—Regular Article—

HPLC Determination of Five Polyphenols in Rat Plasma after Intravenous Administrating Hawthorn Leaves Extract and Its Application to Pharmacokinetic Study

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A simple and specific HPLC-UV method was developed to simultaneously determine five active compounds including vitexin-4″-*O*-glucoside (VG), vitexin-2″-*O*-rhamnoside (VR), vitexin (VIT), rutin (RUT) and hyperoside (HP) in rat plasma after intravenous administrating the hawthorn leaves extract (HLE). With baicalin as internal standard (I.S.), sample pretreatment involved a one-step extraction with methanol of 0.2 ml plasma. The HPLC assay was carried out using a Phenomsil C18 analytical column with UV detection at 332 nm. The mobile phase consisted of methanol-acetonitrile-tetrahydrofuran-1% glacial acetic acid (6 : 1.5 : 18.5 : 74, v/v/v/v). The calibration curves were liner over the range of 2.030–500.5, 0.1513–75.64, 0.2507–12.54, 0.5128–25.64 and 0.4032–20.16 μg/ml for VG, VR, VIT, RUT and HP, respectively. The relative standard deviations (RSD) of the intra- and inter-day precisions for the analysis of the five analytes were between 1.0 and 8.9% with accuracies (relative error) below 8.2% for the analysis of the five analytes. The average extraction recoveries of five analytes were more than 82.67±4.74%. The HPLC method herein described was fully validated and successfully applied to the pharmacokinetic studies after intravenous administration of HLE solution to rats over three doses.

Key words——HPLC; hawthorn leaves extract (HLE); polyphenol; rat plasma; pharmacokinetics

INTRODUCTION

The leaves of *Crataegus pinnatifida* Bge. var. *major* (hawthorn leaves), a well-known traditional Chinese medicine (TCM) used for the treatment of qi-stagnancy and blood stasis, chest distress, palpitation, loss of memory, dizziness and tinnitus,[1] which mainly contain polyphenols including vitexin-4″-*O*-glucoside (VG), vitexin-2″-*O*-rhamnoside (VR), vitexin (VIT), rutin (RUT), hyperoside (HP).[2,3] Recently, many *in vitro* studies reported the pharmacological actions such as VG for the antioxidant effect,[4] VR for inhibiting DNA synthesis in MCF-7 human breast cancer cells,[5] and VIT for inducing apoptosis and suppress tumor growth[6] and so on. In addition, much attention has been paid to the *in vivo* pharmacokinetics of HP, VR and VG by HPLC,[7,8] UP-LC-ESI-MS/MS[9] and LC/MS/MS[10] method. However, the previous researches mostly concentrated on the pharmacokinetic studies of the pure compounds. As the pharmacological actions of ploy-phenolic compounds possessed were attributable to a synergistic effect of multiple components[11] and therefore it is of great importance to determination of multiple components of HLE entering into the body for evaluating the efficacy and investigating the action mechanism. Although some papers focus on the simultaneous determination of VG and VR in rat plasma,[12,13] the external standard method could lead to the analytical errors and was unsuitable to the determination of polyphenolic components *in vivo*. Hence, it is necessary to develop a method using an internal standard to investigate the HLE pharmacokinetics in plasma samples. Recently, Zhang, W. J., *et al*[14] reported the pharmacokinetic data on four compounds of hawthorn leaves, but to our knowledge, there is little valuable method for the simultaneous determination of five compounds in biological fluids after an intravenous administration of HLE at different doses.

The aim of this study was to establish a rapid and sensitive HPLC method to fully evaluate the HLE in rat *via* the simultaneous determination of VG, VR, VIT, RUT and HP, and the pharmacokinetic studies

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of the five polyphenols will be helpful to clinical application of the HLE.

MATERIALS AND METHODS

Plant Material Dried hawthorn leaves were collected from Shenyang (Liaoning, China) on October 22, 2009. Voucher specimens were maintained at Liaoning University of Traditional Chinese Medicine, China.

Reagents and Chemicals VG, VR, VIT and HP were isolated from hawthorn leaves in our laboratory according to the recent methods in literature.[3,9,15] Their chemical structures were confirmed by ^{1}H and ^{13}C, and purities of them were over 99% by HPLC analysis. RUT and the internal standard (I.S.), baicalin, were provided by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The chemical structures of five compounds and I.S. were shown in (Fig. 1(A-F)). Methanol, acetonitrile and tetrahydrofuran (all of HPLC grade) were obtained from Xinxing (Chemical Reagent Plant, Shanghai, China), and the water

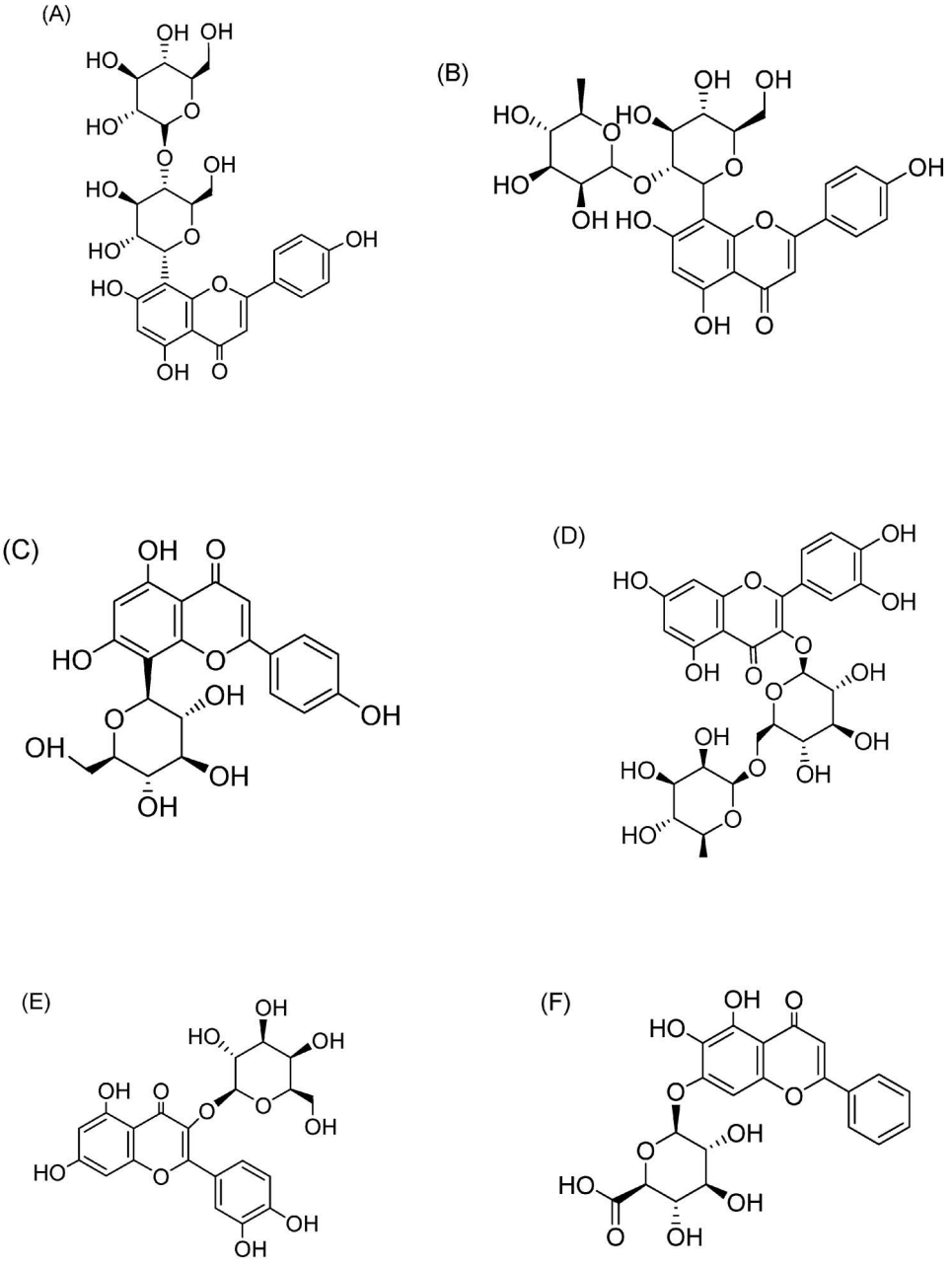

Fig. 1. Chemical Structures
(A) vitexin-4″-*O*-glucoside; (B) vitexin-2″-*O*-rhamnoside; (C) vitexin; (D) rutin; (E) hyperoside; and (F) baicalin.

used in all experiments was purified by a Milli-Q Biocel Ultrapure Water System (Millipore, Bedford, MA, USA). All other chemicals were of analytical reagent grade purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

Chromatographic System The experiment was performed on an Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary Pump (G1310A), a vacuum degasser (G1322A), a UV-VIS spectrophotometric detector (G1314A) and Chemstation software (Agilent). The analytes were determined at room temperature on an analytical Phenomsil C18 column (4.6 mm×150 mm, i.d., 5 μm, Feinami Technologies, Beijing, China) protected by a KR C18 guard column (8.0 mm×35 mm, i.d., 5 μm, Dalian Create Science and Technology Co., Ltd., China). The mobile phase for HPLC analysis consisted of a mixture of methanol-acetonitrile-tetrahydrofuran-1% glacial acetic acid (6∶1.5∶18.5∶74, v/v/v/v), which was filtered and degassed under reduced pressure prior to use. The analysis was carried out at a flow rate of 1 ml /min with the detection wavelength of 332 nm.

Preparation of HLE Solution The dried hawthorn leaves (1 kg) were cut in small pieces and extracted twice by refluxing with 70% ethanol (1∶15 and then 1∶10, w/v) for 2 h, and the extraction solutions were combined, filtered, concentrated under reduced pressure, and then passed through an AB-8 macro-porous resin column (10 cm×120 cm, Shanghai, China). Initially, eluted with 15 l water to eliminate the impurity, subsequently, eluted with 5 l of 70% ethanol to extract the polyphenolic compounds. The eluate was then evaporated under reduced pressure until dryness at 40℃. Preparing a conical flask with lid, the dosing HLE was added, extracted with a mixture of methanol-water (50∶50, v/v) in the ultrasonic bath for 30 min, filtered. After evaporation, the residue was dissolved in physiological saline to give a solution containing 0.2 g HLE in each milliliter. The administration solution was filtered with 0.45 μm membrane filter and stored at 4 ℃ before use. The prepared HLE solution was analyzed by HPLC. The contents corresponding to VG, VR, VIT, RUT and HP were 12.4, 4.96, 0.665, 0.703 and 2.68 mg/ml, respectively.

Preparation of Standards and Quality Control Samples The stock solutions of VG, VR, VIT, RUT, HP and I.S. were prepared by precisely weighing the reference standards of five compounds and dissolving in methanol to yield the concentrations of 3691, 601.5, 194.4, 223.6, 224.2 and 9.26 μg/ml, respectively. A series of standard mixture working solutions with concentrations 8.120–2002 μg/ml for VG, 0.6052–302.6 μg/ml for VR, 1.004–54.15 μg/ml for VIT, 2.052–102.6 μg/ml for RUT and 1.612–80.64 μg/ml for HP were obtained by diluting the mixture of the stock standard solutions with methanol. The working solution of I.S. (2.315 μg/ml) was prepared by diluting I.S. stock solution with methanol. All solutions were stored at 4℃. Six calibrators of VG (2.030, 4.060, 10.15, 25.37, 100.5 and 500.5 μg /ml), VR (0.1513, 0.3026, 0.7564, 3.026, 15.13 and 75.64 μg/ml), VIT (0.2507, 0.5015, 1.003, 2.006, 5.015 and 12.54 μg/ml), RUT (0.5128, 1.026, 2.051, 4.102, 10.26 and 25.64 μg/ml) and HP (0.4032, 0.8064, 1.613, 3.226, 8.064 and 20.16 μg/ml) were prepared by 200 μl drug-free rat plasma spiked with the appropriate amount of the standard mixture working solutions (50 μl) and I.S. working solution (50 μl). Quality control (QC) samples were prepared low, middle and high concentrations at (6.090, 250.2 and 400.4 μg/ml for VG, 0.4539, 37.82 and 60.51 μg/ml for VR, 0.5014, 6.269 and 10.48 μg/ml for VIT, 1.519, 12.51 and 20.51 μg/ml for RUT, 0.9707, 10.08 and 16.13 μg/ml for HP) in bulk and aliquots were stored at −20℃ until analysis.

Plasma Sample Preparation To aliquots (200 μl) of plasma, 20 μl of acetic acid, 50 μl of I.S., baicalin (2.315 μg/ml), and 1 ml methanol were added, followed by vortex mixing for 1 min and centrifuging at 890×*g* for 15 min. The supernatant was collected and evaporated to dryness at 50℃ under a gentle stream of nitrogen. The dried residue was then reconstituted in 100 μl of mobile phase, and centrifuged at 15,092×*g* for 10 min, and an aliquot (20 μl) of the supernatant was injected into the HPLC system for analysis.

Method Validation

Selectivity The selectivity was determined by comparing chromatograms of different blank plasma obtained from rat with those of corresponding standard plasma samples spiked with VG, VR, VIT, RUT, HP and I.S., and plasma sample after intravenous administration doses of HLE solution.

Linearity, Limits of Detection (LOD) and Quantification (LOQ) The linearities were evaluated over the concentration range of 2.030–500.5, 0.1513–

75.64, 0.2507–12.54, 0.5128–25.64 and 0.4032–20.16 μg/ml for VG, VR, VIT, RUT and HP, respectively. For the calibration curve, the ratios of the chromatographic peaks area of analytes to I.S. were plotted *versus* the nominal concentrations in the standard plasma samples. The regression equation was obtained by weighted ($1/c^2$) least square linear regression. LOD and LOQ were determined by stepwise dilution of the QC sample at low concentration level using a signal-to-noise ratio of 3 and 10, respectively.

Precision and Accuracy The precision and accuracy of the method were evaluated with QC samples at low, middle and high three concentrations and using five replicates on three consecutive days. The intra- and inter-assay precisions were assessed by determining the QC samples at three concentration levels of each compound. For the intra-day validation, five replicates of the QC plasma samples were analyzed on the same day, whilst the inter-day values were carried out over three consecutive days. The accepted criteria for each QC sample were that the precision and accuracy should not exceed 15%, except at the LOQ where it should not exceed 20%. The precision was expressed as the relative standard deviation (RSD) and the accuracy as the relative error (RE).

Extraction Recovery The extraction recoveries of VG, VR, VIT, RUT and HP were determined by comparing the peak area of each compound from QC samples ($n=6$) that were at low, middle and high three concentrations with the mean peak areas of those obtained from blank plasma extracts that were spiked post-extraction at the corresponding concentrations

Stability For the assessment of five analytes in plasma after storage, a fresh sample was drawn and separated in five aliquots. Stabilities were assessed by three in house control (short-term, long-term and freeze-thaw stability).

Short-term Stability Five aliquots of QC samples at low, middle and high concentration unextracted QC samples were kept at ambient temperature (25 °C) for 4 h in order to determine the short-term stability of VG, VR, VIT, RUT and HP in rat plasma. Then, the samples were processed and analyzed. The concentrations obtained were compared with the nominal values of QC samples. The samples were initially analyzed within 4 h at ambient temperature (25 °C) of preparation to obtain reference concentrations, consequently, short-term stability had been evaluated.

Long-term Stability Five aliquots each of low, middle and high concentration was determined by analyzing the unextracted QC plasma samples stored at −20°C for 3 months. The samples were processed and analyzed, and then concentrations obtained were compared with the nominal values.

Freeze-thaw Stability The stabilities of plasma samples were determined after three freeze and thaw cycles. In each cycle, the QC samples were stored at −20°C for 24 h and thawed unassisted at room temperature. When completely thawed, the sample was refrozen within 24 h. The cycle was repeated twice and the samples were analyzed after the third cycle. Stability was assessed by comparing the mean concentration of the stored QC samples with the mean concentration of freshly prepared QC samples.

Animals and Pharmacokinetic Study Male Wistar rats (250–300 g) were obtained from the Experimental Animal Center of Liaoning University of Traditional Chinese Medicine (Shenyang, China) and housed in an environmentally controlled breeding room, fed with a standard laboratory food and water *ad libitum* for a week before starting the experiments, subsequently, fasted for 12 h before drug administration, and water was freely available for rats during experiments. Animal experiments were carried out in accordance with the Guidelines for Animal Experimentation of Liaoning University of Traditional Chinese Medicine, and the procedure was approved by the Animal Ethics Committee of this institution.

Three groups (five rats/group) randomly assigned were given administration HLE solution *via* tail vein injection at doses of 1.25, 2.5 and 5 ml/kg (equal to 15.5, 31.0, 62.0 mg/kg of VG, 6.20, 12.4, 24.8 mg/kg of VR, 0.831, 1.66, 3.33 mg/kg of VIT, 0.879, 1.76, 3.52 mg/kg of RUT and 3.35, 6.70, 13.4 mg/kg of HP, respectively). Blood samples (0.4 ml) were collected into heparinized tubes from the orbital sinus at times of 0.05, 0.083, 0.167, 0.5, 0.83, 1.33, 2.0, 3.0, 4.5, 6.5, 9 h after intravenous administration and then centrifuged at 890×*g* for 15 min, immediately. Then, the plasma was transferred into clean test tubes and stored at −20°C until analysis.

Data Aanalysis All data were subsequently processed with the computer program Microsoft Office Excel 2003 and 3p97 (Practical Pharmacokinetic Program, 1997, China) to determine the compartment models and pharmacokinetic parameters.

RESULTS

Method Validation Typical chromatograms obtained from blank plasma, blank plasma spiked with five standard analytes and I.S., and plasma sample obtained at time of 0.5 h after intravenous administration of HLE were shown in Fig. 2, demonstrating that there was no remarkable interference from endogenous components for the analysis of five analytes, and well-acceptable selectivity was obtained by this method. The retention times of VG, VR, VIT, RUT, HP and I.S. were approximately 7.49, 9.69, 13.2, 15.5, 19.0, 21.6 min, respectively. The total run time was 30 min.

All the linear regressions of VG, VR, VIT, RUT and HP in rat plasma displayed good linear relationships over the range of 2.030–500.5, 0.1513–75.64, 0.2507–12.54, 0.5128–25.64 and 0.4032–20.16 μg/ml, respectively. The slope and intercept of the calibration graphs were calculated by weighted ($1/c^2$) least square linear regression. The mean values of regression equation of the analytes in rat plasma were: $y=0.006x+0.0075$ ($r=0.9942$, VG), $y=0.0564x+0.0344$ ($r=0.9946$, VR), $y=0.0641x+0.0012$ ($r=0.9908$, VIT), $y=0.0304x-0.0056$ ($r=0.9917$, RUT), $y=0.0457x-0.0105$ ($r=0.9905$, HP), where y is the peak area ratio of the analyte to I.S., and x referred to the concentration of the analytes in plasma (μg/ml).

The LOQs, defined as the lowest concentration on the calibration curves and determined at a signal-to-noise ratio (S/N>10), were 2.030 μg/ml for VG, 0.1513 μg/ml for VR, 0.2507 μg/ml for VIT, 0.5128 μg/ml for RUT and 0.4032 μg/ml for HP, with both precision and accuracy not exceeding 15%. As to the LOD, the same procedures were performed with con-

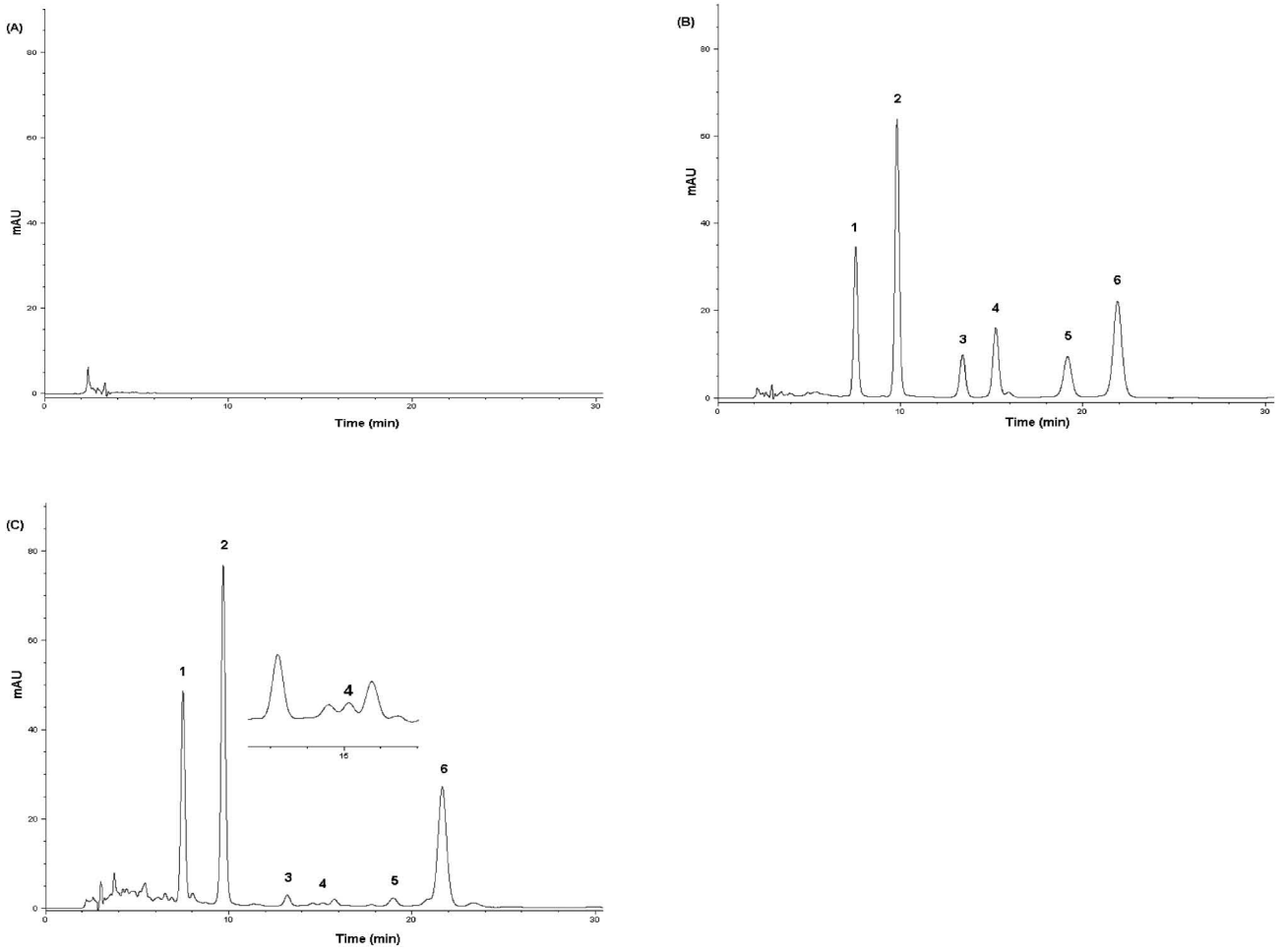

Fig. 2. HPLC Chromatograms

(A) representative chromatograms of blank plasma; (B) plasma spiked with vitexin-4″-*O*-glucoside, vitexin-2″-*O*-rhamnoside, vitexin, rutin, hyperoside and baicalin; (C) plasma sample at 0.5 h after an intravenous administration of HLE solution at dose of 2.5 ml/kg (equivalent to 31.0, 12.4, 1.66, 1.76 and 6.70 mg/kg). Peak 1: vitexin-4″-*O*-glucoside; Peak 2: vitexin-2″-*O*-rhamnoside; Peak 3: vitexin; Peak 4: rutin; Peak 5: hyperoside; Peak 6: baicalin (I.S.).

Table 1. Precision and Accuracy for the Determination

	Added C (μg/ml)	Intra-day			Inter-day		
		Found C (μg /ml)	RSD (%)	RE (%)	Found C (μg/ml)	RSD (%)	RE (%)
VG	6.090	6.213±0.230	3.2	3.4	6.232±0.213	2.1	3.9
	250.2	252.4±2.11	1.0	1.0	252.7±2.95	1.3	1.1
	400.4	406.4±4.68	1.1	1.6	406.4±4.88	1.6	1.6
VR	0.4539	0.4614±0.134	4.9	0.9	0.4639±0.114	4.9	1.8
	37.82	38.03±0.490	1.7	1.4	37.79±0.660	1.7	0.8
	60.51	61.38±1.98	2.0	2.3	61.86±1.28	2.0	1.2
VIT	0.5014	0.5114±0.0209	3.3	2.9	0.5151±0.0173	1.4	3.0
	6.269	6.671±0.252	2.5	0.9	6.634±0.161	2.2	2.1
	10.48	10.82±1.18	7.7	4.2	10.81±0.831	4.3	6.1
RUT	1.519	1.79±0.133	2.8	1.3	1.68±0.0433	1.8	0.8
	12.51	12.82±0.374	2.8	1.4	12.79±0.371	2.1	1.1
	20.51	21.62±0.602	3.5	2.6	20.92±0.780	3.9	4.5
HP	0.9707	1.082±0.133	8.9	8.2	1.080±0.102	7.3	4.7
	10.08	10.36±0.640	5.8	3.6	10.49±0.584	2.7	4.9
	16.13	16.44±0.623	3.7	2.5	16.51±0.680	1.0	2.9

Vitexin-4″-*O*-glucosid (VG), vitexin-2″-*O*-rhamnoside (VR), vitexin (VIT), rutin (RUT) and hyperoside (HP) in rat plasma (intra-day: $n=5$; inter-day: $n=3$ days with 5 replicates per day).

tents of analytes in plasma samples (S/N=3), was 0.4024 μg/ml for VG, 0.03353 μg/ml for VR, 0.08714 μg/ml for VIT, 0.1293 μg/ml for RUT and 0.06631 μg/ml for HP, respectively.

Intra-day and inter-day precision and accuracy were determined by determination of QC samples at three concentrations as described above. Analytical accuracy and precision data are shown in Table 1. The precision of the five analytes at low, middle and high concentrations were below 8.9 and 7.3% for intra-day and inter-day assays, respectively, with RE from 0.8 to 8.2%. The precision (RSD%) determined at each concentration level was required not exceeding 15% and accuracy (RE%) within ±15% of the actual value which conforms to the criteria for the analysis of biological sample according to guidance of USFDA.[16]

The extraction recoveries of VG, VR, VIT, RUT and HP were no less than 82.67±3.94% (Table 2), and that of I.S. was 97.90±4.51%, suggesting that the precision and accuracy of this method were acceptable.

The results of short-term stability, long-term stability and freeze-thaw stability are listed in Table 3, which indicating that no significant degradation occurred during chromatography, extraction and sample storage processes for five analytes plasma samples.

Table 2. Recovery for the Determination

	Added C (μg/ml)	Recovery (%)	RSD (%)
VG	6.090	84.94±3.75	6.3
	250.2	91.07±3.28	3.2
	400.4	93.21±0.78	2.8
VR	0.4539	86.69±2.09	10.6
	37.82	90.64±1.14	4.7
	60.51	96.36±3.72	2.3
VIT	0.5014	86.23±1.35	3.9
	6.269	87.29±2.35	6.7
	10.48	85.99±4.59	3.9
RUT	1.519	82.67±4.74	5.7
	12.51	89.77±2.42	2.6
	20.51	88.37±1.33	3.4
HP	0.9707	85.59±2.07	8.5
	10.08	94.01±1.04	4.9
	16.13	91.47±4.93	3.6

Vitexin-4″-*O*-glucosid (VG), vitexin-2″-*O*-rhamnoside (VR), vitexin (VIT), rutin (RUT) and hyperoside (HP) in rat plasma ($n=6$).

Application to Pharmacokinetic Study Pharmacokinetic data were processed by 3p97 software (The Chinese Society of Mathematical Pharmacology, Beijing, China). The validated method was successfully applied to monitor the concentrations and pharmacokinetic studies of the five compound in rat plasma after intravenous administration of HLE so-

lution with the dose of 1.25, 2.5, 5 ml/kg. The plasma concentration-time profiles of VG, VR, VIT, RUT and HP are shown in Fig. 3. The corresponding results of the pharmacokinetic parameters are shown in Table 4.

Table 3. Stability

	Added C (μg/ml)	Accuracy (%, mean±S.D.)		
		Short-term stability	Long-term stability	Freeze-thaw stability
VG	6.090	98.13±2.03	99.68±3.47	101.6±1.92
	250.2	101.1±3.78	104.8±4.36	102.5±3.25
	400.4	99.76±2.51	97.63±3.42	100.8±2.14
VR	0.4539	101.4±3.69	102.8±1.66	100.4±4.21
	37.82	97.65±3.11	99.86±2.94	101.6±4.74
	60.51	99.86±4.68	96.32±2.98	103.9±2.98
VIT	0.5014	100.6±2.11	104.0±2.45	98.36±4.12
	6.269	102.4±5.01	98.66±3.25	103.8±5.07
	10.48	98.68±2.01	102.4±5.33	101.9±3.53
RUT	1.519	99.55±4.02	95.11±6.01	101.3±2.46
	12.51	102.0±2.06	105.8±4.23	98.65±6.32
	20.51	101.4±2.03	105.8±5.44	102.0±1.97
HP	0.9707	100.7±5.98	96.38±4.11	103.0±2.33
	10.08	107.3±4.36	103.8±2.01	102.4±3.96
	16.13	98.63±2.45	102.0±4.21	100.3±3.28

Vitexin-4″-*O*-glucosid (VG), vitexin-2″-*O*-rhamnoside (VR), vitexin (VIT), rutin (RUT) and hyperoside (HP) in rat plasma at three QC levels (n=5).

DISCUSSION

Method Development In the chromatographic separation, the main attention is not only on the separation within the five analytes, but also that of the analytes with endogenous compounds. The selection of mobile phase components was critical in our experiment because of the existence of other compounds and the analytes with the similarity in polarity and chemical structures. To obtain suitable retention time and good separation for the analysis, the mobile phase was chosen after several trials in various proportions with methanol-acetonitrile-tetrahydrofuran-water (1.5–6 : 1.5 : 18.5 : 78.5–74, v/v/v/v). In addition, to improve the peak shape, 0.5–1% glacial acetic acid was added in, however, only when 1% glacial acetic acid was applied to the mobile phase, a good peak shape and good separation were obtained. Finally, an isocratic elution of methanol-acetonitrile-tetrahydrofuran-1% glacial acetic acid (6 : 1.5 : 18.5 : 74, v/v/v/v) was used.

Gradient elution was usually used for the separation of flavonoids in plant tissues, and suitable to the compounds that are difficult to separate during a short period of run time, and therefore several

Table 4. Pharmacokinetic Parameters

	Dose (mg/kg)	Parameters				
		$T_{1/2\alpha}$ (h)	$T_{1/2\beta}$ (h)	V_c (l/kg)	CL (l/h/kg)	$AUC_{0\to t}$ (mg·h/l)
VG	15.5	0.620±0.031	5.13±0.062	0.105±0.0084	0.0713±0.0012	148.1±4.73
	31.0	0.720±0.071	5.40±0.074	0.103±0.0011	0.0778±0.011	399.8±5.65
	62.0	0.680±0.011	5.55±0.059	0.119±0.010	0.0793±0.0061	1048±11.8
VR	6.20	0.249±0.0038	0.866±0.075	0.114±0.0041	0.349±0.023	17.76±2.26
	12.4	0.467±0.0042	2.69±0.31	0.215±0.0050	0.191±0.0039	64.99±3.00
	24.8	1.17±0.022	6.09±1.34	0.256±0.0065	0.142±0.030	176.2±5.90
VIX	0.831	0.0286±0.0070	0.355±0.029	0.193±0.010	0.930±0.052	0.8934±0.0403
	1.66	0.0520±0.0085	0.331±0.083	0.206±0.011	0.902±0.0053	2.150±0.185
	3.33	0.415±0.0083	2.28±0.62	0.190±0.0028	0.371±0.014	8.970±1.16
RUT	0.879	ND	ND	ND	ND	ND
	1.76	0.0235±0.0014	0.936±0.022	0.0814±0.025	0.370±0.075	1.623±0.424
	3.52	0.0652±0.075	1.07±0.39	0.123±0.031	0.323±0.013	3.484±0.400
HP	3.35	0.0250±0.0074	1.05±0.13	0.0496±0.043	0.252±0.092	1.987±0.022
	6.70	0.298±0.0034	2.22±0.020	0.209±0.0055	0.170±0.033	5.877±1.04
	13.4	0.455±0.020	4.12±1.0	0.122±0.052	0.0668±0.031	29.70±1.00

Vitexin-4″-*O*-glucosid (VG), vitexin-2″-*O*-rhamnoside (VR), vitexin (VIT), rutin (RUT) and hyperoside (HP) in rats (mean±S.D., n=5) after an intravenous administration of HLE solution at dose of 1.25, 2.5 and 5 ml/kg (equivalent to 15.5, 31.0, 62.0 mg/kg of VG, 6.20, 12.40, 24.80 mg/kg of VR, 0.83, 1.66, 3.33 mg/kg of VIT, 0.879, 1.76, 3.52 mg/kg of RUT and 3.35, 6.70, 13.40 mg/kg of HP, respectively). "ND": Not detectable.

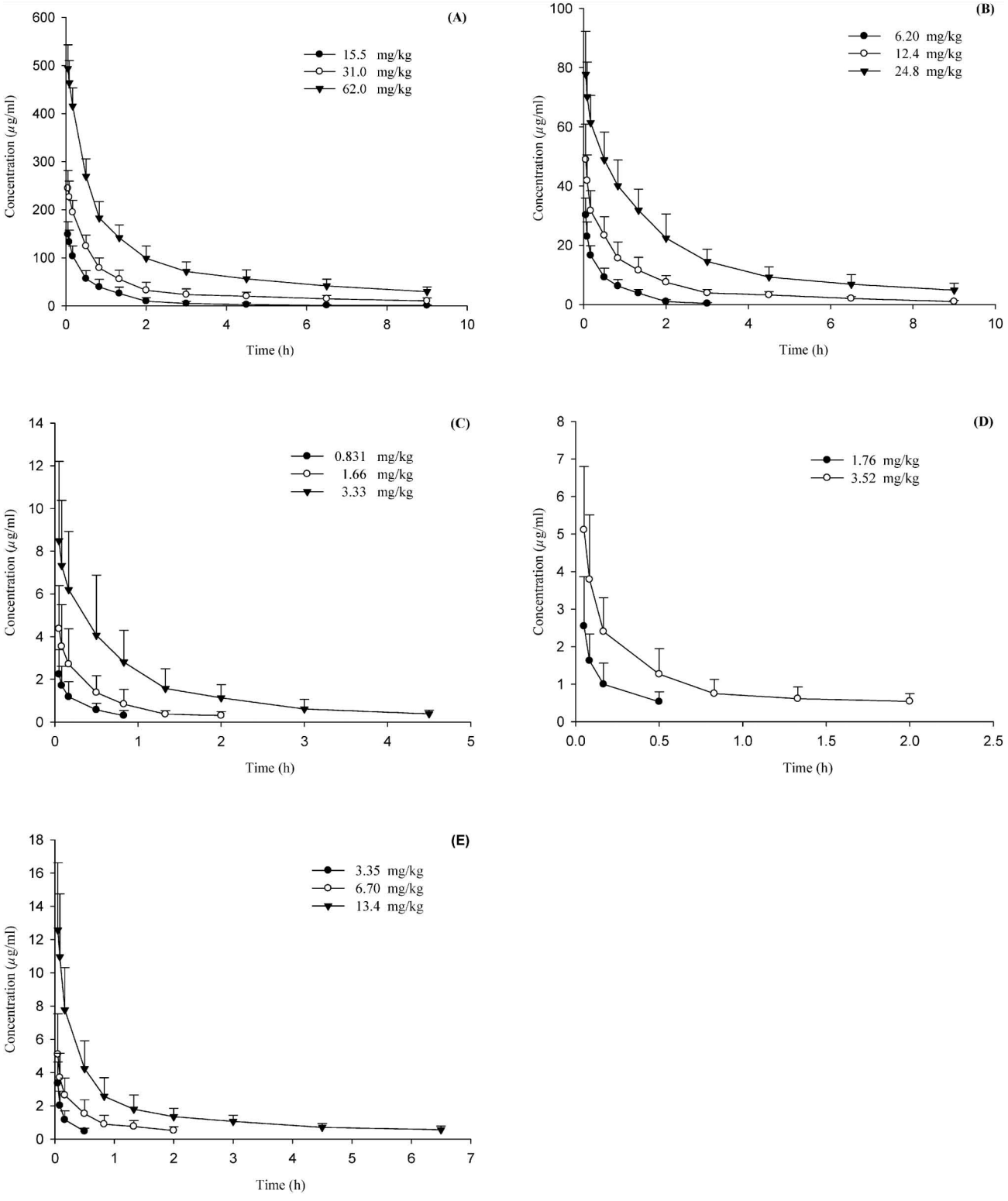

Fig. 3. Pharmacokinetic Curves

(A) mean plasma concentration-time curves of vitexin-4″-*O*-glucoside; (B) vitexin-2″-*O*-rhamnoside; (C) vitexin; (D) rutin, (E) hyperoside in rats (mean±S.D., $n=5$) after an intravenous administration of HLE solution of doses of 1.25, 2.5 and 5 ml/kg, equivalent to 15.5, 31.0, 62.0 mg/kg of VG, 6.20, 12.4, 24.8 mg/kg of VR, 0.831, 1.66, 3.33 mg/kg of VIT, 0.879, 1.76, 3.52 mg/kg of RUT and 3.35, 6.70, 13.4 mg/kg of HP, respectively. These sampling points, which couldn't be detected, i.e., plasma concentration under LOQ, are not included in the mean plasma concentration-time curves.

methods with gradient elution have been tried, according to the literature.[3,17] Nevertheless the methods were unsuccessful for producing a serious baseline drift and retention time shifting. To achieve a complete separation of VG, VR, VIT, RUT, HP and I.S. from the other metabolites, a rapid and isocratic chromatographic procedures were developed and optimized. The peaks of the analytes in the plasma were identified by comparing their retention times with those of the standards. Typical retention times of VG, VR, VIT, RUT, HP and I.S. were 7.49, 9.69, 13.2, 15.5, 19.0, 21.6 min, respectively. The total run time was 30 min.

The UV absorption spectra of VG, VR, VIT, RUT and HP have two maximum absorptions at 270 and 330 nm, 270 and 332 nm, 269 and 331 nm, 257 and 358 nm and 256 and 359 nm, respectively, belonging to the two characteristic absorption regions of flavoinds, and I.S. at 277 and 314 nm. In order to improve the sensitivity of I.S., we tried to select the wavelength of 255 and 277 nm, respectively. The interferences from endogenous substances in the plasma were observed when the wavelength was set at 255 and 277 nm, especially for low concentration analytes when the wavelength was set at 277 nm. To obtain the high sensitivity for each compound, immediately, the wavelength of 330 nm[12] and 360 nm[7] have been considered. I.S. presented a weak absorption when the wavelength was set at 360 nm indicating that it was unsuitable to the determination of the five analytes. When the wavelength was set at 330 nm, both the analytes and the I.S. have the good absorption. Therefore, 332 nm was chosen as the detection wavelength, and proved to be suitable for the assay.

Selection of I.S. To select a suitable internal standard, several components including baicalin, quercitrin and hesperidin having structural or chemical similarity to the analytes, were considered. However, the resolutions between quercitrin and HP, VIT and RUT with hesperidin were unsatisfied under severally chromatographic conditions. Baicalin, being structurally or chemically similar to the analyte and no interference around it in the whole process, finally, was chosen as the internal standard for the assay.

Sample Preparation Considering the chemical features of the five analytes and I.S., several extraction solvents including methanol, acetonitrile, methanol-ethyl acetate in different ratios were tried to precipitate the protein. Finally, methanol was used as the precipitant because of the high extraction recovery and precision. The reason was that methanol was not only advantageous to the protein precipitation but also to the extract of five analytes and I.S. To obtain the higher extraction efficiency, 0, 10, 20, 30 μl of acetic acid were tried and added in the plasma to avoid the dissociation of polyphenols. Eventually, 20 μl of acetic acid was added to the plasma.

Application to Pharmacokinetic Study To obtain more pharmacokinetic information of VG, VR, VIT, RUT and HP, the pharmacokinetic studies after intravenous administration at three different doses were developed, thus the some pharmacokinetic parameters can be elucidated *via* multiple dosing. The weight of $1/c^2$ was chosen by comparing the goodness of fit of three doses. According to F test, AIC and R^2, a three-compartment open model (Weight$=1/c^2$) gave the best fit to the plasma concentration-time curves obtained in rats. After administered with 1.25, 2.5 and 5 ml/kg of HLE solution, area under the plasma concentration-time curve from 0 h to the time of last measurable concentration ($AUC_{0\to t}$) of VG in rats increased in direct proportion with the dosage. The terminal half-life ($t_{1/2\beta}$) had no good correlation with the administered dose, suggesting that the pharmacokinetics of VG in rats obeyed linear processes, and the results conform to the previous reference.[12] Whereas, the $AUC_{0\to t}$ values of VR, VIT, RUT and HP in rats were not proportional to the administered doses. In addition, the terminal half-life ($t_{1/2\beta}$) was longer in higher doses, meaning that the pharmacokinetics of VR, VIT, RUT, and HP in rats obeyed nonlinear processes. The pharmacokinetic characters of HP were in coincidence with the published reports,[15] while VR presented a different pharmacokinetic process comparing the previous literature.[8] This could be induced partly by the metabolic enzyme of drug or carrier of drug membrane-permeable process saturated in high concentration or an administration of a large dose that could lead to retardation of the drug elimination and prolongation of its effect,[18] and for VIT, the mechanism was similar. RUT obeyed nonlinear processes that could be related to tissue protein-binding displacement.[19]

VG and VR with higher concentration in HLE solution could be detected up to 9 h after intravenous administration, whereas VIT and HP were detectable only up to 0.83 h in low dosage, 4.5 h in high dosage in rats, and RUT were no found in low dosage and de-

tectable only up to 2 h in high dosage in rats, and the reason was that RUT probably underwent conjugation or hydrolysis *in vivo*.[19] Furthermore, VIT and RUT were eliminated most rapidly in high dosage with $t_{1/2\beta}$ at 2.283 and 1.065 h among the five tested compounds, and the CL for VIT and RUT were much higher than that of VG, VR and HP, indicating that VIT and RUT were transported quickly from blood into tissues and organs, and eliminated from blood rapidly.[20]

In this study, the HLE, compared with pure compounds of VR and HP, presented a relatively long half-time of elimination phase (6.09 *vs* 0.941 h, 4.12 *vs* 1.15 h) and about 2–3 fold enhancement of $AUC_{0\rightarrow t}$ (176.2 *vs* 74.48 mg·h/l, 29.70 *vs* 9.603 mg·h/l) after administrating the nearly same dosages.[8,15] The above results indicated that the pharmacokinetic process of VR and HP in HLE were different from pure compound in rats, and the elimination postponed and $AUC_{0\rightarrow t}$ increased, which prolonged the potency in the *in vivo*. The differences in the pharmacokinetic parameters between HLE and pure compounds were maybe caused by the co-occurring components in HLE which could have inhibited the metabolic clearances or transporters. Hence, the compounds in HLE might play an important role in affecting the elimination of each analyte in rat plasma. In a word, this investigation was helpful to better understand the pharmacokinetic profile of HLE in rat after an intravenous administration of HLE at different doses, and further studies are needed in the future for fully understanding its action mechanism and efficacy.

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