Journal of Chromatographic Science 2012;50:885–892
doi:10.1093/chromsci/bms086 Advance Access publication June 18, 2012

Article

Simultaneous Determination of Oleanolic Acid, *p*-Coumaric Acid, Ferulic Acid, Kaemperol and Quercetin in Rat Plasma by LC–MS-MS and Application to a Pharmacokinetic Study of *Oldenlandia diffusa* Extract in Rats

Neng Li, Changhui Liu, Suiqing Mi, Ningsheng Wang, Xia Zheng, Yingyi Li, Xiaotao Huang, Shaoling He, Hao Chen and Xinhua Xu

Guangzhou University of Chinese Medicine, Institute of Clinical Pharmacology

*Author to whom correspondence should be addressed. Email: stephenchliu@hotmail.com

Received 1 October 2011; revised 8 March 2012

A simple, rapid and sensitive liquid chromatography tandem mass spectrometry method is presented for the simultaneous determination of oleanolic acid, *p*-coumaric acid, ferulic acid, kaemperol and quercetin in rat plasma. Glycyrrhetinic acid was used as an internal standard, and sample pretreatment consisted of a liquid–liquid extraction. Chromatographic separation was achieved on a Gemini 110A C18 column (50 × 2.0 mm i.d., 5 μm) by gradient elution with a mobile phase consisting of methanol, acetonitrile and 0.01% formic acid in water. Tandem mass spectrometric detection was conducted using multiple reaction monitoring under negative ionization mode. Calibration curves offered linear ranges of two orders of magnitude with r > 0.99. The method was validated in terms of matrix effect, intra-day and inter-day precision, accuracy, linearity, specificity and stability. The relative standard deviation of intra-day and inter-day variations ranged from 2.66 to 14.74% and 1.9 to 14.55%. No substantial endogenous interference from blank plasma was observed. The method has been successfully applied to a pharmacokinetic study of *Oldenlandia diffusa* extract after oral administration in rats.

Introduction

Oldenlandia diffusa is one of the most extensively used Chinese herbal remedies in modern Chinese practice and officially listed as an ingredient of Chinese patent medicine. The plant is a member of the Rubiaceae family and known as *Hedyotis diffusa* (botanical name), *Herba hedyotis diffusae* (pharmaceutical name) and Bai Hua She She Cao (common name). It is primarily distributed in Southern China and has been used medicinally for many centuries in China (1). *Oldenlandia diffusa* has been used for the treatment of hepatitis, tonsillitis, sore throat, appendicitis, urethral infection and malignant tumors of the liver, lung and stomach (2). Many pharmacological studies of *Olenlandia diffusa* have demonstrated that it has antitumor, immunomodulatory (3, 4), anti-mutagenic (5, 6), anti-inflammatory, hepatoprotective (7), anti-oxidative (8, 9) and neuroprotective (10) activities.

Oldenlandia diffusa includes many components. Oleanolic acid (OA), *p*-coumaric acid (p-CA), ferulic acid (FA), kaemperol and quercetin are five components existing in *Oldenlandia diffusa* (10–13). OA, present in *Oldenlandia diffusa* at high abundance, is one of the pentacyclic triterpenoids that exist widely in plants (11). Studies indicate that the biological activities of OA are multiple, including hepatoprotective, anti-tumor, chemo-preventive, anti-angiogenic, and anti-inflammatory, antioxidant and pro-apoptotic effects (14–16). FA is a known antioxidant with various pharmacological properties (17), and is generally effective as an antibiotic (18). Furthermore, it has anti-microbial, anti-inflammatory, anti-thrombosis and anti-cancer activities (14). Additionally, it protects against coronary disease, lowers cholesterol in serum and liver and increases sperm viability (19). P-CA has chemoprotectant and anti-oxidant effects (20), and it is believed to reduce the risk of stomach cancer by reducing the formation of carcinogenic nitrosamines (21, 22). Kaemperol and quercetin are flavonoids that have many biological activities, including anti-cancer, anti-inflammatory and reverse tumor cells an multiple drug resistance effects (23). Collectively, this indicates that p-CA, FA, kaemperol, quercetin and OA may be the five primary bioactive components of *Oldenlandia diffusa*.

Several methods have been described to determine *Oldenlandia diffusa* extract. Liang *et al.* (24) used high-performance liquid chromatography (HPLC) to analyze ursolic acid, oleanolic acid, asperuloside, E-6-O-p-coumaroyl scandoside methyl ester and E-6-O-p-coumaroyl scandoside methyl ester-10-O-methyl ether in *Oldenlandia diffusa*. Razborsek *et al.* (25) used gas chromatography–mass spectrometry (GC–MS) after derivatization to determine OA in Lamiaceae. Song *et al.* (26) and Ji *et al.* (27) developed a sensitive LC–MS method to determine the plasma concentration of OA in human and rodents, respectively. However, there is no method established for the simultaneous measurement of these five constituents in *Oldenlandia diffusa*; therefore, simultaneous determination of these five bioactive components in biological fluids for pharmacokinetic investigations is required. In this article, a simple, rapid and sensitive method for the simultaneous determination of OA, p-CA, FA, kaemperol and quercetin in rat plasma and its application to a pharmacokinetic study of *Oldenlandia diffusa* extract in rats are described.

Materials and Methods

Herbal materials and chemicals

Oldenlandia diffusa was purchased from a pharmacy called Xing Yuan Chun in Guangzhou, and authenticated by Prof. Rong Zhang. The *Oldenlandia diffusa* extract was prepared by refluxing with 75% ethanol and the powder was obtained by rotary evaporation under vacuum at 55°C, and the extraction ratio was approximately 10%. The contents of all five components in the *Oldenlandia diffusa* extract are 0.175% for OA, 0.837% for p-CA, 0.021% for FA, 0.024% for kaemperol and 0.171% for quercetin, respectively. OA, p-CA, FA, kaemperol, quercetin and

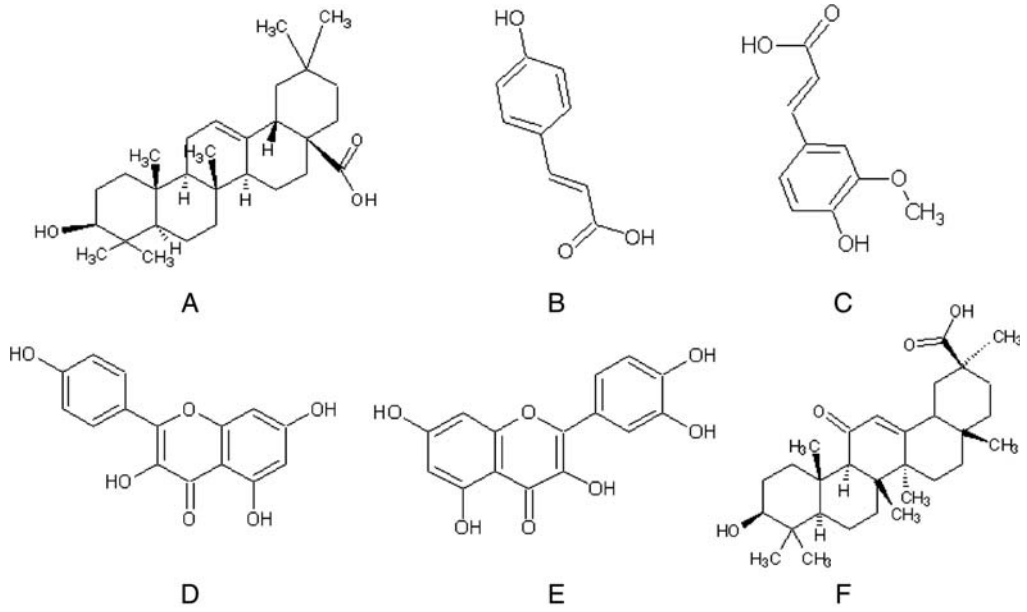

Figure 1. Structures of OA (A); p-CA (B); FA (C); kaemperol (D); quercetin (E); GA (IS) (F).

glycyrrhetinic acid (GA, internal standard) (Figure 1) were all obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC-grade methanol, formic acid and acetonitrile were products of Merck (Darmstadt, Germany). HPLC-grade ethyl acetate was obtained from Fisher. Deionized water was purified using an ultrapure water purification system (Research/Unique-R20). The other reagents were of the highest quality available.

Preparation of calibration standards and quality control samples

Primary stock solutions of OA, p-CA, FA, kaemperol and quercetin were prepared in methanol. Working standard solutions of OA, p-CA, FA, kaemperol and quercetin were prepared by combining aliquots of each primary stock solution and diluting with methanol. The working solution for GA (5 μg/mL) was prepared by diluting an aliquot of stock solution with methanol.

Rat plasma calibration standards of OA (1.0, 2.5, 5.0, 10.0, 25.0, 50 and 100 ng/mL), FA, kaemperol, quercetin (0.5, 1.0, 2.5, 5.0, 12.5, 25.0 and 50.0 ng/mL) and p-CA (50, 100, 250, 500, 1,000, 2,500 and 5,000 ng/mL) were prepared by spiking the working standard solutions into a pool of drug-free rat plasma. Quality control (QC) samples of OA (1.5, 10.0 and 100 ng/mL), FA, kaemperol, quercetin (1.5, 5.0 and 50.0 ng/mL) and p-CA (50, 500, 5,000 ng/mL) was prepared in bulk by adding 10 μL of the appropriate working standard solutions to drug-free rat plasma. All standard solution was stored at 4°C when not in use.

Sample preparation

Plasma samples (100 μL) spiked with 10 μL of internal standard working solution and 10 μL of 0.1M hydrochloric acid were extracted with 1 mL of ethyl acetate in 1.5-mL polypropylene tubes by vortex mixing for 15 min and centrifuging at 8,000 × g for 10 min at 4°C. The upper organic phase (900 μL) was transferred and evaporated to dryness in a water bath at 40°C under a nitrogen stream. The residues were reconstituted in 100-μL aliquots of methanol and centrifuged at 12,000 × g for 10 min at 4°C, and 5 μL of supernatants were injected into LC–MS-MS for analysis.

Chromatographic and mass spectrometric conditions

Analyses were performed on an Agilent Series 1200 series liquid chromatography system consisting of a G1311A Quart pumps, a G1322A degasser, a G1313A ALS and a G1316A TCC (Agilent Technologies; Santa Clara, CA) interfaced to an API4000 triple quadrupole mass spectrometer (Applied Biosystem/MDS-SCIEX; Foster City, CA) with an electrospray Turbo Ion source (ESI) in negative mode. The operation conditions were optimized by infusing diluted stock solutions of each analyte as follows: nebulizer gas (gas 1), nitrogen, 30 psi; turbo gas (gas 2), nitrogen, 20 psi; curtain gas, nitrogen, 15 psi; collision gas, nitrogen, 6; ion spray voltage, −4,500 V; source temperature, 350°C with multiple reaction monitoring (MRM). The separation was performed on a Gemini C18 110A column (5 μm, 2.0 mm i.d. × 50 mm, Phenomenex), and the column temperature was kept at 40°C. The mobile phase consisted of 0.01% formic acid in water (solvent A), methanol (solvent B) and acetonitrile (solvent C) was used with a flow rate of 0.25 mL/min. The following gradient program was used: A:B:C (82:8:10, v/v/v) at 0–1.1 min; A:B:C (10:10:80, v/v/v) at 1.6–3.6 min; A:B:C (5:5:90, v/v/v) at 6.6–11.6 min; A:B:C (82:10:8, v/v/v). Detection of the ions was performed in MRM mode and unit mass resolution was set in both mass-resolving quadrupoles Q1 and Q3. The following precursor to product ion transitions of the analytes were obtained by optimizing parameters of declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP): OA, m/z 455.3 → 455.3; p-CA, m/z 162.9 → 118.9; FA, m/z 192.9 → 133.9; kaemperol, m/z 284.8 → 184.9; quercetin, m/z 300.9 → 151.0; GA, m/z 469.2 → 425.5. The product ion

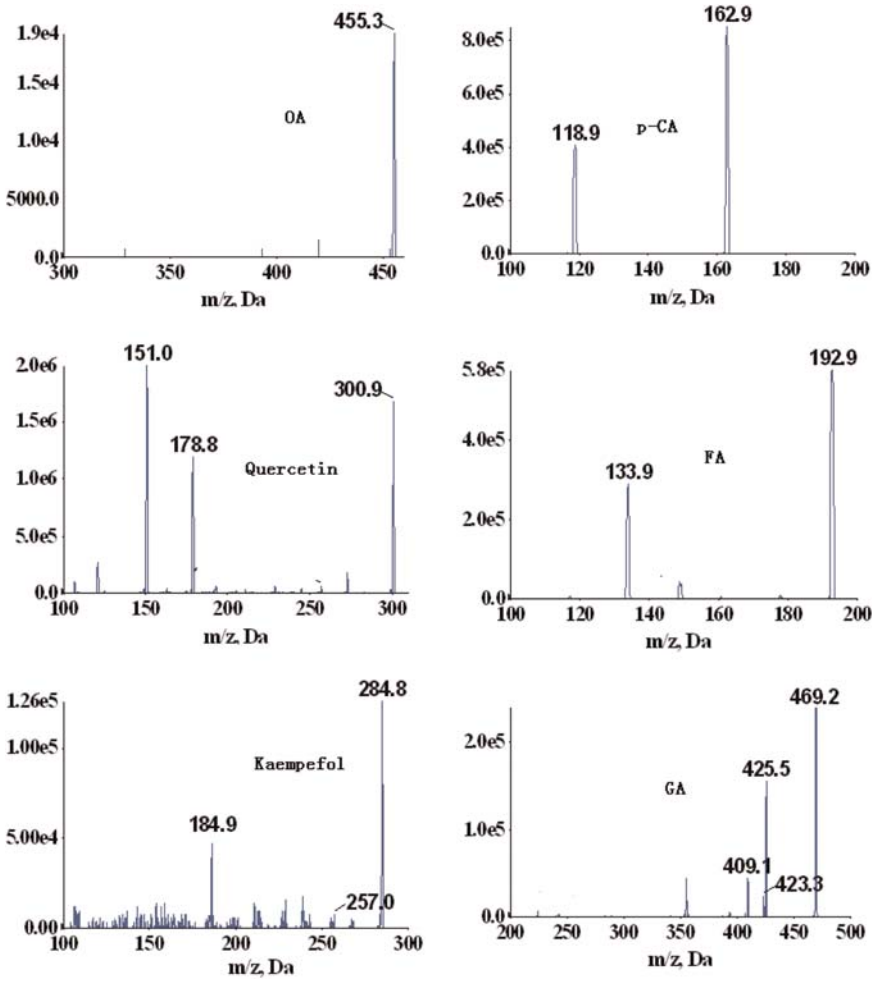

Figure 2. MS-MS product ion mass spectra of OA, p-CA, FA, kaemperol, quercetin and GA (IS).

Table I
Optimized Mass Parameters for p-CA, FA, Kaemperol, Quercetin and OA and GA (IS)

Sample	MRM (*m*/*z*)	Dwell time (ms)	DP (V)	EP (V)	CE (V)	CXP (V)
OA	455.3 → 455.3	100	−112.0	−7.8	−52.0	−15.0
p-CA	162.9 → 118.8	100	−28.2	−7.8	−10.0	−7.1
FA	192.9 → 133.9	100	−29.2	−4.6	−13.0	−10.0
Kaemperol	284.8 → 184.9	100	−84.1	−7.6	−38.0	−17.2
Quercetin	300.9 → 151.0	100	−80.9	−11.6	−26.7	−11.7
GA	469.2 → 425.5	100	−83.9	−5.1	−56.6	−10.0

tandem mass spectra of the deprotonated molecules of OA, p-CA, FA, kaemperol and quercetin are shown in Figure 2. The specific parameters for each analyte are shown in Table I.

Method Validation

Selectivity and sensitivity

Selectivity is the ability of the analytical method to differentiate and quantify the analyte in the presence of other components in the sample. For selectivity, analyses of blank plasma samples should be obtained from at least six rats. Each blank sample should be tested for interference.

The lower limit of quantification (LLOQ) was defined as the lowest concentration of an analyte in a sample that can be quantitatively determined with acceptable precision and accuracy [% bias and coefficient of variation (CV) less than or equal to 20%]. The limit of detection (LOD) was defined as the lowest concentration of an analyte that the bioanalytical procedure could reliably differentiate from background noise with a signal-to-noise ratio of three.

Calibration curve

The linearity for OA, p-CA, FA, kaemperol and quercetin was evaluated over the range of 1–100, 50–5,000, 0.5–50, 0.5–50 and 0.5–50 ng/mL. Calibration curves of OA, p-CA, FA, kaemperol and quercetin with seven levels were prepared by adding serial diluted working standards to pooled blank plasma. A linear regression model has constructed based on the peak area ratio (y) of the analyte to internal standard versus nominal concentration (x).

Matrix effect and recovery

Matrix effect was examined to ensure that there was no substantial endogenous interference from blank plasma that

affected the measurements of OA, p-CA, FA, kaemperol and quercetin in the rat plasma. Matrix effects caused by ionization competition among OA, p-CA, FA, kaemperol and quercetin and endogenous co-eluting components were assessed at three concentrations by comparing the peak areas of the OA, p-CA, FA, kaemperol and quercetin from blank plasma (A1) and the neat QC standards (A2).

The absolute recoveries of OA, p-CA, FA, kaemperol, and quercetin from plasma were calculated by comparing the peak areas from the standards spiked before and after extraction in plasma at three concentration levels. The accuracy of the assay was expressed by (mean observed concentration − spiked concentration) / (spiked concentration) × 100%.

Precision and accuracy

Stability

The stock solutions of OA, p-CA, FA, kaemperol and quercetin were tested at 4°C for 30 days. The freeze-thaw stability was evaluated by analyzing the QC plasma samples at three concentration levels after three cycles from −70°C to room temperature. The autosampler stability was assessed by keeping the processed QC plasma samples at room temperature for 24 h. Long-term storage stability was evaluated by freezing the QC plasma samples at −70°C for seven or 14 days, and then comparing the concentrations with those of QC plasma samples before the storage period.

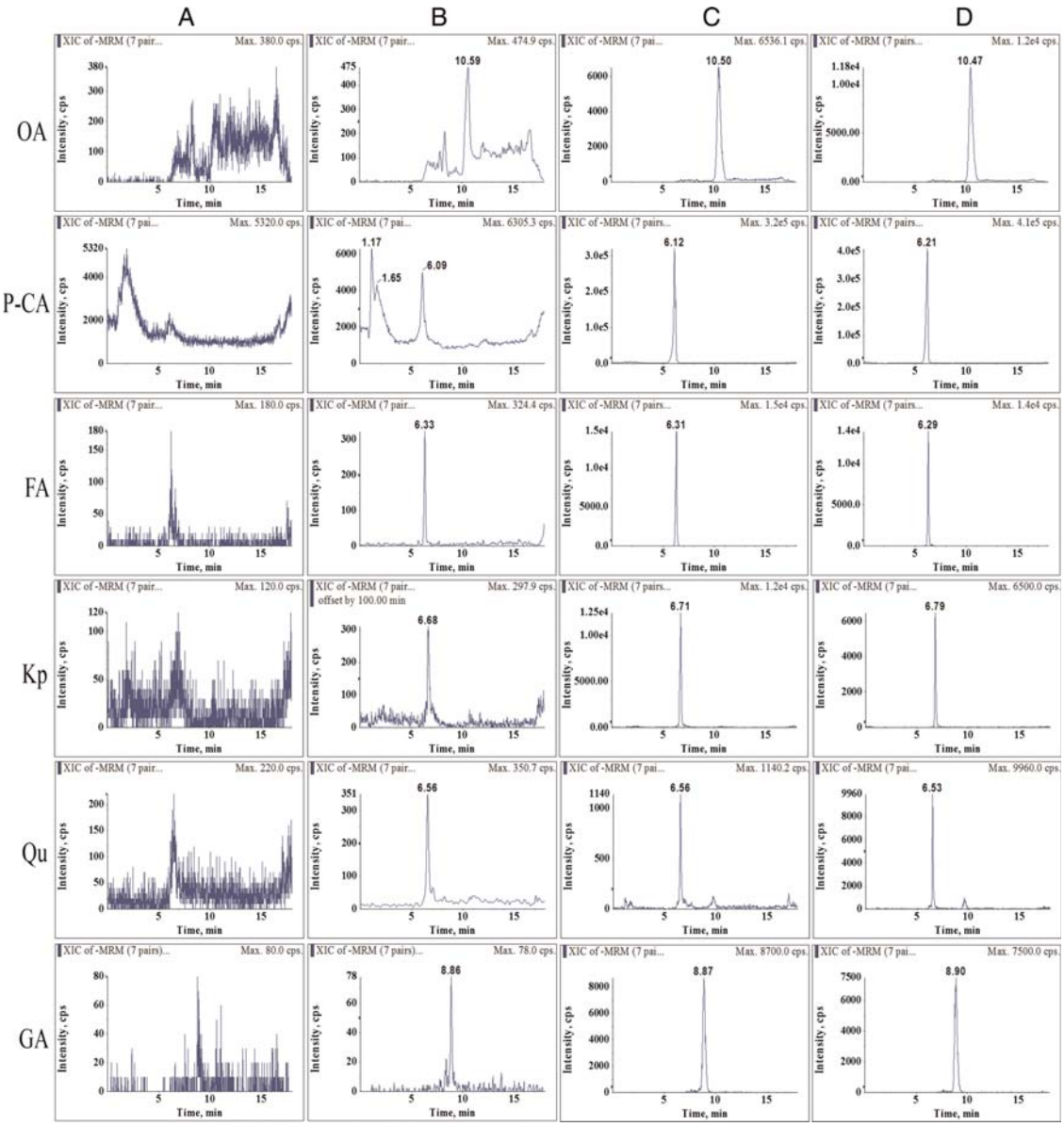

Figure 3. Representative MRM chromatograms for blank plasma (A); blank plasma spiked with 0.5 ng/mL OA, 0.1 ng/mL p-CA, 0.5 ng/mL FA, 0.5 ng/mL kaemperol, 0.5 ng/mL quercetin and 0.5 ng/mL GA (IS) (B); blank plasma spiked with 50 ng/mL OA, 500 ng/mL p-CA, 50 ng/mL FA, 10 ng/mL kaemperol and 2.5 ng/mL quercetin with 500 ng/mL GA (IS) (C); plasma samples of 45 min after oral administration of *Oldenlandia diffusa* extract with 500 ng/mL GA (D).

Application of the method and pharmacokinetic study

The application of the developed assay was demonstrated in a pharmacokinetic study of *Oldenlandia diffusa* extract after oral administration to rats. The Experimental Animal Center of Guangdong province (Guangzhou, China) supplied six male Sprague-Dawley rats, weighing 300 ± 20 g. The Animal Ethics Committee of Guangzhou University of Chinese Medicine approved the study. Rats were fasted for 12 h before dosing, with free access to water. The rats were weighed and administered a 10 g/kg dose of *Oldenlandia diffusa* extract (equivalent to 17.5 mg/kg for OA, 83.7 mg/kg for p-CA, 2.1 mg/kg for FA, 2.4 mg/kg for kaemperol and 17.1 mg/kg for quercetin, respectively). *Oldenlandia diffusa* extract dissolved in 0.5% CMC-Na. Serial blood samples (~0.25 mL each at 0, 10, 20, 30, 45, 60, 120, 180, 240, 360, 480 and 600 min after administration) were collected in heparinzed tubes by puncture of the orbital sinus of the rats. Plasma was obtained by centrifugation at 1,500 × g for 10 min at 4°C and stored in polypropylene tubes at −70°C until assay.

Data analysis

Data are expressed as mean ± standard deviation (SD). The pharmacokinetic parameters were estimated by a non-compartmental analysis. The maximum plasma concentrations (C_{max}) and the time to reach maximum plasma concentrations (T_{max}) were determined by visually inspecting the experimental data. Elimination rate constant (K_{el}) and absorption rate constant (K_a) were calculated from slopes by regression analysis. Moreover, the half-life ($t_{1/2}$) was calculated based on the following equation: $t_{1/2\beta} = 0.693/K_{el}$, $t_{1/2\alpha} = 0.693/K_a$. The area under the plasma concentration-time curve from time zero to the last measurable concentration ($AUC_{0\to t}$) was calculated using the linear trapezoidal rule and was extrapolated to infinity ($AUC_{0\to\infty}$) according to the relationship $AUC0_{\to\infty} = AUC_{0\to t} + C_t/k_{el}$, where C_t is the last concentration evaluated in plasma.

Results and Discussion

Selectivity

No endogenous interfering substances were observed at the retention times of OA, p-CA, FA, kaemperol, quercetin or GA in chromatograms obtained from control blank plasma samples from six rats. Therefore, it was expected that a high and acceptable selectivity could be obtained by this method. Representative chromatograms are shown in Figure 3.

Sensitivity

The LOD was defined as the amount that could be detected with a signal-to-noise ratio of three. The LODs for OA, p-CA, FA, kaemperol and quercetin were 0.2, 0.05, 0.1, 0.1 and 0.1 ng/mL, respectively. The LLOQs were 0.5, 0.1, 0.5, 0.5 and 0.5 ng/mL for OA, p-CA, FA, kaemperol and quercetin.

Linearity of calibration

All linear regressions of OA, p-CA, FA, kaemperol and quercetin in rat plasma displayed good linear relationships over the ranges of 1–100, 50–5,000, 0.5–50, 0.5–50 and 0.5–50 ng/mL, respectively. The mean values of regression equations of the analytes in rat plasma were: $y = 0.0134x + 0.0483$ ($n = 8$, r = 0.9995, OA), $y = 0.0518x + 5.1106$ ($n = 8$, r = 0.9974, p-CA), $y = 0.0424x + 0.0064$ ($n = 8$, r = 0.9992, FA), $y = 0.0051x + 0.0091$ ($n = 8$, r = 0.9959, kaemperol) and $y = 0.0177x + 0.0419$ ($n = 8$, r = 0.9942, quercetin).

Table II
Mean Extraction Recovery of OA, p-CA, FA, Kaemperol and Quercetin in Rat Plasma ($n = 5$)

Sample	Concentration (ng/mL)	Recovery (mean ± SD %)	RSD (%)
OA	1.5	64.43 ± 3.56	5.53
	10	62.88 ± 1.78	2.84
	100	61.36 ± 3.62	4.50
p-CA	50	77.63 ± 2.38	3.06
	500	70.11 ± 2.34	3.34
	4,000	77.70 ± 2.83	3.64
FA	1.5	73.06 ± 5.84	7.98
	5	74.58 ± 3.29	4.42
	50	72.88 ± 4.05	5.56
Kaemperol	1.5	66.17 ± 5.92	8.94
	5	61.83 ± 4.32	6.98
	50	72.69 ± 4.07	5.61
Quercetin	1.5	61.33 ± 3.47	5.66
	5	69.95 ± 1.14	1.63
	50	63.54 ± 4.58	7.21

Table III
Matrix Effects in the Assay of OA, p-CA, FA, Kaemperol and Quercetin or from Rat Plasma ($n = 5$).

Sample	Concentration (ng/mL)	Measured concentration (ng/mL) (mean ± SD)	A1/A2 (mean SD%)	± RSD (%)
OA	1.5	1.42 ± 0.07	94.67 ± 4.7	4.96
	10	9.62 ± 0.04	96.2 ± 0.4	0.42
	100	100.80 ± 2.72	100.8 ± 2.72	2.69
p-CA	50	45.91 ± 4.38	91.8 ± 8.76	9.45
	500	511.075 ± 47.92	102.2 ± 9.58	9.38
	5,000	5,260.30 ± 116.61	105.2 ± 2.33	2.21
FA	1.5	1.37 ± 0.08	91.33 ± 5.3	5.80
	5	4.64 ± 0.36	92.8 ± 7.2	7.77
	50	51.76 ± 5.99	103.5 ± 11.9	11.59
Kaemperol	1.5	1.46 ± 0.08	96.7 ± 5.3	5.48
	5	5.41 ± 0.43	108.2 ± 8.6	7.99
	50	49.91 ± 4.08	99.8 ± 8.2	8.17
Quercetin	1.5	1.57 ± 0.11	104.6 ± 7.3	6.97
	5	4.51 ± 0.46	90.2 ± 9.2	10.20
	50	47.51 ± 5.79	95.0 ± 11.6	12.19

Recovery

Results from the determination of the absolute recovery of OA, p-CA, FA, kaemperol and quercetin from rat plasma are shown in Table II ($n = 5$ for each concentration). The mean recoveries for OA (1.5, 10 and 100 ng/mL) were 64.43, 62.88 and 61.36%; for p-CA (50, 500 and 5,000 ng/mL) were 77.63, 70.11 and 77.70%, respectively; for FA (1.5, 5 and 50 ng/mL) were 73.06, 74.58 and 72.88%, respectively; for kaemperol (1.5, 5 and 50 ng/mL) were 66.17, 61.83 and 72.69%, respectively; for quercetin (1.5, 5 and 50 ng/mL) were 61.33, 69.95 and 63.54%, respectively.

Matrix effect

Matrix effect caused by ionization competition among OA, p-CA, FA, kaemperol and quercetin or endogenous co-eluting

Table IV
Precision and Accuracy Values for OA, p-CA, FA, Kaemperol and Quercetin from the Assay QC Standards

	Intra-day ($n = 5$)				Inter-day ($n = 5$)		
Sample	Spiked concentration (ng/mL)	Measured concentration (ng/mL) (mean ± SD)	RSD (%)	Bias (%)	Measured concentration (ng/mL) (mean ± SD)	RSD (%)	Bias (%)
	0.5	0.59 ± 0.06	10.12	18.0	0.57 ± 0.08	14.04	14.0
OA	1.5	1.53 ± 0.12	7.84	2.0	1.57 ± 0.08	5.09	4.7
	10	10.65 ± 0.79	7.45	6.5	9.45 ± 0.72	7.60	−5.5
	100	98.51 ± 4.45	4.52	−1.5	104.63 ± 5.95	5.69	4.6
	0.1	0.095 ± 0.014	14.74	−5.0	0.11 ± 0.016	14.55	10.0
p-CA	50	51.59 ± 4.04	7.83	3.2	49.69 ± 3.34	6.73	−0.6
	500	513.8 ± 14.78	2.88	2.8	531.55 ± 10.10	1.90	6.3
	5,000	4,362.62 ± 115.87	2.66	−12.7	5,192.73 ± 247.79	4.77	3.8
	0.5	0.46 ± 0.04	10.32	−8.0	0.52 ± 0.05	10.18	4.0
FA	1.5	1.43 ± 0.08	5.61	−4.7	1.38 ± 0.07	5.07	−8.0
	5	4.43 ± 0.19	4.31	−11.4	4.61 ± 0.36	7.80	−7.8
	50	44.49 ± 1.95	4.38	−11.0	52.49 ± 2.02	3.86	4.9
	0.5	0.52 ± 0.06	11.24	4.0	0.52 ± 0.05	8.63	4.0
Kaemperol	1.5	1.58 ± 0.09	5.66	5.3	1.47 ± 0.11	7.14	−2.0
	5	4.79 ± 0.44	9.14	−4.2	4.71 ± 0.39	8.28	−5.8
	50	48.54 ± 4.11	8.48	−2.9	48.21 ± 3.08	6.40	−3.6
	0.5	0.51 ± 0.07	13.83	2.0	0.57 ± 0.06	11.31	14.0
Quercetin	1.5	1.41 ± 0.06	4.25	−6.1	1.55 ± 0.13	8.38	3.3
	5	4.90 ± 0.62	12.57	−2.0	4.74 ± 0.36	7.59	−5.2
	50	50.39 ± 7.00	13.9	0.8	49.19 ± 3.86	7.85	−1.6

Table V
Results from Determination of the Stability of OA, p-CA, FA, Kaemperol and Quercetin ($n = 5$)

		24 h at room temperature			Three freeze and thaw cycles			7 or 14 days at −70°C		
Sample	Spiked concentration (ng/mL)	Measured concentration (ng/mL) (mean ± SD)	RSD(%)	Bias(%)	Measured concentration (ng/mL) (mean ± SD)	RSD(%)	Bias(%)	Measured concentration (ng/mL) (mean ± SD)	RSD(%)	Bias(%)
OA	1.5	1.41 ± 0.08	5.67	−6.1	1.51 ± 0.05	3.31	0.6	1.47 ± 0.07	4.76	2.0
	10	10.61 ± 0.59	5.59	6.1	9.91 ± 0.80	8.12	−0.9	9.37 ± 0.70	7.48	−6.3
	100	97.28 ± 6.21	6.38	−2.7	97.30 ± 10.31	10.60	−2.7	97.81 ± 1.92	1.96	−2.2
p-CA	50	44.47 ± 3.62	8.13	−11.1	46.53 ± 6.82	14.65	−6.9	53.58 ± 3.23	6.02	7.2
	500	472.06 ± 12.97	2.75	−5.6	491.75 ± 31.28	6.36	−1.6	559.73 ± 19.68	3.52	11.9
	5,000	4,734.56 ± 532.57	11.24	−5.3	4,942.36 ± 286.94	5.81	−1.2	4,707.25 ± 145.35	3.08	−5.9
FA	1.5	1.58 ± 0.13	8.23	5.3	1.48 ± 0.05	3.38	−1.3	1.51 ± 0.06	3.97	0.7
	5	4.78 ± 0.25	5.16	−4.4	4.66 ± 0.36	7.67	−6.8	4.90 ± 0.24	4.93	−2.0
	50	48.62 ± 6.00	12.33	−2.8	49.43 ± 3.50	7.09	−1.1	52.61 ± 4.45	8.45	5.2
Kaemperol	1.5	1.43 ± 0.13	9.09	−4.6	1.53 ± 0.11	7.81	2.0	1.56 ± 0.13	8.17	4.0
	5	5.00 ± 0.37	7.38	0.0	5.07 ± 0.40	7.85	1.4	5.17 ± 0.52	10.02	3.4
	50	48.25 ± 1.78	3.68	−3.5	49.86 ± 4.88	9.78	−0.3	48.92 ± 3.34	6.82	−2.2
Quercetin	1.5	1.55 ± 0.17	10.96	3.3	1.44 ± 0.12	8.33	−4.0	1.58 ± 0.09	5.69	5.3
	5	5.26 ± 0.13	2.46	5.2	4.92 ± 0.68	13.81	−1.6	4.95 ± 0.57	11.44	−1.0
	50	49.60 ± 4.32	8.72	−0.8	52.36 ± 5.93	11.32	4.7	52.55 ± 4.00	7.62	5.1

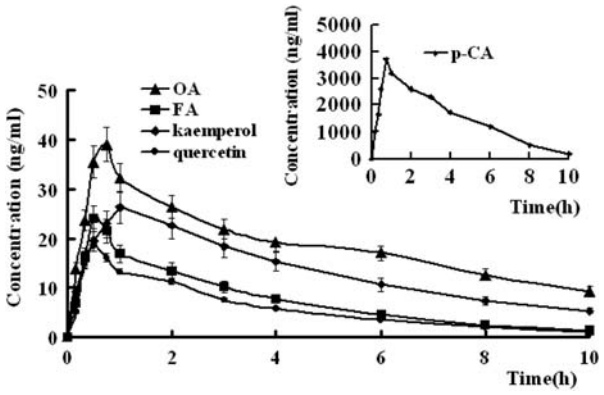

Figure 4. Mean plasma concentration-time profile for OA, p-CA, FA, kaemperol and quercetin in rat plasma after oral administration of *Oldenlandia diffusa* extract.

components were evaluated at three concentrations ($n = 5$). The results are shown in Table III.

Precision and accuracy

The precision and accuracy of the method were assessed in plasma by performing replicated analyses of spiked samples against calibration standards. The intra-day and inter-day precision and accuracy of the method are shown in Table IV.

Stability

Stability data is summarized in Table V, which indicates that the five analytes are stable in plasma for at least three freeze-thaw cycles. Moreover, the results of the stability showed that all

Table VI
Mean Pharmacokinetic Parameters of OA, p-CA, FA, Kaemperol and Quercetin in Rat Serum ($n = 6$) after Oral Administration of *Oldenlandia diffusa* Extract

Parameter	Compounds				
	OA	p-CA	FA	Kaemperol	Quercetin
$AUC_{0\to\infty}$ (ng/h/mL)	267.39 ± 44.36	15,957.16 ± 779.71	80.83 ± 10.28	166.84 ± 9.13	62.5 ± 2.46
C_{max} (ng/mL)	41.42 ± 6.62	3,765.41 ± 131.27	24.35 ± 2.93	26.60 ± 1.77	18.27 ± 0.82
t_{max} (h)	0.54 ± 0.05	0.80 ± 0.11	0.55 ± 0.07	1.08 ± 0.05	0.62 ± 0.04
K_{el} (h)	0.17 ± 0.01	0.46 ± 0.03	0.32 ± 0.03	0.18 ± 0.01	0.32 ± 0.02
K_a (h)	6.14 ± 0.34	0.81 ± 0.19	4.59 ± 0.61	2.73 ± 0.23	4.65 ± 0.45
$t_{1/2\alpha}$ (h)	0.11 ± 0.03	0.86 ± 0.07	0.17 ± 0.03	0.26 ± 0.02	0.15 ± 0.01
$t_{1/2\beta}$ (h)	4.18 ± 0.27	1.50 ± 0.11	2.19 ± 0.21	3.95 ± 0.28	2.21 ± 0.18

investigated compounds are stable for 24 h at room temperature and one or two weeks when kept frozen at −70°C. This indicated that OA, p-CA, FA, kaemperol and quercetin were stable in biological fluids under our experimental conditions, so the method was suitable for pharmacokinetic research in rats.

Application of the analytical method in pharmacokinetics study

The mean plasma concentration-time profiles for OA, p-CA, FA, kaemperol and quercetin are shown in Figure 4. The T_{max} was 0.54 ± 0.05, 0.80 ± 0.11, 0.55 ± 0.07, 1.08 ± 0.05 and 0.62 ± 0.04 h for OA, p-CA, FA, kaemperol and quercetin, respectively. The $t_{1/2\beta}$ of OA, p-CA, FA, kaemperol and quercetin was 4.18 ± 0.27, 1.50 ± 0.11, 2.19 ± 0.21, 3.95 ± 0.28 and 2.21 ± 0.18 h, respectively. The pharmacokinetic parameters are listed in Table VI. The information described herein might be helpful for further studies on the pharmacokinetics of *Oldenlandia diffusa*.

Conclusion

This paper described a simple, rapid, sensitive and validated LC–MS-MS bioanalytical method for simultaneous determination of OA, p-CA, FA, kaemperol and quercetin in rat plasma after oral administration of *Oldenlandia diffusa* extract, as well as an investigation of their pharmacokinetic studies. The assay provided adequate recovery with good precision and accuracy. The pharmacokinetic results contribute to a better understanding of the pharmacological action mechanism, and provide a firm basis for evaluating the clinical efficacy of *Oldenlandia diffusa*.

Acknowledgments

The National Natural Science Funds (No: 81102883), Guangdong Province Natural Science Funds (No: S2011010005540), Outstanding Doctoral Thesis Funds (No: sybzzxm201045) and 211 the National Programs of Development Fun for Fund for Colleges and Universities supported this work. The authors also gratefully appreciated Bing Wong (University of Toronto, Canada) for editing this manuscript.

References

1. Ganbolda, M., Braker, J., Ma, R., Jones, L., Carew, M.; Cytotoxicity and bioavailability studies on a decoction of *Oldenlandia diffusa* and its fractions separated by HPLC; *Journal of Ethnopharmacology*, (2010); 131: 396403.
2. Xu, G.J., Xu, L.S., Wang, Z.T.; *Species systematization and quality evaluation of commonly used Chinese traditional drugs. South-China edition*. Fujian Science and Technology Press, Fuzhou, China, (1997), pp. 658.
3. Chung, H.S., Jeong, H.J., Hong, S.H., Kim, M.S., Kim, S.J., Song, B.K., *et al.*; Induction of nitric oxide synthase by *Oldenlandia diffusa* in mouse peritoneal macrophages; *Biological and Pharmacological Bulletin*, (2002); 25: 1142–1146.
4. Yoshida, Y., Wang, M.Q., Liu, J.N., Shan, B.E., Yamashita, U.; Immunomodulating activity of Chinese medicinal herbs and *Oldenlandia diffusa* in particular; *International Journal of Immunopharmacology*, (1997); 19: 359–370.
5. Wong, B.Y., Lau, B.H., Yamasaki, T.; Inhibition of dexamethasone-induced cytochrome P450-mediated mutagenicity and metabolism of aflatoxin B1 of Chinese medicinal herbs; *European Journal of Cancer Prevention*, (1993); 2: 351–356.
6. Wong, B.Y., Lau, B.H., Jia, T.Y., Wan, C.P.; *Oldenlandia diffusa* and Scutellaria barbata augment macrophage oxidative burst and inhibit tumor growth; *Cancer Biotherapy and Radiopharmaceuticals*, (1996); 11: 51–56.
7. Lin, C.C., Ng, L.T., Yang, J.J., Hsu, Y.F.; Anti-inflammatory and hepatoprotective activity of peh-hue-juwa-chi-cao in male rats; *American Journal of Chinese Medicine*, (2002); 30: 225–234.
8. Lu, C.M., Yang, J.J., Wang, P.Y., Lin, C.C.; A new acylated flavonol glycoside and antioxidant effects of *Hedyotis diffusa*; *Planta Medica*, (2000); 66: 374–377.
9. Kim, D.H., Lee, H.J., Oh, Y.J., Kim, M.J., Kim, S.H., Jeong, T.S., *et al.*; Iridoid glycosides isolated from *Oldenlandia diffusa* inhibit LDL-oxidation; *Archives of Pharmacal Research*, (2005); 28: 1156–1160.
10. Kim, Y., Park, E.J., Kim, J., Kim, Y., Kim, S.R., Kim, Y.Y.; Neuroprotective constituents from *Hedyotis diffusa*; *Journal of Natural Products*, (2001); 64: 75–78.
11. Lu, H.C., He, J.; A study on chemical constituents of *Oldenlandia diffusa* (Wild) Roxb; *Natural Product Research and Development*, (1996); 8: 34–37.
12. Lu, C.M., Yang, J.J., Wang, P.Y., Lin, C.C.; A new acylated flavonol glycoside and antioxidant effects of *Hedyotis diffusa*; *Planta Medica*, (2000); 66: 374–377.
13. Ren, F.Z., Liu, G.S., Zhang, L., Niu, G.Y.; Studies on chemical constituents of *Hedyotis diffusa* Willd; *Journal of Chinese Pharmaceutical*, (2005); 40: 502–504.
14. Liu, J.; Pharmacology of oleanolic acid and ursolic acid; *Journal of Ethnopharmacology*, (1995); 49: 57–68.
15. Liu, J.; Oleanolic acid and ursolic acid: Research perspectives; *Journal of Ethnopharmacology*, (2005); 100: 92–94.
16. Kim, D.K., Baek, J.H., Kang, C.M., Yoo, M.A., Sung, J.W., Chung, H.Y.; Apoptotic activity of ursolic acid may correlate with the inhibition of initiation of DNA replication; *International Journal of Cancer*, (2000); 87: 629–636.
17. Graf, E.; Antioxidant potential of ferulic acid; *Free Radical Biology and Medicine*, (1992); 13: 435–448.

18. Beschia, M., Leonte, A., Oancea, I. Phenolic components with biological activity in vegetable extracts; *Bulletin of the University of Galati*, (1982); 6: 59–63.
19. Mussatto, S.I., Dragone, G., Roberto, I.C.; Ferulic p-coumaric acid extraction by alkaline hydrolysis of brewer's spent grain; *Industrial Crop and Products*, (2007); 25: 231–237.
20. Ou, S., Kwok, K.C.; Ferulic acid: Pharmaceutical functions, preparation and applications in foods; *Journal of the Science of Food and Agriculture*, (2004); 84: 1261–1269.
21. Faveri, D.D., Torre, P., Aliakbarian, B., Domínguez, J.M., Perego, P., Converti, A.; Response surface modeling of vanillin production by *Escherichia coli* JM109pBB1; *Biochemical Engineering Journal*, (2007); 36: 268–275.
22. Torres, J.L., Rosazza, J.P.N.; Microbial transformations of p-coumaric by Bacillusmegateriumand CurvuLaria lunata; *Journal of Natural Products*, (2001); 64: 1408–1414.
23. Ferguson, L.R., Zhu, S., Harris, P.J.; Antioxidant and antigenotoxic effects of plant cell wall hydroxycinnamic acids in cultured HT-29 cells; *Molecular Nutrition and Food Research*, (2005); 49: 585–593.
24. Liang, Z., He, M., Fong, W., Jiang, Z., Zhao, Z.; A comparable, chemical and pharmacological analysis of the traditional Chinese medicinal herbs *Oldenlandia diffusa* and *O. corymbosa* and a new valuation of their biological potential; *Phytomedicine*, (2008); 15: 259–267.
25. Razborsek, M.I., Voncina, D.B., Dolecek, V., Voncina, E.; Determination of oleanolic, betulinic and ursolic acid in lamiaceae and mass spectral fragmentation of their trimethylsilylated derivatives; *Journal of Chromatography A*, (2008); 67: 433–440.
26. Song, M., Huang, T.J., Wang, Y., Jiang, L., Wu, X.L., Zhang, Z.X., *et al.*; Determination of oleanolic acid in human plasma and study of its pharmacokinetics in Chinese healthy male volunteers by HPLC tandem mass spectrometry; *Journal of Pharmaceutical and Biomedical Analysis*, (2006); 40: 190–196.
27. Ji, H.Y., Shin, B.S., Jeong, D.W., Park, E.J., Park, E.S., Yoo, S.D., *et al.*; Interspecies scaling of oleanolic acid in mice, rats, rabbits and dogs and prediction of human pharmacokinetics; *Archives of Pharmacal Research*, (2009); 32: 251–257.