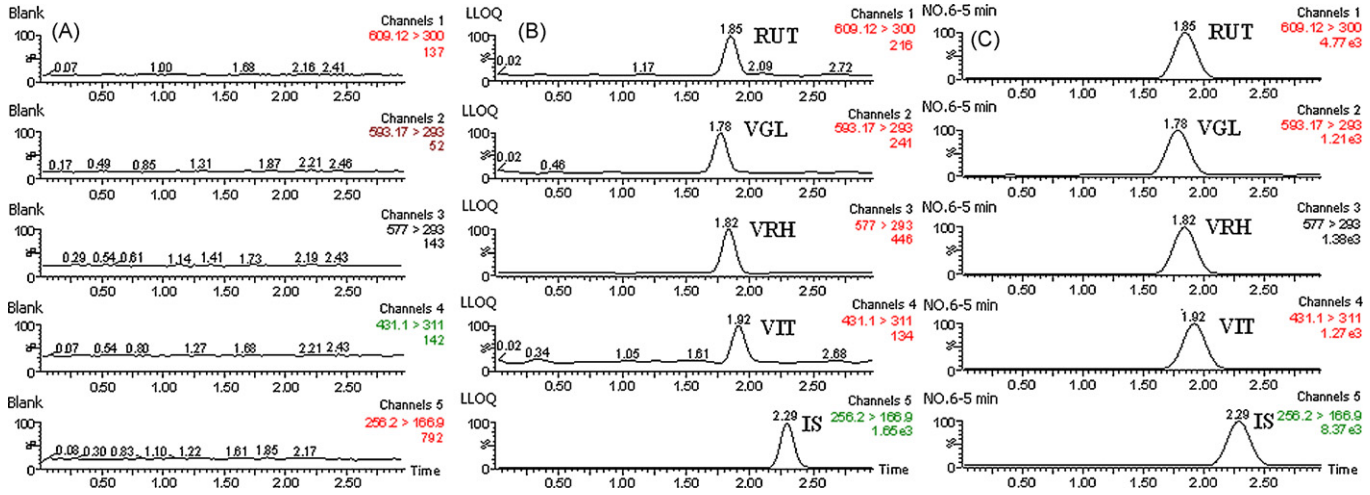

Fig. 2. Chromatograms for VGL (channel 2), VRH (channel 3), RUT (channel 1), VIT (channel 4) and IS (channel 5) in rat plasma samples: (A) a blank plasma sample; (B) a blank plasma sample spiked with analytes at the LLOQ and IS (20 ng/mL); (C) a plasma sample from a rat at 5 min after a single intravenous administration of HLF.

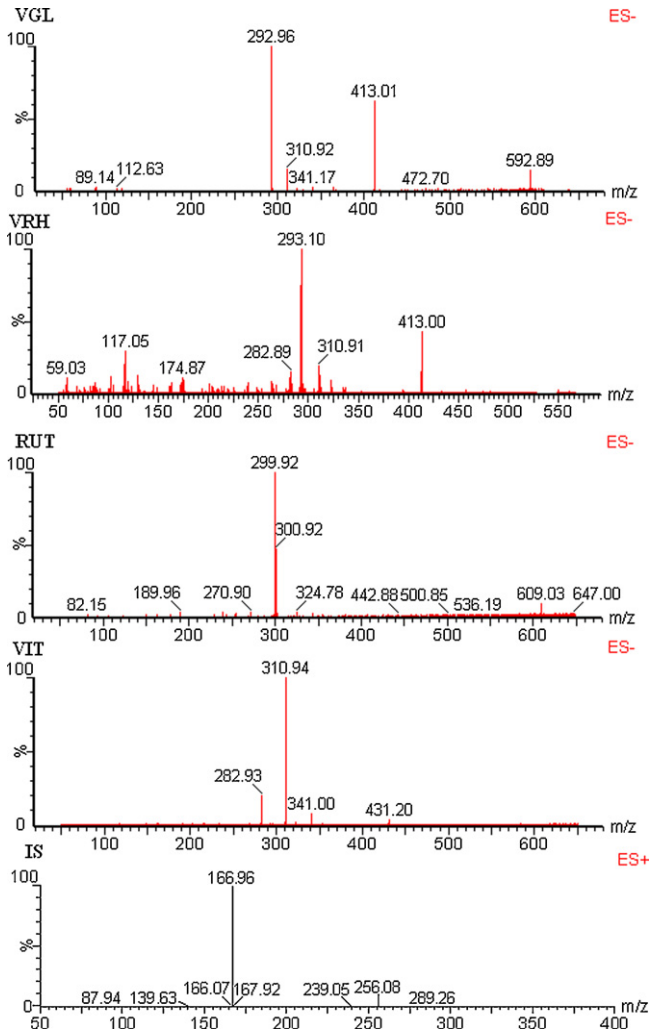

Fig. 3. MS/MS spectra of VGL, VRH, RUT, VIT and IS.

2.5. Method validation

2.5.1. Selectivity and matrix effect

To investigate the selectivity, chromatograms of rat blank plasma were compared with those of LLOQ plasma samples and plasma samples after intravenous administration. Matrix effects on the ionization of analytes were evaluated by comparing the peak area of analytes in the samples spiked post-extraction (A) with that of analyte standard solutions dried directly and reconstituted with the same volume of initial mobile phase (B). Three concentrations of the analytes, each in six replicates, were studied. When the peak area ratio (A/B × 100)% of the analytes and the internal standard solution was between 85% and 115%, the matrix effect may be considered as negligible.

2.5.2. Linearity and LLOQ

To evaluate the linear relationship of the method, eight different concentrations of analytes in plasma were prepared by spiking blank plasma with 20 μL analytes and IS using the above extraction procedure on three successive days. The plasma concentrations of the calibration curves ranged from 10 to 40,000 ng/mL for VGL, 10 to 50,000 ng/mL for VRH, 8 to 1000 ng/mL for RUT and 16 to 2000 ng/mL for VIT, respectively. The calibration curves were established and fitted by least-squares regression using $1/C^2$ as the weighting factor of the peak area ratios of the four constituents to the IS versus the respective standard concentration. The concentrations of analytes in QCs or the test samples were calculated using the regression parameters obtained from the calibration curves. The LLOQ was defined as the lowest concentration on the calibration curve with acceptable precision and accuracy (six replicates with a RSD below 20% and RE within ±20%).

2.5.3. Accuracy, precision and recovery

Accuracy and precision were assessed by analyzing QCs using six replicates at three concentration levels on three different validation days. Precision was calculated as the RSD within a single run and between different runs. The accuracy was expressed as the relative error (RE) and calculated from the nominal concentration (C_{nom}) and the value of the analytical concentration (C_{ana}) as follows: RE (%) = $[(C_{ana} - C_{nom})/(C_{nom})] \times 100$. The intra- and inter-run precisions should not exceed 15% and the accuracy was required to be within ±15%, except for the low QC samples where the precision should be below 20% and the accuracy within ±20%.

The extraction recoveries of the four flavonoids (VGL, VRH, RUT and VIT) at three QC levels were determined by comparing the mean peak areas of analytes obtained from plasma samples with four analytes spiked before extraction with those spiked after extraction, which represented 100% recovery. The extraction recovery of the IS was determined in a similar way using the medium QC samples as a reference.

2.5.4. Stability

Investigation of the stability was designed to cover the anticipated conditions that plasma samples might be exposed to in this study. The stability of QC plasma samples at three concentrations was evaluated in two ways: (1) the stability of the analytes in the plasma sample, before extraction, stored at 25 °C (room temperature), −20 °C (storage temperature) and during three freeze–thaw

Table 2
Matrix effects and the extraction recoveries of the four analytes ($n = 6$).

Analytes	Spiked-concentration (ng/mL)	Matrix effects (%)	RSD (%)	Recovery (%)	RSD (%)
VGL	20	104.11 ± 14.35	13.77	89.10 ± 9.72	10.91
	20,000	105.98 ± 5.53	5.23	80.68 ± 11.71	14.52
	32,000	95.53 ± 7.43	7.78	86.01 ± 9.81	11.41
VRH	20	106.16 ± 7.07	6.66	94.96 ± 12.11	12.75
	25,000	100.34 ± 10.93	10.89	88.55 ± 10.79	12.19
	40,000	93.71 ± 4.05	4.32	88.19 ± 4.97	5.63
RUT	16	98.72 ± 14.59	14.78	87.64 ± 4.98	5.69
	500	96.13 ± 7.30	7.60	78.55 ± 7.30	9.30
	800	93.21 ± 2.16	2.31	72.05 ± 6.55	9.09
VIT	32	106.87 ± 6.65	6.23	75.27 ± 11.30	14.99
	1000	95.51 ± 4.39	4.60	93.76 ± 10.18	10.86
	1600	94.45 ± 2.69	2.84	81.87 ± 4.57	5.58

Table 3
Regression data and LLOQs of the analytes determined.

Analytes	Linear range (ng/mL)	Linear equation	Correlation coefficient (r)	LLOQ (ng/mL)
VGL	10–40,000	$y = 1.101 \times 10^{-3}x + 3.100 \times 10^{-2}$	0.996	10
VRH	10–50,000	$y = 3.032 \times 10^{-3}x + 3.101 \times 10^{-2}$	0.998	10
RUT	8–1000	$y = 4.021 \times 10^{-3}x + 2.100 \times 10^{-2}$	0.997	8
VIT	16–2000	$y = 1.061 \times 10^{-3}x + 2.104 \times 10^{-2}$	0.995	16

cycles; (2) the stability of the analytes after extraction from plasma stored at 4 °C. The rat plasma samples containing the four analytes were stored at 25 °C for 2 h, −20 °C for 30 days, as well as three freeze–thaw cycles, and the processed samples at 4 °C for 4 h, respectively. Freeze–thaw stability was evaluated by exposing them to three freeze–thaw (−20 °C for 24 h, −20 °C for 12 h, −20 °C for 12 h) (25 °C for 30 min per time, three times) cycles before sample preparation. In addition, the stability of the stock solution and working standard at 4 °C for 3 months and at 25 °C for 24 h was also assessed.

3. Results and discussion

3.1. IS and the extraction procedure

A stable isotope-labeled analyte is the ideal IS for LC–MS/MS assay to correct for unknown losses during the procedure used. However, the availability of labeled compounds is limited, because they are not naturally present in the sample to be studied and exhibit similar behavior to the analytes during the sample extraction, chromatographic elution and mass spectrometric detection. In fact, the components of TCMs are extremely complex. The identities of some are even unknown. It is difficult to decide whether the analyte analogs are present or not in analytical samples. Thus, it is more difficult to select a suitable IS for simultaneous determination of four flavonoids. To solve the problem, a chemical reference standard may be more suitable as the IS, if it has a high response and is rapidly eluted under the same LC–MS/MS conditions as the analytes. Therefore, diphenhydramine was used as the IS throughout the investigations and in the final pharmaceutical study [23].

Liquid–liquid extraction (LLE) was first carried out during sample preparation. It produced a relatively clean sample and reduced the possibility of introducing highly polar materials into the column and MS system, although low analyte recoveries and a high variability were obtained. LLE is preferred for the extraction of less-polar flavonoids (flavonoid aglycones) that are soluble in water-immiscible organic solvents [24]. The four analytes (flavonoid glycosides) are polar and not amenable to solvent extraction. However, solvent-induced protein precipitation (PP) was found to be effective in removing proteins from rat plasma, due to a low probability of workup losses during sample preparation, and it was fully compatible with the UPLC–ESI-MS/MS analysis. In this work, several organic solvents were tested for their suitability for the precipitation of protein and extraction of the four analytes from rat plasma. They included methanol, acetonitrile, 6% perchloric acid and methanol-ethyl acetate (1:2, v/v) and their volume was one to four times that of the plasma sample. The results showed that the most efficient protein precipitation and highest extraction recovery were obtained when methanol was chosen. The extraction recoveries of the four analytes and IS were at least above 70% and no interferences were found at the retention times of analytes and IS when the plasma sample–methanol ratio was 1:3 and 1:4 (v/v), respectively. According to student's t-test, the recovery of the analytes did not exhibit a significant difference ($p > 0.05$). Considering the detection sensitivity, the methanol volume added was three times that of the plasma sample. Based on these results, we established the sample extraction procedure as described in Section 2.3.2.

3.2. Optimization of UPLC–MS

In view of the higher efficiency and linear velocities, the use of 1.7 μm particles of stationary phase allowed UPLC to push the limits of both peak capacity and speed of analysis. Different mobile phases were evaluated to improve the UPLC separation and enhance the sensitivity of the MS detection. Researchers [25] have studied the optimization of a liquid chromatography method based on simultaneous ESI-MS for analysis of flavonoid glycosides. In the negative-ion mode, gradient elution using a mobile phase composed of acetonitrile–water (containing 0.1% formic acid) was finally chosen for separation and resulted in an excellent peak shape and a shorter analysis time. A small amount of formic acid in the mobile phase improved the ionization of the IS and, subsequently, improved the sensitivity. The additives in the mobile phase also helped reduce the interference produced by matrix effects [26]. In addition, the starting mobile phase was used to reconstitute the

Table 4
The intra- and inter-day precisions and accuracies of the analytes in rat plasma ($n = 6$).

Analytes	Spiked Conc. (ng/mL)	Intra-run			Inter-run		
		Measured Conc. (ng/mL)	Precision (%, RSD)	Accuracy (%, RE)	Measured Conc. (ng/mL)	Precision (%, RSD)	Accuracy (%, RE)
VGL	20	19.55 ± 2.72	13.91	−2.24	21.16 ± 1.72	8.14	5.8
	20,000	20,835.19 ± 1979.77	9.47	4.18	20,937.57 ± 1120.66	5.35	4.69
	32,000	29,636.76 ± 1943.49	6.56	−7.39	31,299.31 ± 1521.62	4.86	−2.19
VRH	20	21.06 ± 3.16	15.01	5.31	20.65 ± 2.01	9.74	3.26
	25,000	24,181.81 ± 2714.02	11.22	−3.27	24,554.18 ± 322.49	1.31	−1.78
	40,000	38,462.81 ± 4626.29	12.03	−3.84	40,746.24 ± 1979.98	4.86	1.87
RUT	16	16.89 ± 2.54	15.04	5.53	16.59 ± 1.11	6.67	3.68
	500	547.56 ± 43.43	7.93	9.51	505.93 ± 39.50	7.81	1.19
	800	820.09 ± 65.47	7.98	2.51	825.09 ± 45.40	5.50	3.14
VIT	32	32.64 ± 4.41	13.51	1.98	32.28 ± 1.32	4.09	0.86
	1000	1033.84 ± 101.02	9.77	3.38	1043.27 ± 61.03	5.85	4.32
	1600	1656.41 ± 149.50	9.03	3.53	1608.31 ± 51.90	3.27	0.52

The "Conc." indicated the plasma concentration.

Table 5
The stability of the analytes in rat plasma and plasma extracts (mean ± SD, $n = 3$).

Analytes	Conc. added (ng/mL)	−20 °C for 30 days in plasma		Three freeze–thaw cycles in plasma		25 °C for 2 h in plasma		4 °C for 4 h in plasma extract	
		Conc. ass (ng/mL)	RSD (%)	Conc. ass (ng/mL)	RSD (%)	Conc. ass (ng/mL)	RSD (%)	Conc. ass (ng/mL)	RSD (%)
VGL	20	19.38 ± 1.38	7.12	21.71 ± 2.16	9.98	20.12 ± 1.93	9.61	21.81 ± 2.53	11.61
	20,000	19,596.65 ± 1747.27	8.92	21,310.69 ± 2026.58	9.51	19,501.65 ± 2214.87	11.36	19,353.29 ± 1829.77	9.45
	32,000	30,544.75 ± 2061.20	6.75	32,690.26 ± 3013.64	9.22	29,778.91 ± 1357.76	4.56	30,742.58 ± 3414.38	11.11
VRH	20	19.17 ± 8.28	8.28	19.98 ± 2.87	14.39	19.87 ± 2.10	10.55	19.34 ± 2.21	1.46
	25,000	24,516.95 ± 3239.11	13.21	25,570.08 ± 2499.23	9.77	26,157.71 ± 1654.17	6.32	24,412.01 ± 1679.12	6.88
	40,000	39,363.59 ± 2274.79	5.78	39,671.37 ± 3886.80	9.81	43,017.26 ± 1627.07	3.78	38,949.68 ± 1576.88	4.05
RUT	16	14.82 ± 2.20	14.82	14.65 ± 1.57	10.75	16.87 ± 2.64	15.62	15.39 ± 1.72	11.09
	500	511.04 ± 59.85	11.71	504.98 ± 75.47	14.95	492.97 ± 63.09	12.80	533.11 ± 63.19	11.85
	800	784.68 ± 41.35	5.27	819.28 ± 77.35	9.44	788.22 ± 82.65	10.49	810.85 ± 82.14	10.13
VIT	32	29.64 ± 3.61	12.21	31.83 ± 2.17	6.81	31.93 ± 2.98	9.33	31.68 ± 5.60	17.68
	1000	1041.49 ± 139.07	13.35	1044.23 ± 128.77	12.33	1116.84 ± 163.99	14.68	1023.02 ± 145.8	14.25
	1600	1574.88 ± 106.77	6.78	1643.19 ± 178.51	10.86	1650.45 ± 222.32	13.74	1720.38 ± 176.58	10.26

The "Conc. added" indicated the plasma concentration (ng/mL) at the initial time point and the "Conc. ass" and "RSD" indicated the plasma concentration assayed (ng/mL, mean ± SD) and relative standard deviation of the recovery under different storage conditions ($n = 3$), respectively.

residues completely and improve the response of analytes indirectly.

As shown in Fig. 2, five channels were used for recording. The elution order and retention time of the analytes and IS were as follows: channel 1 for RUT (1.85 min), channel 2 for VGL (1.78 min), channel 3 for VRH (1.82 min), channel 4 for VIT (1.92 min) and channel 5 for IS (2.29 min). All the analytes were rapidly eluted with retention times less than 2.3 min, and the total run time of each sample was 3.0 min which met the requirement for the high-throughput determination of biosamples. The very narrow chromatographic peaks generated by UPLC resulted in an increase in the chromatographic sensitivity and selectivity.

To obtain better responses for the analytes and IS, the MS conditions should be optimized by direct injection of their standard solutions into the mass spectrometer. We recommend the negative-ion mode for analysis of flavonoid glycosides in biological samples because of its better sensitivity and limited fragmentation. The effects of desolvation temperature, source temperature, capillary voltage, and desolvation gas flow were examined. We established the optimum conditions as described in Section 2.2.2. In negative-ion ESI mode, the $[M-H]^-$ signal was observed, and acquisition of mass spectrometry data for the four analyte standards was performed. IS data were obtained in positive ionization mode $[M+H]^+$. At first, acquisition in negative-ion mode for IS was also tried, but no obvious $[M-H]^-$ ion signal was observed. Finally, two different ionization modes using ESI were employed to monitor the analytes and IS. The product ion spectra of VGL, VRH, RUT, VIT and IS were shown in Fig. 3. In the MS spectra of VIT, ions of $[M-H-120]^-$ ($[M-H-C_4H_8O_4]^-$) at m/z 311 were observed, which are consistent with the characteristic ions of a C-glycosidic flavonoid [27]. The fragmentation of VGL gave rise to intense ions at m/z 413 and m/z 292.96 corresponding to $[M-H-glc]^-$ and subsequent $[413-C_4H_8O_4]^-$. VRH was observed to lose its terminal rhamnose unit giving a product ion at m/z 413 followed by elimination of a $C_4H_8O_4$ group to give a product ion at m/z 293.10. RUT gave a higher signal ($[M-H]^-$) at m/z 609.03, and gave rise to intense ions at m/z 299.92 corresponding to the loss of the rutinose unit. Diphenhydramine exhibited a major fragment ion at m/z 166.96, produced from the loss of a neutral fragment of $[HOCH_2CH_2N(CH_3)_2]$.

The mass transitions chosen for quantification were m/z 592.89 → 292.96 for VGL, m/z 577.00 → 293.10 for VRH, m/z 609.03 → 299.92.00 for RUT, m/z 431.20 → 310.94 for VIT and m/z 256.08 → 166.96 for IS.

3.3. Method validation

3.3.1. Selectivity and matrix effect

The selectivity of the method was tested by analysis of (A) a blank plasma sample, (B) a blank plasma sample spiked with analytes at the LLOQ (10 ng/mL for VGL, 10 ng/mL for VRH, 8 ng/mL for RUT and 16 ng/mL for VIT) and IS (20 ng/mL) and (C) a plasma sample from a rat at 5 min after a single intravenous administration of 20 mg/kg HLF. There was no significant chromatographic interference around the retention times of the analytes and IS (Fig. 2). The retention times of VGL, VRH, RUT, VIT and IS were 1.78, 1.82, 1.85, 1.92, and 2.29 min, respectively.

Matrix effects of VGL, VRH, RUT and VIT in rat plasma were shown in Table 2. There was no significant matrix effect on the analytes and IS in this method.

3.3.2. Linearity and LLOQs

All calibration curves exhibited good linearity with coefficients of correlation (r) within the range 0.995–0.998. The LLOQs (chromatographic spectra were shown in Fig. 2) were appropriate for

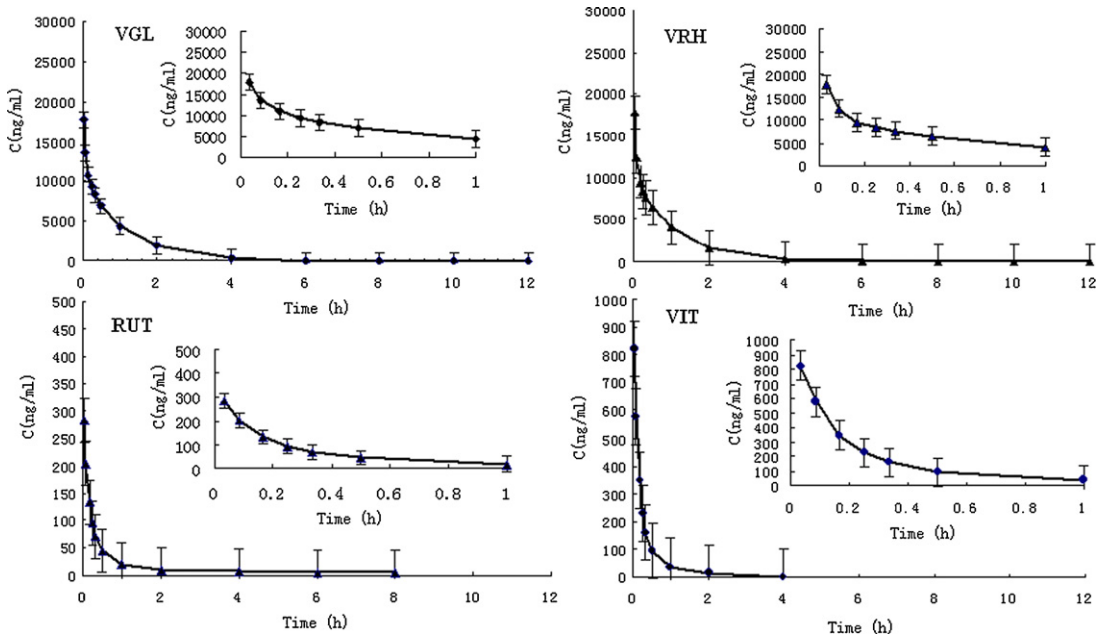

Fig. 4. Plasma concentration-time profiles of VGL, VRH, RUT and VIT after intravenous administration of HLF (20 mg/kg).

quantitative detection of analytes in the pharmacokinetic studies. Linear ranges, regression equations, correlation coefficients and LLOQs obtained from typical calibration curves were shown in Table 3. Calibration curves were linear and r^2 values were all higher than 0.991.

3.3.3. Precision, accuracy and recovery

As shown in Table 4, the intra- and inter-run precisions (RSD) of these analytes were no more than 15.04% and 9.74% at the lowest concentration and for the other concentrations were no more than 12.03% and 7.81%. The accuracies (RE) ranged from −7.39% to 9.51%. The results in Table 4 demonstrated that the values were all within the acceptable range and the method showed good precision and accuracy.

The extraction recoveries determined for VGL, VRH, RUT and VIT were shown in Table 2. At three concentration levels of the four analytes, the absolute extraction recoveries were all in the range 72.05% to 94.92%. The extraction recovery of the IS was more than 85%. These results demonstrated that the values were all within the acceptable ranges.

3.3.4. Stability

The stabilities of the four analytes in rat plasma sample and plasma extract ($n = 3$) at different conditions were shown in Table 5. The results indicated that all analytes in rat plasma samples were stable when stored at 25 °C for 2 h, −20 °C for at least 30 days and after three freeze–thaw cycles at low, medium and high concentrations, respectively. Therefore, the plasma samples should be processed within 2 h at 25 °C or 30 days at −20 °C. The stability experiment also indicated that the stock solution stored at 4 °C was stable for at least 3 months. The working standard solutions stored at 25 °C were stable for at least 24 h.

3.4. Pharmacokinetic application

The validated method was sensitive enough to allow measurement of all the compounds in rat plasma after intravenous administration of a HLF solution (20 mg/kg). It was found that, using DAS software, the best fit pharmacokinetic model to estimate the pharmacokinetic parameters was the three compartment model with a weight of $1/C^2$ for intravenous administration, and there were some differences in the distribution and elimination among VGL, VRH, VIT and RUT. The plasma concentration-time profiles of the analytes in rat plasma were shown in Fig. 4. Figure shows that VIT was eliminated quickly and nothing could be detected after 4 h. Both VGL and VRH were eliminated over after 12 h, while RUT could be detected in plasma after 8 h. The estimated pharmacokinetic parameters were listed in Table 6. The pharmacokinetic behaviors of VGL and VRH in rats after intravenous administration of HLF were similar owing to their similar chemical structures. The pharmacokinetic behaviors of VIT and RUT were markedly different from those of components mentioned above possibly owing to different types of glycosidic bonds and glycosylation sites of the analytes. It could be confirmed by their plasma concentration–time curves and some pharmacokinetic parameters of the four active ingredients. In addition, the pharmacokinetic parameters of VGL and VRH

Table 6
Pharmacokinetic parameters of analytes after intravenous administration of 20 mg/kg HLF (mean ± SD, $n = 6$).

Parameter	VGL	VRH	RUT	VIT
$t_{1/2\alpha}$ (h)	0.11 ± 0.043	0.28 ± 0.13	0.13 ± 0.066	0.13 ± 0.056
$t_{1/2\beta}$ (h)	0.87 ± 0.48	0.84 ± 0.37	4.37 ± 2.25	1.53 ± 0.37
AUC_{0-t} (μg h/L)	15,602.08 ± 7951.91	13,680.95 ± 6940.14	144.16 ± 30.35	253.01 ± 121.93
$AUC_{0-\infty}$ (μg h/L)	15,626.16 ± 7948.55	13,700.22 ± 6949.12	157.34 ± 25.79	258.04 ± 122.76
MRT_{0-t} (h)	1.17 ± 0.13	1.19 ± 0.16	1.27 ± 0.45	0.38 ± 0.15
$MRT_{0-\infty}$ (h)	1.19 ± 0.14	1.20 ± 0.15	1.99 ± 0.91	0.42 ± 0.14
CL (L/h/kg)	0.002 ± 0.001	0.002 ± 0.001	130.11 ± 22.18	97.44 ± 52.65
V (L/kg)	0.003 ± 0.002	0.004 ± 0.002	322.33 ± 153.60	47.46 ± 23.61
C_{max} (μg/L)	17,706.47 ± 9543.27	19,255.28 ± 7808.50	329.66 ± 119.73	889.99 ± 482.58

were a little different from those reported in the literature [20,22], possibly due to differences in dosage, components and the route of administration.

4. Conclusion

A novel UPLC–ESI-MS/MS method has been established for simultaneous determination of four flavonoid glycosides of HLF in rat plasma. This is the first UPLC–MS/MS quantitative assay for investigating the pharmacokinetics of HLF in rats. Using this method, the quantification was found to be fast, sensitive and precise, and there were no interferences from endogenous substances. The method is suitable for pharmacokinetic studies of vitexin-4″-O-glucoside, vitexin-2″-O-rhamnoside, rutin and vitexin in rats following a single intravenous administration.

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