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Identification and determination of major flavonoids in rat serum by HPLC–UV and HPLC–MS methods following oral administration of *Dalbergia odorifera* extract

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Abstract

The major flavonoids in rat serum after oral administration of *Dalbergia odorifera* extract were analyzed qualitatively and quantitatively by high performance liquid chromatography (HPLC) and its coupling to mass spectrometry (HPLC–MS). Utilizing HPLC–MS technique, 18 flavonoids including five isoflavones, four isoflavanones, four neoflavones, two flavanones, two chalcones, one isoflavanonol were identified in free form in serum sample based on comparison with the authentic standards. Furthermore, the amounts of the four prominent flavonoids, (3*R*)-4′-methoxy-2′,3,7-trihydroxyisoflavanone, vestitone, formononetin and sativanone were determined in serum by HPLC–UV with internal standard method. The method was validated and utilized in pharmacokinetic studies of these four analytes. This is the first report on identification and determination of the major flavonoids in rat serum after oral administration of *D. odorifera* extract and the results provided a firm basis for clarifying the pharmacological effect of *D. odorifera* and evaluating the clinical applications of this medicinal herb.

Keywords: *Dalbergia odorifera*; Flavonoids; (3*R*)-4′-methoxy-2′,3,7-trihydroxy-isoflavanone; Vestitone; Formononetin; Sativanone

1. Introduction

Flavonoids are polyphenolic substances isolated from a wide range of plants with many health-related properties, which are especially based on their antioxidant activity and inhibitory role in various stages of tumor development [1,2]. Recently, much attention has been paid on the absorption and metabolism of flavonoids. By far, high performance liquid chromatography (HPLC) and its coupling to mass spectrometry (HPLC–MS) have grown into the most widely employed techniques for the measurement of flavonoids in biological fluids as well as in plant materials because of their high level of sensitivity and selectivity. A number of papers dealing with studies on the metabolism of flavonoids by these techniques have been published [3–17].

The heartwood of *Dalbergia odorifera* T. Chen. (Leguminosae) is a traditional Chinese medicine, named as Jiangxiang in Chinese [18]. As a widely used medicinal herb to treat cardiovascular diseases, *D. odorifera* is known to be rich in flavonoids [19–21], while little work has been done on the metabolism of the flavonoids of *D. odorifera*. In our previous work, we purified 22 flavonoids including 6 isoflavones, 5 neoflavones, 4 isoflavanones, 3 flavanones, 2 chalcones, 1 isoflavanonol and 1 pterocarpan from *D. odorifera*, and their structures were listed in Fig. 1. These flavonoids showed various in vitro benefits to human health, such as anti-inflammatory, anticoagulant, antitumor, antihyperlipidic, antinephritic, antioxidant and vasodilative effects [22–28]. However, to what extent in vitro effects produced by these flavonoids could be extrapolated to the in vivo situation was poorly understood. Therefore, investigation on the metabolism of *D. odorifera* flavonoids in vivo is paramount to understand their role in human health and evaluate the clinical efficacy of this medicinal herb.

The objective of the current research was to analyze the major flavonoids qualitatively and quantitatively in rat serum after oral administration of *D. odorifera* extract with HPLC–UV

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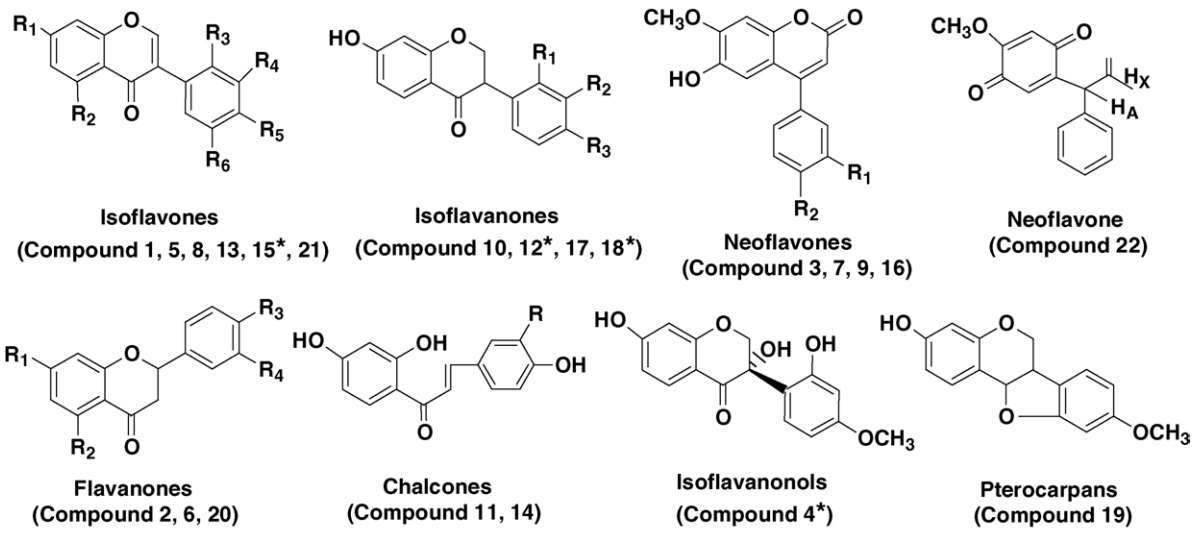

Type	No.	Name	R_1	R_2	R_3	R_4	R_5	R_6
Isoflavone	1	3'-hydroxydaidzein	OH	H	H	OH	OH	H
	5	koparin	OH	H	OH	OH	OCH_3	H
	8	2',7-dihydroxy-4',5'-dimethoxyisoflavone	OH	H	OH	H	OCH_3	OCH_3
	13	2'-hydroxyformononetin	OH	H	OH	H	OCH_3	H
	15*	formononetin	OH	H	H	H	OCH_3	H
	21	prunetin	OCH_3	OH	H	H	OH	H
Isoflavanone	10	violanone	OCH_3	OH	OCH_3	—	—	—
	12*	vestitone	OH	H	OCH_3	—	—	—
	17	3'-O-methylviolanone	OCH_3	OCH_3	OCH_3	—	—	—
	18*	sativanone	OCH_3	H	OCH_3	—	—	—
Neoflavone	3	3'-hydroxymelanettin	OH	OH	—	—	—	—
	7	melanettin	H	OH	—	—	—	—
	9	stevenin	OH	H	—	—	—	—
	16	dalbergin	H	H	—	—	—	—
	22	4-methoxydalbergione	—	—	—	—	—	—
Flavanone	2	butin	OH	H	OH	OH	—	—
	6	liquiritigenin	OH	H	OH	H	—	—
	20	pinocembrin	OH	OH	H	H	—	—
Chalcone	11	butein	OH	—	—	—	—	—
	14	isoliquiritigenin	H	—	—	—	—	—
Isoflavanonol	4*	(*3R*)-4'-methoxy-2',3,7-trihydroxyisoflavanone	—	—	—	—	—	—
Pterocarpan	19	medicarpin	—	—	—	—	—	—

Fig. 1. Structures of flavonoids **1–22** identified from *D. odorifera* (*compounds **4**, **12**, **15** and **18** are quantified in serum by HPLC–UV method).

and HPLC–MS techniques. Based on HPLC–MS analysis, 18 flavonoids were unambiguously identified in free form in serum sample by comparing their retention times, UV and MS spectra with those of authentic compounds. Furthermore, the four prominent flavonoids in serum, i.e., (3*R*)-4′-methoxy-2′,3,7-trihydroxyisoflavanone, vestitone, formononetin and sativanone, were quantified by HPLC–UV with internal standard method, so as to a limited view of their pharmacokinetic pro-

files. To our knowledge, there were no previous reports on the determination of the flavonoid of *D. odorifera* in biological fluids. It was the first report on the identification and determination of major biologically active flavonoids in rat serum after oral administration of *D. odorifera* extract.

2. Experimental

2.1. Herbal materials

The heartwood of *D. odorifera* was purchased from Tong Ren Tang Pharmaceutical Co. Ltd. (Beijing, PR China). The crude drug (1 kg) was extracted twice by refluxing with 10 l of 75% ethanol for 1 h, then collected and combined the filtrates. After evaporating ethanol, the residue was pushed through a preactivated 3 kg macroreticular resin column. The column was eluted with 10 l of 20% ethanol and 20 l of 60% ethanol, respectively. The 60% ethanol fraction was evaporated in vacuo, and the residue (112 g) was stored at −20 °C until administration to rat. To calculate the administered dose of (3*R*)-4′-methoxy-2′,3,7-trihydroxyisoflavanone, vestitone, formononetin and sativanone, their contents in *D. odorifera* extract were quantitatively analyzed by HPLC–UV with external standard method, which were 10.98, 23.16, 12.70 and 22.39 μg/mg, respectively.

2.2. Chemicals and reagents

The reference standards of 22 flavonoids were isolated by the author from *D. odorifera*, their structures were fully characterized by using chemical and spectroscopic methods (UV, IR, NMR, MS). The purity of the standards was checked by HPLC-DAD at three different wavelengths (230, 275 and 350 nm), and the results suggested their purity to be above 95%. The internal standard (I.S.), isorhamnetin, was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, PR China). Acetonitrile and methanol were of HPLC grade (J.T. Baker, Phillipsburg, NJ, USA). The deionized water was purified by Milli-Q water system (Millipore, Bedford, MA, USA) and was filtered with 0.45 μm membranes. All other chemicals were of analytical reagent grade.

2.3. Instrumentation and conditions

HPLC analysis was carried out on an Agilent 1100 Series HPLC (Palo Alto, CA, USA) with diode-array detector using a Zorbax SB-C_{18} column (250 mm × 4.6 mm, 5 μm) connected with a Zorbax SB-C_{18} guard column (20 mm × 4 mm, 5 μm). The mobile phase consisted of (A) acetonitrile and (B) 0.3% aqueous acetic acid (v/v) using a gradient elution of 25–30% A at 0–20 min, 30–50% A at 20–37 min and 80% A at 37–45 min. Re-equilibration duration was 10 min between individual runs. The flow rate was 1.0 ml/min and the temperature of the column oven was 40 °C. Detection wavelength was set at 275 nm and UV spectra from 190–400 nm were also recorded for peak identification.

Mass spectra were acquired using a LCQ mass spectrometer (ThermoFinnigan, San Jose, CA), which is an ion-trap mass spectrometer equipped with electrospray ionization source. Nitrogen was used as the sheath and auxiliary gas, and helium was used as the damping and collision gas. All the mass spectra were acquired in the negative ion mode with ion spray voltage at 5.0 kV, capillary temperature at 350 °C, capillary voltage at −12 V, sheath gas flow rate at 50 (arbitrary units), auxiliary gas flow rate at 15 (arbitrary units) and tube lens offset at −10 V. The mass spectra were recorded in the single ion monitoring (SIM) mode in the range of *m/z* 80–1000 and from 4–45 min of the run duration.

2.4. Preparation of stock solutions, calibration standards and quality control samples

For identification by HPLC–MS, methanol stock solution containing known concentrations of all standards (20–70 μg/ml) and *D. odorifera* extract solution with initial mobile phase (676 μg/ml) were prepared and aliquot of 10 μl were subjected to the HPLC–MS analysis, respectively. For quantification by HPLC–UV, standards of (3*R*)-4′-methoxy-2′,3,7-trihydroxyisoflavanone, vestitone, formononetin, sativanone and the I.S. were accurately weighted and dissolved in the initial mobile phase, then diluted to appropriate concentrations. To achieve the working solutions for calibration curves, the standard solution (100 μl) and I.S. (100 μl) was spiked to drug-free rat serum (500 μl), the resulting serum contained 0.150–30.0 μg/ml of (3*R*)-4′-methoxy-2′,3,7-trihydroxyisoflavanone, 0.200–40.0 μg/ml of vestitone, 0.075–15.0 μg/ml of formononetin, 0.222–22.2 μg/ml of sativanone and 12.0 μg/ml of I.S. Quality control samples were similarly prepared at high, mid and low concentrations for the four analytes, and used to assess accuracy and precision of the assay method. All solutions were stored at 4 °C and found to be stable within 4 days.

2.5. Sample preparation

For identification of flavonoids by HPLC–MS: 2 ml of ethyl acetate were added to 500 μl of rat serum, the supernatant was evaporated to dryness under a stream of nitrogen, after 10 min vortexing and 10 min centrifugation at 3000 rpm, the residue was dissolved in 100 μl initial mobile phase and stored at 4 °C until use. For determination of (3*R*)-4′-methoxy-2′,3,7-trihydroxyisoflavanone, vestitone, formononetin and sativanone by HPLC–UV, the serum sample preparation was identical to the above procedure except that the I.S. (100 μl) was spiked in the first step.

2.6. Animals

Male Sprague–Dawley rats (200–220 g) were obtained from the Laboratory Animal Center of Peking University Health Science Center (Beijing, PR China). They were kept in environmentally controlled breeding room for 3 days before starting the experiments and fed with standard laboratory food and water ad libitum and fasted overnight before the test.

2.7. Method validation for the determination of four prominent flavonoids in rat serum by HPLC–UV

Calibration standards were prepared by adding appropriate amounts of stock solutions to drug-free rat serum and then serially diluting the standards with additional blank serum in order to obtain various concentrations of the four analytes. Three samples at each concentration were analyzed. The internal ratios (analyte peak area/I.S. peak area) were calculated for each point, and calibration curves were constructed by weighted ($1/y$) least squares linear regression analysis of internal ratios versus concentrations. The limit of quantification (LOQ) was determined as the lowest concentration of the analyte in serum that could be quantified with an inter-assay coefficient of variation (CV) lower than 20% and an accuracy between 80 and 120%. The precision and accuracy of the assay were evaluated by analyzing the QC samples with high, mid and low concentrations. The intra-day variance was determined by assaying the six replicates on the same day and inter-day variance was assayed on four consecutive days. Precision was expressed as the relative standard deviation (R.S.D.). Accuracy was determined by comparing the calculated concentration using calibration curves to the known concentration. The extraction recoveries were calculated in serum samples spiked with the four flavonoids at three levels of concentration (high, medium and low). The peak area ratios of spiked serum samples were compared with those of the standards prepared in initial mobile phase at the same concentration to provide the recovery values. Stability of the four analytes in serum was assessed by five replicate analyses of the three in-house control samples. The samples were initially analyzed within 2 h of preparation to obtain reference concentrations, and then were used to assess the stability of analytes during storage at 4 °C after 4 days. Aliquots of the three samples were also stored at −20 °C, then thawed at ambient temperature and reassayed. The three aliquots were refrozen and thawed twice more to provide data from three freeze-thaw cycles for a period of 2 weeks.

2.8. Application of the method and pharmacokinetic study

The utility of the analytical method was demonstrated in the in vivo conditions. Each rat was administered an oral dose of 1.5 g/kg of *D. odorifera* extract, which indicated that the amounts of the four flavonoids administered were 16.47, 34.74, 19.05 and 33.59 mg/kg, respectively. A blood sample was collected at times of 5, 10, 30, 45, 60, 80, 100, 120, 240, 480, 720 and 1440 min after dosing. Within 30 min after blood withdrawal, the samples were centrifuged and the separated serum samples were frozen in polypropylene tubes at −20 °C prior to analysis. For identification of flavonoids by HPLC–MS, a blood sample collected at 30 min after dosing was performed as described under Section 2.6 and 20 μl of the solution was analyzed. For determination by HPLC–UV, the blood samples collected at 0–24 h after dosing were performed as described under Section 2.6 and 30 μl of each solution was analyzed. The serum concentrations of the four analytes at different time point were evaluated by means of linear regression analysis. All statistical analysis was performed using Microsoft Excel 2000. The relevant pharmacokinetic parameters were calculated using the computer program 3p97 (the Chinese Society of Mathematical Pharmacology).

3. Results

3.1. Identification of flavonoid aglycones in rat serum by HPLC–MS

UV chromatograms at 275 nm and MS total ion current (TIC) chromatograms of the flavonoid standards (A), *D. odorifera* extract (B), the blank rat serum (C) and the rat serum sample collected at 30 min after oral administration of *D. odorifera* extract (D) were shown in Fig. 2. The corresponding retention times, UV λ_{max} and MS data were summarized in Table 1. It demonstrated that there was no significant interference from endogenous components for the analysis. As shown in Table 1 and Fig. 2, based on direct comparison with the reference standards, all 22 flavonoids including 6 isoflavones, 5 neoflavones, 4 isoflavanones, 3 flavanones, 2 chalcones, 1 isoflavanonol and 1 pterocarpan were identified in *D. odorifera* extract and 18 of them were identified in serum sample. Compounds **19–22** not observed in serum might be due to their minor amounts in *D. odorifera* extract. It indicated that the majority of flavonoid aglycones could present in free form in serum after oral administration of *D. odorifera* extract.

3.2. Determination of the four prominent flavonoids in rat serum by HPLC–UV

As shown in HPLC–MS chromatograms (Fig. 2D), (3*R*)-4′-methoxy-2′,3,7-trihydroxyisoflavanone (peak 4), vestitone (peak 12), formononetin (peak 15) and sativanone (peak 18), were the prominent flavonoid aglycones identified in serum after oral administration, especially (3*R*)-4′-methoxy-2′,3,7-trihydroxyisoflavanone, which was the most dominant component in serum sample. Thus, the amounts of these four components were determined by HPLC–UV with internal standard method. Chromatographic profiles of blank rat serum, blank rat serum spiked with the four analytes and I.S., and rat serum collected at 30 min after oral administration of *D. odorifera* extract were shown in Fig. 3. The retention times of (3*R*)-4′-methoxy-2′,3,7-trihydroxyisoflavanone (**1**), vestitone (**2**), formononetin (**3**), sativanone (**4**) and I.S. were 10.40, 20.92, 31.13, 34.22 and 25.71 min, respectively. A baseline separation of these compounds was obtained under the specified chromatographic conditions. No interfering peaks were detected.

This method was validated to meet the acceptance criteria of industrial guidance for the bioanalytical method validation [29]. As shown in Table 2, the test range of calibration curve was found to be adequate for the concentrations observed from the analysis of collected serum samples and the four analytes showed good linearity ($r^2 > 0.99$) in each concentration range. Table 3 showed the precision and accuracy data for the QC samples. The intra-day accuracy of the four analytes ranged from 90 to 102% with R.S.D. less than 10%, the inter-day accuracy ranged from 80 to 100% with R.S.D. less than 10%, which indicating that the

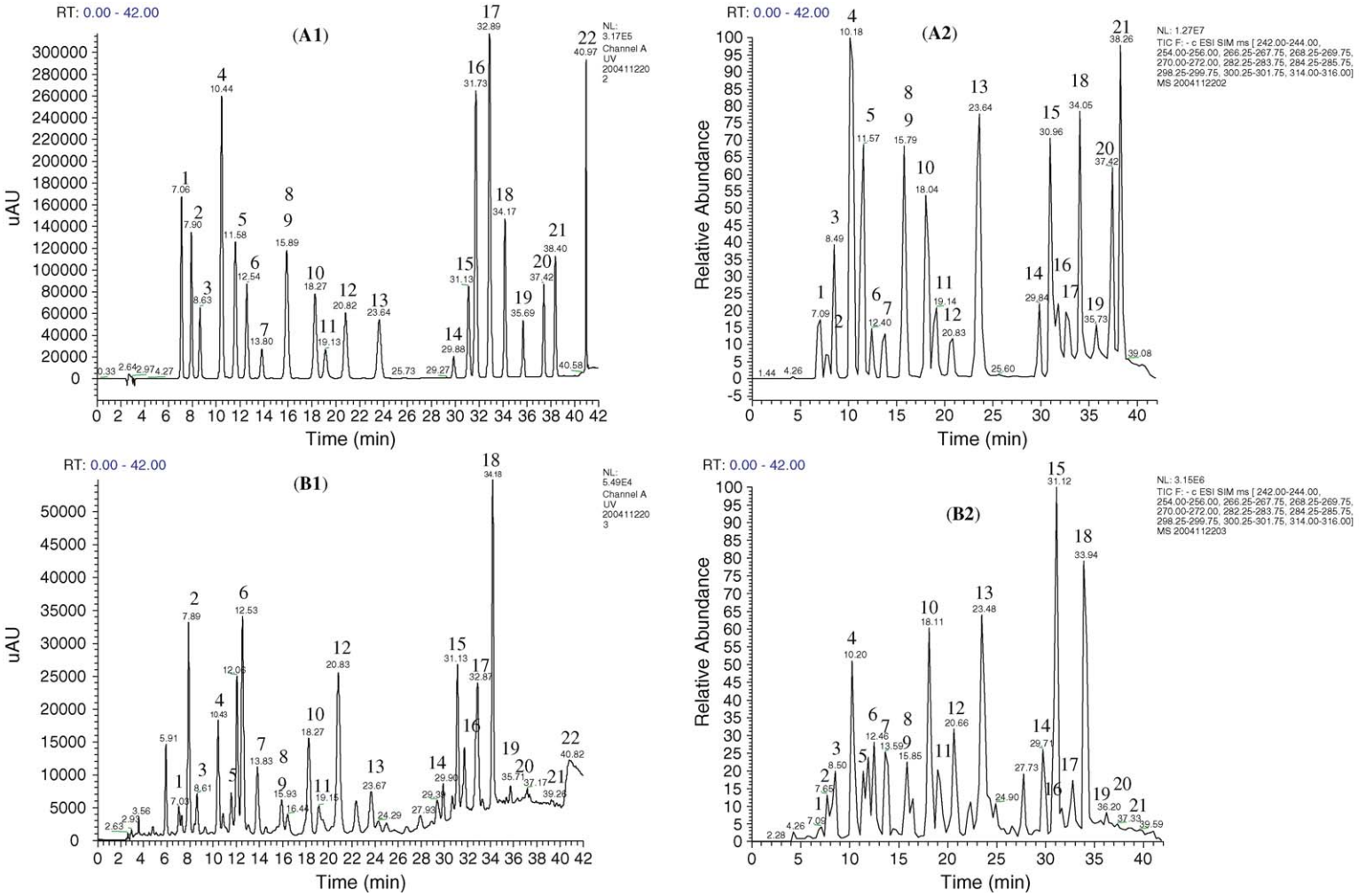

Fig. 2. Typical LC–MS chromatograms. UV chromatograms at 275 nm (A1, B1, C1 and D1); MS total ion current chromatograms (A2, B2, C2 and D2). (A1) and (A2) of the flavonoid standards; (B1) and (B2) of *D. odorifera* extract; (C1) and (C2) of the blank rat serum; (D1) and (D2) of the serum sample collected at 30 min after oral administration of *D. odorifera* extract.

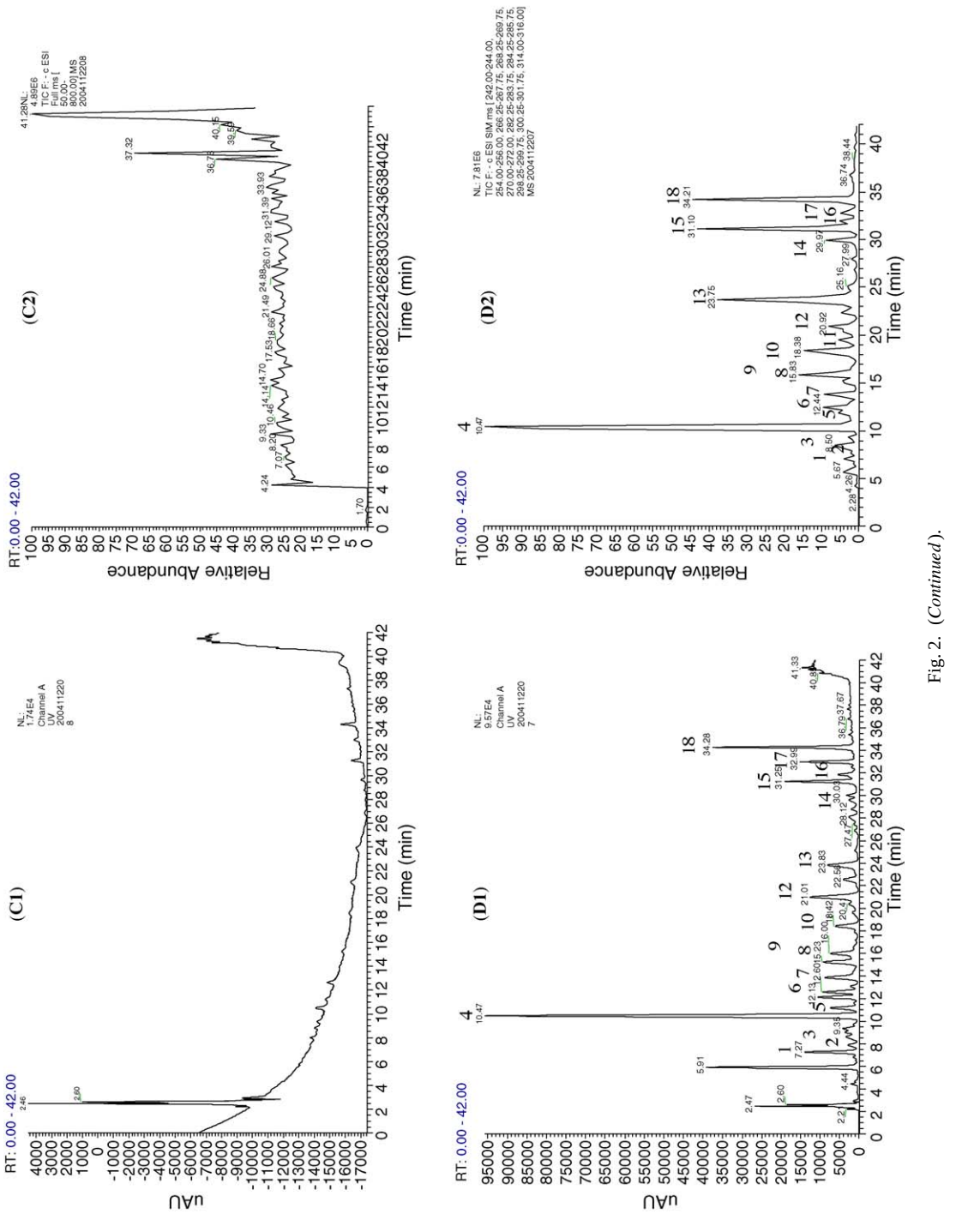

Fig. 2. (*Continued*).

Table 1
Retention time (t_R), MS data and UV λ_{max} values for the main flavonoids identified by HPLC–MS

No.	t_R (min)	$[M–H]^-$ (*m/z*)	λ_{max} (nm)	Identity
1	7.06	269	220292	3′-Hydroxydaidzein
2	7.90	271	232278	Butin
3	8.63	299	340	3′-Hydroxymelanettin
4[a]	10.44	301	280	(3*R*)-4′-methoxy-2′,3,7-trihydroxyisoflavanone
5	11.58	299	240290	Koparin
6	12.54	255	230276310	Liquiritigenin
7	13.80	283	294	Melanettin
8[b]	15.89	283	286350	2′,7-Dihydroxy-4′,5′-dimethoxyisoflavone
9[b]	15.89	313	290	Stevenin
10	18.27	315	274310	Violanone
11	19.13	271	262	Butein
12[a]	20.83	285	278312	Vestitone
13	23.64	283	248290	2′-Hydroxyformononetin
14	29.88	255	240372	Isoliquiritigenin
15[a]	31.13	267	250302	Formononetin
16	31.73	267	232352	Dalbergin
17	32.89	329	232274320	3′-*O*-Methylviolanone
18[a]	34.17	299	230276310	Sativanone
19[c]	35.69	269	228286	Medicarpin
20[c]	37.42	255	232290	Pinocembrin
21[c]	38.40	283	260	Prunetin
22[c]	40.97	[d]	260	4-Methoxydalbergione

[a] Compounds **4**, **12**, **15** and **18** were quantified in serum by HPLC–UV method.
[b] Compounds **8** and **9** had the identical retention time.
[c] Compounds **19**–**22** were not be detected in serum sample.
[d] MW = 270, while the molecular ion is not detectable by ESI–MS.

Table 2
Calibration curves for the four flavonoids of *D. odorifera* in serum ($n = 7$)

Analyte[a]	Regression equation[b]	r^2	Linear range (μg/ml)	LOQ (μg/ml)
1	$y = 0.1206x+0.00024$	0.9975	0.150–30.0	0.050
2	$y = 0.1456x+0.00138$	0.9914	0.200–40.0	0.067
3	$y = 0.1628x+0.00051$	0.9924	0.075–15.0	0.015
4	$y = 0.1309x+0.00364$	0.9979	0.222–22.2	0.074

[a] **1** = (3*R*)-4′-methoxy-2′,3,7-trihydroxyisoflavanone, **2** = vestitone, **3** = formononetin, **4** = sativanone.
[b] In the regression equation $y = ax + b$, x refers to the concentration of the analyte in serum (μg/ml); y refers to the peak area ratio of the analyte and I.S.

Table 3
Validation of the intra-day and inter-day assays

Analyte[a]	Spiked (μg/ml)	Intra-day ($n = 6$)			Inter-day ($n = 4$)		
		Measured (μg/ml)	Accuracy (%)	Precision (R.S.D., %)	Measured (μg/ml)	Accuracy (%)	Precision (R.S.D., %)
1	30.00	29.05 ± 0.23	96.84	1.8	28.94 ± 0.45	96.46	2.6
	6.00	5.69 ± 0.17	94.82	3.0	5.51 ± 0.23	91.88	4.2
	0.60	0.56 ± 0.03	93.05	6.1	0.54 ± 0.03	89.92	5.5
2	40.00	38.19 ± 1.35	95.48	3.5	36.69 ± 1.73	91.72	4.7
	8.00	7.74 ± 0.12	96.78	5.4	7.61 ± 0.30	95.09	6.8
	0.80	0.73 ± 0.06	90.89	8.7	0.66 ± 0.05	82.39	9.3
3	15.00	15.18 ± 0.46	101.19	3.1	13.96 ± 0.59	93.07	4.2
	3.00	2.93 ± 0.05	97.65	5.0	2.77 ± 0.17	92.31	6.1
	0.30	0.29 ± 0.01	95.36	4.2	0.27 ± 0.02	91.01	5.9
4	22.20	20.34 ± 1.04	91.63	5.1	20.10 ± 0.92	90.53	4.5
	4.44	4.25 ± 0.10	95.71	2.5	4.13 ± 0.26	93.09	6.3
	0.44	0.40 ± 0.03	91.05	6.8	0.38 ± 0.03	84.84	5.7

[a] **1** = (3*R*)-4′-methoxy-2’,3,7-trihydroxyisoflavanone, **2** = vestitone, **3** = formononetin, **4** = sativanone.

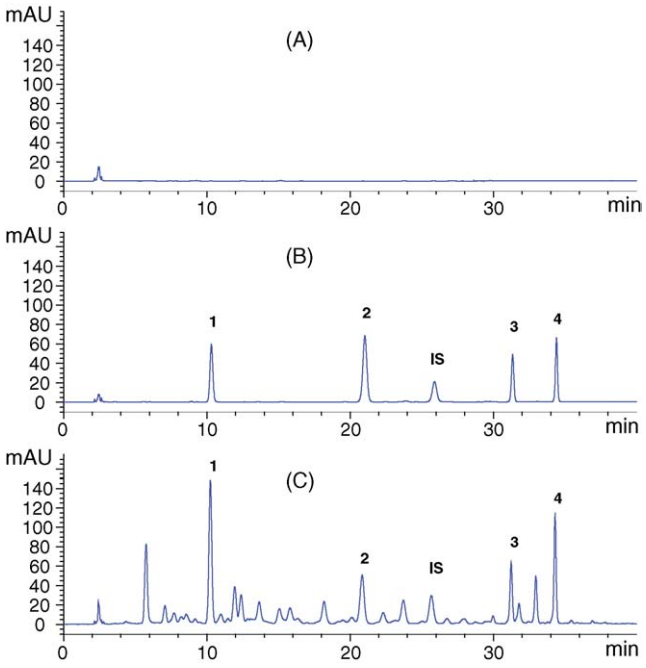

Fig. 3. Typical chromatograms for the determination of (3*R*)-4′-methoxy-2′,3,7-trihydroxyisoflavanone (**1**), vestitone (**2**), formononetin (**3**) and sativanone (**4**) in serum samples: (A) chromatogram of a blank rat serum sample; (B) chromatogram of a rat serum sample spiked with (**1**)–(**4**) and I.S.; (C) chromatogram of the rat serum sample collected at 30 min after oral administration of *D. odorifera* extract.

overall reproducibility of the method was acceptable. As shown in Table 4, the recoveries for the four analytes ranged from 80 to 100%, while R.S.D. values were lower than 10%. The stability results suggesting that there was no significant degradation of the four analytes for 4 days when stored at 4 °C and for up to 2 weeks when stored at −20 °C (Table 5).

This validated analytical method was employed to study the pharmacokinetic profiles of (3*R*)-4′-methoxy-2′,3,7-trihydroxyisoflavanone, vestitone, formononetin and sativanone in rat serum after oral administration of *D. odorifera* extract. The

Table 4
Recovery of the four flavonoids of *D. odorifera* in serum ($n = 3$)

Analyte[a]	Spiked (μg/ml)	Recovery (%)	R.S.D. (%)
1	30.00	93.51 ± 3.08	3.2
	6.00	89.33 ± 4.93	2.5
	0.60	90.28 ± 5.17	5.7
2	40.00	86.81 ± 4.11	4.7
	8.00	87.78 ± 2.81	5.2
	0.80	82.06 ± 6.40	7.7
3	15.00	87.86 ± 4.91	5.9
	3.00	91.65 ± 6.08	6.6
	0.30	84.21 ± 4.62	5.4
4	22.20	86.69 ± 5.63	2.9
	4.44	84.61 ± 7.91	3.5
	0.44	83.30 ± 5.43	6.2

[a] **1** = (3*R*)-4′-methoxy-2′,3,7-trihydroxyisoflavanone, **2** = vestitone, **3** = formononetin, **4** = sativanone.

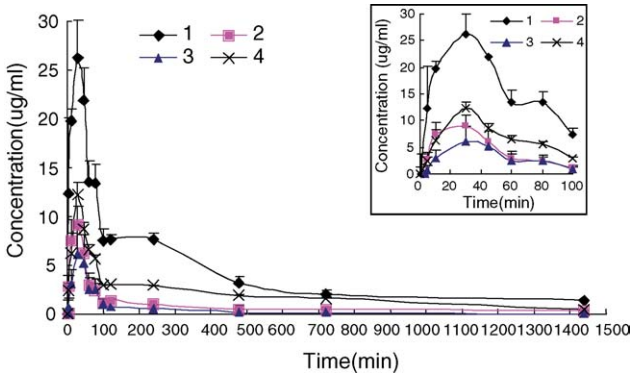

Fig. 4. Mean serum concentration–time profile for the four analytes in rat serum after oral administration of *D. odorifera* extract to rats. Each point with bar represents mean ± S.D. ($n = 5$) as the percentage of the administered dose.

mean serum concentration–time profiles of (3*R*)-4′-methoxy-2′,3,7-trihydroxyisoflavanone, vestitone, formononetin and sativanone ($n = 5$) were shown in Fig. 4. These four analytes exhibited consistent tendency in serum concentration–time profiles,

Table 5
Stability of the four flavonoids of *D. odorifera* in serum

Analyte[a]	Concentration (μg/ml)	Storage at 4 °C		Freeze-thaw cycles	
		Mean concentration obtained (μg/ml)	R.S.D. (%)	Mean concentration obtained (μg/ml)	R.S.D. (%)
1	30.00	29.14	3.4	29.62	5.1
	6.00	6.11	4.7	6.13	3.0
	0.60	0.56	5.6	0.53	7.9
2	40.00	39.43	6.5	36.73	6.8
	8.00	7.26	6.1	6.65	7.5
	0.80	0.69	7.9	0.70	9.4
3	15.00	15.08	4.4	13.65	6.7
	3.00	2.77	4.8	2.53	6.1
	0.30	0.28	6.5	0.27	7.2
4	22.20	21.14	4.0	22.32	4.4
	4.44	4.32	5.7	4.10	6.0
	0.44	0.40	4.3	0.40	7.3

[a] **1** = (3*R*)-4′-methoxy-2′,3,7-trihydroxyisoflavanone, **2** = vestitone, **3** = formononetin, **4** = sativanone.

Table 6
Mean pharmacokinetic parameters for the four flavonoids in rat serum ($n = 5$) after oral administration of *D. odorifera* extract

Parameter	Value[a]			
	1	**2**	**3**	**4**
t_{max} (min)	20.97	22.31	21.27	21.56
C_{max} (μg/ml)	26.31	6.67	5.40	10.35
AUC0 → ∞ (μg min/ml)	5930.87	1332.42	501.00	2841.27
$t_{1/2}$ α (min)	19.45	17.80	15.69	16.76
$t_{1/2}$ β (min)	436.32	1102.58	260.01	526.23
V_d (ml)	83.50	555.28	353.50	386.66
CL (ml/min)	0.67	6.32	9.22	2.86
Lag time (min)	0.92	0.33	4.08	3.34

[a] **1** = (3*R*)-4′-methoxy-2′,3,7-trihydroxyisoflavanone; **2** = vestitone; **3** = formononetin; **4** = sativanone.

which might be due to their similarity in structure features. The pharmacokinetic characteristics of the four analytes complied with the two-compartment model with the first-order absorption and the pharmacokinetic parameters were listed in Table 6. The four analytes were rapidly absorbed with peak concentrations occurring at around 22 min, and slowly eliminated as the serum concentration of each analyte was still much higher than LOQ after 24 h. The values of clearance (CL) of the four analytes indicated that compounds **3** and **2** were more easily cleared from the rat body than compounds **1** and **4**. The apparent distribution volume (V_d) of the four analytes were much larger than the blood volume of rat, hence it could be speculated that they were widely distributed in the organs. The pharmacokinetic results could be aid in clarifying the pharmacological effect of *D. odorifera* and ensuring the clinical efficacy of this medicinal herb.

4. Discussion

For an efficient clean up of the serum sample and a quantitative analysis of the target compounds, various sample preparation method were tested, such as deproteinization with methanol or acetonitrile, liquid–liquid extraction with different organic solvents such as ethyl acetate, ether and acetone, and solid-phase extraction with various sorbents such as C_8 and C_{18} cartridges (Alltech Associates, Deerfield, IL, USA). Liquid–liquid extraction with ethyl acetate was chosen for it not only to ensure the simultaneous extraction of all target compounds but also to cause less interference from the co-eluted endogenous materials. Additionally, the simple extraction procedure and satisfactory extraction recovery made it feasible for quantitative analysis.

The chromatographic conditions were optimized to obtain chromatograms with a good resolution of the target compounds within a short analysis time. Different types of chromatographic columns such as Zorbax SB-C_{18} column, Zorbax XDB column and Zorbax Extend-C_{18} column were tested. The flavonoids in *D. odorifera* showed different retention behaviors on these columns. The analysis time did not vary significantly on three columns, while the resolution of Zorbax SB-C_{18} column was better than the rest two. Thus, Zorbax SB-C_{18} column was used for analysis. Different mobile phase compositions were screened. As a result, acetonitrile and water containing 0.3% acetic acid was chosen as the eluting solvent system, since with it not only the desired separation was achieved but also less damage to the column. For the separation of all target compounds from the matrix, gradient elution was used. Based on the UV absorption maxima of most flavonoids obtained by HPLC-DAD, the monitoring wavelength chosen was 275 nm. These preliminary experiments also showed that the separation could be improved with column temperature at 40 °C and the flow rate of 0.8 ml/min. For MS analysis, the negative ion mode of ESI was selected in the present study, as it easily provided flavonoid fragmentation information. Under the multiple optimized chromatographic conditions, a good separation of all target 22 flavonoids could be achieved within 45 min (see Fig. 2).

It was also worth mentioning that the UV detector was chosen for quantitative analysis as the detection potential provided sufficient sensitivity and accuracy for the four prominent flavonoids in serum samples, whereas the ESI–MS detector was used for qualitative analysis due to its high sensitivity and selectivity in detecting flavonoids from complex biological matrix. The HPLC–ESI–MS technique showed great advantage in the present study as the following examples: compounds **8** and **9** were identical in retention behavior on C_{18} column (t_R = 15.89 min). If only UV detector was used, these two compounds could not be distinguished even the authentic compounds were available. By ESI–MS, the different $[M–H]^-$ ions in mass spectra permitted us to distinguish these two compounds. Compounds **15** and **16** are a pair of isomers with the same molecular ions in mass spectra (*m/z* 267) and adjacent peaks in UV chromatograms ($t_{R\ 15}$ = 31.13 min, $t_{R\ 16}$ = 31.73 min). Based on comparison with the standards, these two isomers were distinguished by UV spectra by means of the diode-array detector. This study demonstrated that LC/UV-DAD/ESI–MS analysis was an excellent technique for identifying flavonoids in complex matrix.

In a word, the ability to simultaneously analyze different types of flavonoids, the convenient and easy-to-use extraction procedure, efficient clean up, and no need of sample radiolabeling revealed that this newly developed method was appropriate for rapid screening and structural characterization of flavonoids, and suggested its profit to the biological and pharmacological research.

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