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Simultaneous determination of three flavonoid *C*-glycosides in mice biosamples by HPLC–ESI-MS method after oral administration of *Abrus mollis* extract and its application to biodistribution studies

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ABSTRACT

A simple HPLC–ESI-MS method was developed for the determination of vicenin-2 (**1**), isoschaftoside (**2**), and schaftoside (**3**) of *Abrus mollis* extract in mice plasma and tissues (heart, liver, spleen, lungs, and kidneys). The separation was achieved by HPLC on a Shim-Pack CLC-ODS column with a mobile phase composed of 0.1% formic acid (mobile phase A, 72%) and methanol–isopropanol (9:1) (mobile phase B, 28%). The electrospray source of the MS was operated in the selective ion monitoring (SIM) mode at *m/z* 593 ($[M-H]^-$) for **1**, and 563 ($[M-H]^-$) for **2** and **3**, respectively. The limit of detection (LOD) of **1**–**3** was in the range of 8.5–12.6 ng/mL for plasma, and 32.5–49.4 ng/g for tissue tested. The limit of quantification (LOQ) was found to be 25 ng/mL for plasma, and 100 ng/g for tissue. The calibration curves were linear in all matrices ($r^2 > 0.994$) in the concentration range of 25–500 ng/mL in plasma or 100–1250 ng/g in tissue, respectively. Intra-day and inter-day precision studies demonstrate that the method is precise with coefficients of variation intra-day and inter-day below 5.9 and 7.4% for all the samples, respectively. The recoveries of three flavonoid *C*-glycosides ranged from 95.3 to 106.4% for plasma, and 92.6 to 107.3% for tissues. Following oral administration of *A. mollis* extract to mice at a dose of 72 mg/kg, the concentrations of **1**–**3** in plasma and tissues were quantifiable in bio-samples collected up to 180 min. The method described is suitable for studies on the distribution of three flavonoid *C*-glycosides of *A. mollis* extract in plasma and different tissues of mice.

1. Introduction

Abrus mollis, as a member of the family of leguminous, is a commonly used traditional Chinese medicine (TCM) in south of China with obvious bioactivities including heat-clearing, dampness-removing, food retention removing and summer-heat relieving according to records of Chinese ancient book "Ling Nan Cai Yao Lu" [1]. Modern pharmacological researches show *A. mollis* could improve microcirculation, significantly promote blood circulation, remove blood stasis in frog mesenteric microcirculation model with 1–1.5 g/mL of water extraction [2], and increase serum superoxide dismutase content, reduce malondialdehyde content, reduce the degree of rat liver fibrosis induced by subcutaneous injection of 40% CCl_4 with intragastric administration of 0.5–2 mg/kg of *A. mollis* capsula [3]. The aerial parts of *A. mollis* are main herb source of some well-known Chinese patent medicines, such as "Jigucao Capsules", for treating acute and chronic hepatitis, and cholecystitis diseases in China [4]. The major bioactive compounds of *A. mollis* were 3 flavonoid *C*-glycosides, including vicenin-2 (apigenin-6, 8-di-*C*-β-D-glucopyranoside, **1**), isoschaftoside (apigenin 6-*C*-α-L-arabinopyranosyl-8-*C*-β-D-glucopyranoside, **2**), and schaftoside (apigenin 6-*C*-β-D-glucopyranosyl-8-*C*-α-L-arabinopyranoside, **3**) (Fig. 1). It was reported that the flavonoid *C*-glycosides possesses the biological activity of antioxidant, antiflammatory, and antiplatelet [5–7]. In our previous studies, compounds **1**–**3** isolated from *A. mollis* show potent hepatoprotective activities evaluated their effects on carbon tetrachloride (CCl_4), Bacillus Calmette–Guerin (BCG) + lipopolysaccharide (LPS), and ethanol induced hepatocytes damage *in vitro* and may be developed as a

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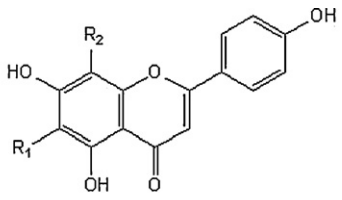

1: R_1 = Glc, R_2 = Glc; **2**: R_1 =Ara, R_2 = Glc; **3**: R_1 = Glc, R_2 = Ara;

Fig. 1. Structure of three flavonoid *C*-glycosides from *A. mollis*. **1**: Vicenin-2, **2**: isoschaftoside, and **3**: schaftoside.

new natural drug for the prevention and treatment of hepatitis and alcoholic liver disease [8–10].

Although *A. mollis* has been applied for hundreds of years in clinic and proven to be very effective against diseases of liver system, until just recently, the pharmacokinetics and biodistribution of active components in blood and tissues were unknown. Due to the sparse data for individual active chemical component, high polar property, complex chemical nature and similar molecular structure of flavonoids in *A. mollis* (especially **2** and **3**), there are only a few methods available to determine total flavonoid contents by UV colorimetric method [11,12] and no reports on quantitative method of single flavonoid *C*-glycosides of *A. mollis in vitro* and *in vivo*. The objective of the present study, therefore, is to develop and validate a method to assay three bioactive flavonoid *C*-glycosides of *A. mollis* extract in mice plasma and tissues that its applicable to routine pharmacokinetic and tissue distribution studies by HPLC–MS equipped with an electro-spray ionization (ESI) mode.

2. Experimental

2.1. Materials

Medicinal raw material sample of *A. mollis* was provided and identified by Senior Engineer Wenzhi Ye, Guangxi Yulin Pharmaceutical Co., Ltd. Three reference flavonoid *C*-glycosides including vicenin-2, isoschaftoside and schaftoside, were isolated from *A. mollis* in our lab and identified based on ^{1}H, ^{13}C NMR and MS spectroscopy and comparisons with literature data [5,8]. The purity of each reference was determined to be over 98% by normalization of the peak area detected by HPLC-UV.

HPLC-grade methanol, isopropanol (Merck, Germany) and formic acid (Appli Chem, Germany) were used for HPLC–ESI-MS analysis. Medicinal alcohol was used for medicinal material extract preparation. Other chemicals were of analytical grade and purchased from Nanjing Chemical Reagent Co., Ltd.

2.2. HPLC–ESI-MS analysis

The analyses were performed on a Shimadzu 2020 LCMS single quadrupole system (Shimadzu Corp., Japan), incorporating a photodiode array detector coupled directly to an electrospray ionization source and a single quadrupole mass analyser. Chromatographic separations were performed on a Shim-Pack CLC-ODS column (250 mm × 4.6 mm, 5 μm, Shimadzu Corp., Japan) protected by a Shim-Pack CLC-ODS guard column (10 mm × 4.6 mm, 5 μm, Shimadzu Corp., Japan) at a column temperature of 30 °C. The injection volume was 10 μL. The isocratic elution was employed using 0.1% acetic acid (mobile phase A) and methanol–isopropanol mixture (9:1, V/V, mobile phase B), and eluted at the ration of 72:28 at the flow rate of 1 mL/min. The column effluent passed through a diode array detector before arriving in the MS interface. The outlet from column was split, and only 0.2 mL/min was delivered in to ESI source. The ion source was operated in negative mode and the mass selective detector (MSD) was operated in selective ion monitoring (SIM) mode at *m/z* 593 ([M−H]$^-$) for **1** and 563 ([M−H]$^-$) for **2** and **3**, respectively, which have been proven by ^{1}H, ^{13}C NMR, and MS spectroscopic analysis in Ref. [8]. The interface conditions were as follows: DL (desolvation line) temperature of 250 °C, block temperature of 200 °C. Interface voltage and Q-array RF (radiation frequency) voltage were according to tuning file. DL voltage and Q-array DC voltage were according to default values by autotuning. The nebulizing gas flow rate was 1.5 L/min, the drying gas flow rate was 15 L/min. Three flavonoid *C*-glycosides were quantified by external standard method.

2.3. Preparation of standard solutions

Mixed stock standard solution (0.1 mg/mL for **1**–**3**) was prepared by weighing out the appropriate amount of **1**–**3** and dissolving them by ultrasonication in methanol. Working standard solutions were then prepared by serial dilution of the mixed stock solution with methanol to obtain the desired concentration range. Plasma or tissue (heart, liver, spleen, lungs and kidneys) calibration standards were prepared by spiking working standard solution into blank mice plasma or 10% (w/v, in normal saline) tissue homogenates to obtain final each standard concentrations of 25, 50, 100, 125, 250, 500 ng/mL in plasma and 100, 250, 500, 750, 1000, 1250 ng/g in tissue. Quality control (QC) samples (50, 125, 500 ng/mL in plasma and 100, 500, 1250 ng/mL in tissue) were prepared in a similar manner with appropriate working standard solutions. All standards and QCs were stored at 4 °C and were brought to room temperature before use.

2.4. Sample preparation

The method of sample preparation in this paper was protein precipitation by methanol *in situ*. In detail, calibration standards, QC, plasma and tissue samples were performed by mixing 100 μL of plasma, 10% tissue homogenates or standards solution with 200 μL of methanol, followed by 5 min vortex (Shanghai Medical University Instrument Plant, Shanghai, China). Then, the samples were centrifuged for 10 min at 10,000 × *g* at 4 °C (Refrigerated Centrifuge 3K30, Sigma, German). A 10 μL aliquot of the supernatant fluid was injected into HPLC–MS for assay.

2.5. Method validation

The specificity was investigated by comparison of the chromatograms for blank bio-samples and bio-samples spiked with mixed standards. Specificity was established by the lack of interfering peaks at the retention time for **1**–**3**.

Several HPLC–MS experiments in SIM mode were performed in order to evaluate the matrix effect. Several blank biosamples prepared from different lots of plasma and tissues preparations were checked under the described chromatographic conditions. The matrix effect was evaluated on the three QCs levels according previously reported papers [13–15]. Matrix effects for the three compounds were determined and calculated by the ratio of the response of methanol precipitated-blank biosamples spiked with mixed stock standard solution over the response of corresponding mixed standard samples prepared by dilution in methanol.

Linearity was tested at six different concentrations in sextuplicate, covering a range of 25–500 ng/mL in plasma and 100–1250 ng/g in tissue for each standard. The calibration curves were calculated by linear regression of the peak area *versus* concentration.

The limit of detection (LOD) under the present chromatographic conditions was defined as the amount of each standard that resulted in a peak of approximately 3 times the noise level (S/N = 3)

Table 1
Calibration curves and LOD of **1**–**3** in plasma, heart, liver, spleen, lungs and kidneys.

Sample matrix	Analyte[a]	Calibration curve[b]	Regression coefficient (r^2)	LOD[c] (ng/mL or g)
Plasma	**1**	$Y = 141.37X - 680.4$	0.9949	8.5
	2	$Y = 124.74X - 1274.5$	0.9990	9.1
	3	$Y = 103.04X - 1483.9$	0.9965	12.6
Heart	**1**	$Y = 38.467X + 1041.4$	0.9979	32.5
	2	$Y = 25.191X + 2644.2$	0.9971	39.3
	3	$Y = 19.694X - 1647.2$	0.9974	42.3
Liver	**1**	$Y = 31.787X + 3189.6$	0.9960	34.5
	2	$Y = 21.815X + 2250.9$	0.9978	51.4
	3	$Y = 21.663X + 221.0$	0.9963	45.9
Spleen	**1**	$Y = 35.780X + 3241.7$	0.9975	42.1
	2	$Y = 28.245X - 274.3$	0.9953	38.3
	3	$Y = 27.274X - 243.0$	0.9966	39.5
Lungs	**1**	$Y = 28.742X + 5494.5$	0.9986	35.3
	2	$Y = 22.768X + 2805.8$	0.9996	44.6
	3	$Y = 13.249X - 74.300$	0.9987	49.4
Kidneys	**1**	$Y = 33.828X + 2845.1$	0.9996	32.7
	2	$Y = 28.283X + 407.4$	0.9982	36.3
	3	$Y = 29.115X + 241.3$	0.9980	34.3

[a] The notation of analyte refers to Fig. 1.
[b] Y and X stand for logarithmic values of peak area and concentration (ng/mL or g), respectively.
[c] Limit of detection (S/N = 3).

by diluting the standard solution. The limit of quantification (LOQ) was assessed as the lowest concentration on the calibration curve that can be quantitatively determined within ±20% accuracy and precision. The LOQ was established based on five replicates on the three consecutive days.

Intra- and inter-day variations were chosen to determine the precision of the developed method. For intra-day variability test, QC samples (50, 125, 500 ng/mL in plasma and 100, 500, 1250 ng/g in tissue) were analyzed in triplicates within one day, whereas for inter-day variability test, QC samples were examined in triplicate for consecutive 5 days. Variations of the peak area were taken as the measures of precision and expressed as percentage relative standard deviations (RSD).

The accuracy of this analytical method was evaluated by analyzing recovery percentages using calibration standards and QC samples in triplicates. Recoveries were calculated by using the ratio of observed concentration to added concentration.

Process stability was assessed by preparing triplicate sets of plasma or tissue homogenate control samples. The disposed samples were run immediately after preparation and again after 6 h of storage at room temperature. Storage stability of **1**–**3** was tested with spiked plasma or tissue homogenates samples stored at −20 °C. They were assayed in triplicates on the day of preparation (baseline) and thereafter at 1 month and 3 months of storage. Stability was also tested by subjecting plasma or tissues control samples to three freeze (−20 °C)–thaw (ambient) cycles.

2.6. Preparation of A. mollis extract

The dried aerial parts of *A. mollis* were grounded into powder. The powered herbs (250 g) were extracted with 2500 mL of 70% ethanol solution under reflux for 2 h, repeated two times. The filtrates were combined and evaporated under 60 °C in vacuum to remove ethanol solvent and yield a residue about 50 g, then the residue was suspended in 250 mL of water and filtered. The solution was chromatographed on a glass column packed with HPD-100 macroporous resin (250 mL), eluted with H_2O (750 mL), 20% aqueous ethanol (750 mL), and 50% aqueous ethanol (1000 mL), successively. The 50% aqueous ethanol elution were collected and evaporated under 60 °C in vacuum, the extracts of *A. mollis* (7.5 g) were obtained. The contents of **1**–**3** in the *A. mollis* extracts were 15.5%, 21.7% and 12.1%, respectively, by means of HPLC-UV methods.

2.7. Application

Kunming mice (male, 8 weeks old, 18–22 g) were purchased from the Experimental Animal Center of China Pharmaceutical University. They had free access to water but were deprived of food for 12 h prior to drug administration. In order to study the pharmacokinetics and tissue distribution of **1**–**3** after administration of *A. mollis* extract, mice ($n = 4$) were orally administered extract solution at a dose of 72 mg/kg. At 15, 30, 60, 90, 120 and 180 min after administration, four animals were sacrificed under ether anesthesia. Blood was collected in heparin-coated tubes and centrifuged at $1000 \times g$ for 5 min. Heart, liver, spleen, lungs, kidneys were removed, weighed and homogenized (10%, w/v) in normal saline solution. All samples were immediately frozen at −20 °C until analysis. Pharmacokinetic parameters for three flavonoid *C*-glycosides were determined from the concentration–time data. A log-linear trapezoidal method 3P97 (Mathematic Pharmacological Committee, Chinese Pharmacological Society, China) was used for the fitting of pharmacokinetic models based on the lowest Akaike's information criterion (AIC) value. The weighing factor was 1.

3. Results and discussion

3.1. Chromatography

To determine the primary ion species for **1**–**3**, negative product ion mass spectra were obtained (Fig. 2) using the corresponding standards. The spectra indicated that the primary ion for **1** at *m/z* 593 ($[M-H]^-$), **2** at *m/z* 563 ($[M-H]^-$) and **3** at *m/z* 563 ($[M-H]^-$), respectively. The ion intensity in negative ion mode was greater than that in positive mode. Therefore, these primary negative ions for the compounds were used in the subsequent analysis.

3.2. Method validation

The chromatograms were free of interference of other compounds after the protein precipitation of plasma and tissue homogenates. The peaks of **1**–**3** were symmetrical and baseline

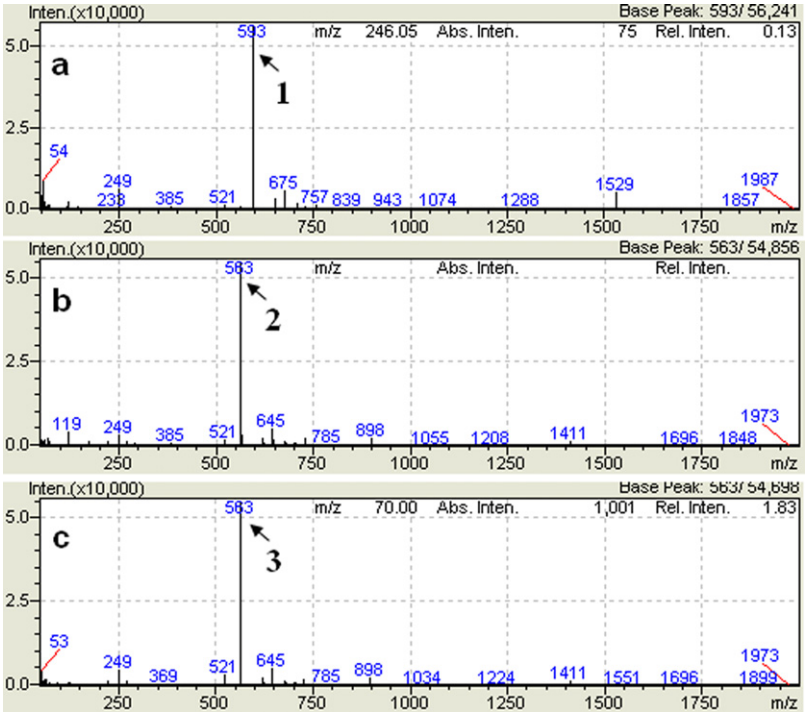

Fig. 2. Negative product ion mass spectra of (a) **1** at *m/z* 593 ([M−H]⁻), (b) **2** at *m/z* 563 ([M−H]⁻) and (c) **3** at *m/z* 563 ([M−H]⁻).

separation was obtained in all matrices (chromatograms of plasma and liver as representative figures in Fig. 3). Blank bio-samples contained no substances that interfere with **1**–**3** elutions at *m/z* 593 and 563. The quality analyzer of LCMS instrument used in this paper is a single quadrupole mass spectrometer. Stable fragment ions could not be produced by increasing collision-induced dissociation (CID) voltage. In addition, the molecular ions at *m/z* 593 ([M−H]⁻) for **1** and 563 ([M−H]⁻) for **2** and **3** have been proven by ^{1}H, ^{13}C NMR,

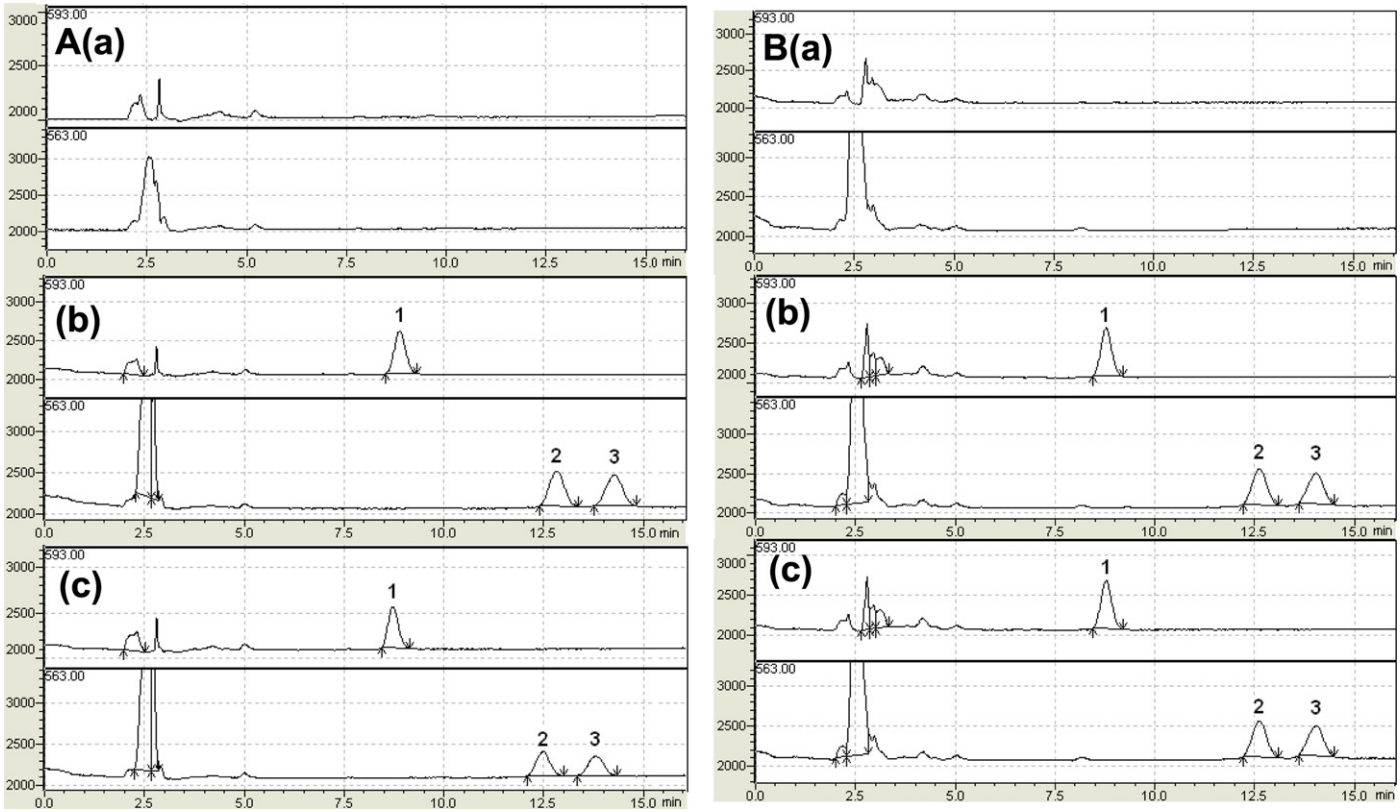

Fig. 3. Representative HPLC–ESI-MS chromatograms of a blank bio-sample (a), blank bio-sample spiked with **1**–**3** (b) and bio-sample at 90 min after oral administration of *Abrus mollis* extract at a dose of 72 mg/kg in mice (c). A: plasma; B: liver. **1**: Vicenin-2, **2**: isoschaftoside, and **3**: schaftoside.

Table 2
Precision and accuracy data of **1**–**3** in plasma, heart, liver, spleen, lungs and kidneys.

Sample matrix	Concentration (ng/mL or g)	Recovery[a] (%)			Precision[b] (%)					
					Intra-day			Inter-day		
		1	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Plasma	50	95.3	96.7	98.9	3.2	4.6	5.4	4.6	6.5	4.3
	125	97.9	106.4	101.9	5.2	2.6	3.3	6.3	5.7	5.3
	500	98.0	103.5	96.4	3.6	3.2	2.2	4.2	6.9	4.2
Heart	100	93.5	99.3	102.2	4.1	3.7	4.3	5.9	7.2	7.1
	500	104.2	98.2	94.6	2.8	1.5	1.8	6.1	5.1	4.5
	1250	102.3	101.3	95.6	1.1	2.5	3.1	5.0	4.2	5.9
Liver	100	99.2	107.3	96.4	4.2	3.7	4.8	6.5	3.9	6.1
	500	100.1	103.2	102.7	2.5	3.5	2.1	5.4	3.3	3.4
	1250	96.8	95.1	101.3	2.2	1.0	2.0	7.4	5.7	3.4
Spleen	100	97.4	95.7	104.2	5.2	3.6	5.9	5.1	7.1	5.7
	500	103.7	97.9	95.2	2.6	4.1	2.7	4.4	4.5	3.5
	1250	104.2	95.2	103.8	0.9	1.3	2.6	3.2	3.9	4.1
Lungs	100	92.6	95.2	98.5	3.6	3.1	2.9	6.7	4.8	6.6
	500	103.2	101.6	98.6	2.7	3.1	3.8	5.8	5.2	5.7
	1250	102.4	103.5	100.2	1.5	2.3	2.2	4.3	3.2	3.0
Kidneys	100	96.3	97.2	101.3	3.2	4.6	5.3	5.4	5.6	5.4
	500	102.3	95.7	96.1	3.1	2.9	1.8	6.8	3.9	3.7
	1250	96.7	102.1	104.3	2.6	0.8	1.1	7.1	4.6	3.2

[a] Recovery = (observed in sample)/(added in sample).
[b] Coefficient of variation.

and MS spectroscopic analysis in our previous research [8]. Therefore, the mass selective detector (MSD) was operated in SIM mode and the fragment ions have not been monitored.

The matrix effect for the three analyzed compounds was evaluated in this paper by the ration of response of spiked biosamples to that of corresponding methanolic samples. No significant matrix effect was observed in control disposed biosamples medium with the resulted ratios ranged from 1.03 to 1.14 in plasma, from 1.01 to 1.21 in tissues, and showed less than 20% variation.

The calibration curves for **1**–**3** in bio-samples were found to be linear over a concentrations range of 25–500 ng/mL in plasma or 100–1250 ng/g in tissue (Table 1). The regression coefficients (r^2) were greater than 0.994. The LOD (S/N = 3) of **1**–**3** was in the range of 8.5–12.6 ng/mL for plasma and 32.5–49.4 ng/g for tissue tested. The lowest concentration with RSD < ±20% was taken as LOQ and was found to be 25 ng/mL for plasma and 100 ng/g for tissue. Accuracy and intra-day and inter-day precision were determined to evaluate reliability of the analytical method. The results are summarized in Table 2. The validation data of the sample preparation and HPLC–ESI-MS procedure in plasma and different tissues demonstrate that the method is accurate and precise with coefficients of variation intra-day and inter-day below 5.9 and 7.4% for all the samples, respectively. The recoveries of three flavonoid *C*-glycosides ranged from 95.3 to 106.4% for plasma and 92.6 to 107.3% for tissue.

The mean values for accuracies were within 4.7% of their expected values, and % variation was less than 5.1 for all the samples, following storage of the compounds in methanol at room temperature for 6 h, indicating no significant decrease in the concentrations for these compounds under the given conditions. These compounds were stable in plasma or tissues for at least three months when stored at −20 °C. Mean accuracies were within 4.6% of the expected values, and % variation was <5.9 and 6.4 respectively, for 1 and 3 months of storage. Thawing and refreezing had no significant effect on accuracy and precision of the results: The mean observed values were within 4.5% of the expected values, and % variations were within 6.9 for the assayed concentration of plasma or tissues.

Several mobile phases have been tested to separate these three flavonoid *C*-glycosides, such as water–methanol, water–acetonitrile, 0.1% acetic acid–methanol and 0.1% acetic acid–acetonitrile. Due to the complex chemical nature and similar molecular structure of flavonoids in *A. mollis*, the elution time it takes for **1**–**3** to come out of the HPLC column separately must be no shorter than 15 min under the step-gradient elution condition used in this paper. With regard to bio-samples disposition, a liquid-phase extraction procedure could not be applied due to the good water solubility of flavonoid *C*-glycosides. Therefore, the use of methanol for deproteinisation was a good choice. Previously a HPLC–MS–MS method for analysis of vicenin-2 in rat plasma following intraperitoneal administration of Lychnophora extract has been published [16]. The method for determination of single flavonoid *C*-glycoside was hampered by the involved and laborious solid-phase extraction procedures required for sample clean-up. From this point of view, the proposed method has the advantage that is a simple, specific and reliable analytical technique to determine simultaneously three flavonoid *C*-glycosides in biological samples by HPLC–ESI-MS, both in plasma and in tissues such as heart, liver, spleen, lungs and kidneys.

3.3. Application

The mean plasma concentration *versus* time profile of three flavonoid *C*-glycosides following an oral administration of *A. mollis* extract is shown in Fig. 4. All curves of plasma concentration *versus* time fitted one-compartment open model with first order rate by simulation, statistical analysis, curve fitting and graph plotting. Area under curve ($AUC_{0\rightarrow\infty}$) and mean retention time ($MRT_{0\rightarrow\infty}$) between time 0 and the infinite were calculated using non-compartmental model of 3p97 software. The pharmacokinetic parameters of **1**–**3** are reported in Table 3. Each of three flavonoid *C*-glycosides was absorbed rapidly and had a short elimination half-life ($t_{1/2(ke)}$ = 69.91, 71.87 and 65.54 min for **1**–**3**,

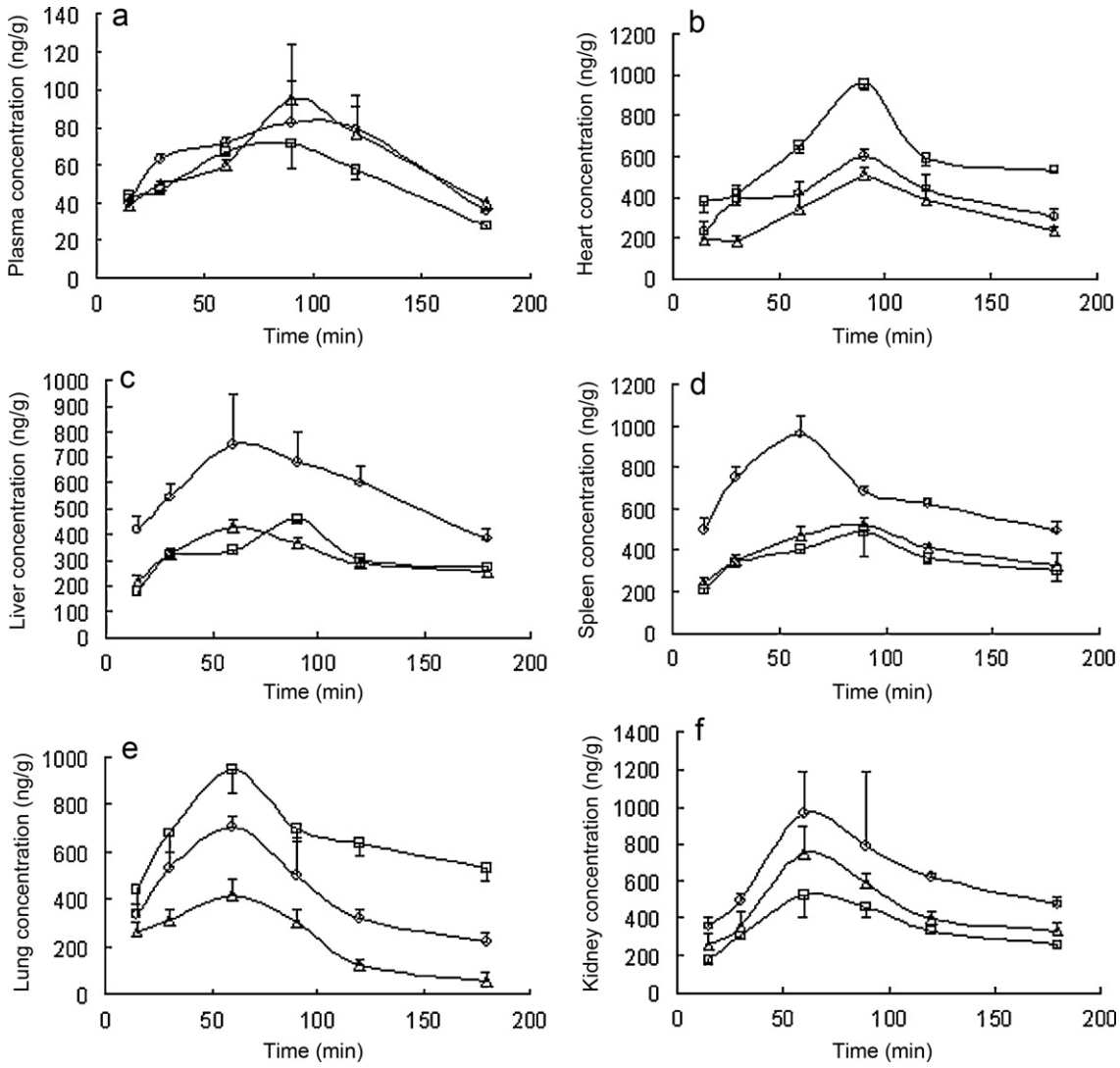

Fig. 4. Mean concentration–time curves of **1** (△), **2** (○) and **3** (□) in bio-samples of mice following an oral administration of *A. mollis* extract at a dose of 72 mg/kg in mice ($n = 4$). (a) Plasma; (b) heart; (c) liver; (d) spleen; (e) lungs; and (f) kidneys. **1**: Vicenin-2, **2**: isoschaftoside, and **3**: schaftoside.

respectively) and rapid elimination rate (Cl_s = 14.00, 19.82 and 14.15 mL/min for **1**–**3**, respectively) from the plasma. Due to the different oral dosage, C_{max}/dosage and $AUC_{0\to\infty}$/dosage were adopted to compare the ability of three flavonoid *C*-glycosides absorbed into plasma after oral administration. The C_{max}/dosage values were 7.16, 5.12 and 8.56 (ng/mL)/(mg/kg) for **1**–**3**, respectively. And the $AUC_{0\to\infty}$/dosage values were 1.32, 0.94 and 1.34 (μg min/mL)/(mg/kg) for **1**–**3**, respectively. Amount and maximum concentration of **1** and **3** absorbed into the systemic blood circulation per dosage after oral administration were similar. Although chemical structures of these three flavonoid *C*-glycosides were similar, the ability of **2** absorbed into plasma was lower than **1** and **3**, and the elimination rate of **2** from plasma was fastest.

Table 3
Pharmacokinetic parameters of three flavonoid *C*-glycosides following an oral administration of *A. mollis* extract at a dose of 72 mg/kg in mice ($n = 4$).

Parameters	Analyte		
	1	**2**	**3**
K_e (1/min)	0.0099	0.0096	0.0106
K_a (1/min)	0.0206	0.0220	0.0309
$t_{1/2(k\alpha)}$ (min)	33.67	31.45	22.42
$t_{1/2(ke)}$ (min)	69.91	71.87	65.54
T_{max} (min)	68.46	66.68	52.74
C_{max} (ng/mL)	79.88	79.93	74.58
Cl_s (mL/min)	14.00	19.82	14.15
$AUC_{0\to\infty}$ (μg min/mL)	14.77	14.67	11.68
$MRT_{0\to\infty}$ (min)	145.90	137.86	132.72

K_e, elimination rate constant; K_a, absorption rate constant; $t_{1/2(k\alpha)}$, absorption half-life; $t_{1/2(ke)}$, elimination half-life; T_{max}, time of maximum concentration; C_{max}, maximum concentration; Cl_s, oral clearance from central compartment; $AUC_{0\to\infty}$, the area under curve between time 0 and the infinite; $MRT_{0\to\infty}$, the mean retention time between time 0 and the infinite.

Table 4
Tissue distribution of three flavonoid *C*-glycosides following an oral administration of *A. mollis* extract at a dose of 72 mg/kg in mice ($n = 4$).

Sample	C_{max} (ng/mL)			$AUC_{0\to\infty}$ (μg min/mL)			$MRT_{0\to\infty}$ (min)		
	1	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Heart	396.44	498.87	781.75	84.55	110.79	196.85	166.66	173.57	220.50
Liver	434.10	728.24	387.33	112.10	185.32	106.60	255.27	268.03	226.74
Spleen	495.63	971.64	440.71	130.85	253.17	125.10	226.71	292.78	241.87
Lungs	439.19	885.84	949.74	41.41	97.48	298.01	85.57	132.37	357.54
Kidneys	115.76	180.24	95.68	127.01	190.06	102.18	181.17	202.50	191.21

The C_{max}, $AUC_{0\to\infty}$ and $MRT_{0\to\infty}$ of different tissues are listed in Table 4. The C_{max} of **1**–**3** in kidneys were lower than that of other tissue. Lower renal toxicity of *A. mollis* extract maybe expected in consideration of the lowest C_{max} of **1**–**3** in kidneys. The AUC of **1** in the spleen was the highest, then the AUC in the kidneys, liver, heart and lungs decreased gradually. The AUC of **2** in different tissue exhibit approximately the same distribution pattern. The AUC of **3** in the lungs was the highest, then the AUC in the heart, spleen, liver and kidneys decreased gradually. The $MRT_{0\to\infty}$ of **1**–**3** in liver were 226.74–268.03 min, indicating long-lasting therapeutic effect on liver disease maybe expected.

4. Conclusion

An HPLC–ESI-MS assay method was developed and validated for the determination of three flavonoid *C*-glycosides of *A. mollis* extract in biological samples including plasma and tissues. Advantages of the method presented in this paper are simple sample preparation and simultaneous determination of three compounds with similar molecular structure. In addition, the sensitivity, selectivity, linearity, accuracy and precision of the assay were adequate, as well as the stability of these three compounds during storage, disposition and assay of samples. This method is applicable for studies on the distribution of three flavonoid *C*-glycosides in plasma and different tissues if mice.

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