An integrated strategy based on UPLC–DAD–QTOF-MS for metabolism and pharmacokinetic studies of herbal medicines: Tibetan “Snow Lotus” herb (Saussurea laniceps), a case study

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An integrated strategy based on UPLC-DAD-QTOF-MS for metabolism and pharmacokinetic studies of herbal medicines: Tibetan “Snow Lotus” herb (Saussurea laniceps), a case study

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ABSTRACT

Ethnopharmacological relevance:
Saussurea laniceps Hand.-Mazz. (SL) has long been used under the herbal name Tibetan “Snow Lotus” for the treatment of rheumatoid arthritis, stomachache and dysmenorrhea in Tibetan folk medicine. Since herbal medicine (HM) is a synergistical system with multiple components, both of the metabolism and pharmacokinetic studies of HM are interdependent.

Aim of the study:
This study aimed to develop an integrated strategy based on UPLC-DAD-QTOF-MS technique for metabolism and pharmacokinetic studies of HM.

Material and methods:
SL was used here as a test herb to verify the feasibility of the proposed strategy. SL was administered to rats, then, the blood plasma, urine and feces was analyzed to determine the metabolic profiles. Using our strategy, umbelliferone and scopoletin were evaluated to be the key bioactive components. Their pharmacokinetic parameters were measured and biotransformation pathways were elucidated.

Results:
After oral administration of SL to rats, 17 components in blood, 10 components in urine and 2 components in feces were identified and characterized using our UPLC-DAD-QTOF-MS method. Umbelliferone, scopoletin and their metabolites were found to be the major components involved in the metabolism process. Literature reports also suggest that umbelliferone and scopoletin are responsible for the therapeutic effects of SL, thus these two components were selected as the active markers for pharmacokinetic study. In the test of validity, the established method presented good linearity with $R^2 > 0.99$. The relative standard deviation value was below 13.9% for precision, and recovery studies for accuracy were found to be within the range 91.8–112.5%.

Conclusion:
The present strategy offers, simultaneously, precision in quantitative analysis (metabolism study) and accuracy in quantitative analysis (pharmacokinetic study) with greater efficiency and less costs, which is therefore reliably used for integrated metabolism and pharmacokinetic studies of HM.

Keywords:
Herbal medicine; Snow Lotus; Saussurea laniceps; Metabolism; Pharmacokinetics; UPLC-DAD-QTOF-MS
1. Introduction

As the use of herbal medicines (HM) increases, there has been a growing awareness that metabolism and pharmacokinetic pattern of HM play as determinants of the HM action in vivo (Bent, 2008). However, HM is a synergistical system which contains multiple components, metabolism and pharmacokinetic studies of HM remain very challenging up to now. Because of the complicated chemical composition of HM, a number of compounds may enter the circulation and then be converted into even more metabolites, leading to the complexity and difficulties for the metabolism studies. To clarify “which are absorbed” (chemical constituents of HM) and “which are produced” (biotransformed metabolites) become the basis for metabolism and pharmacokinetic studies (Xiang et al., 2011; Qiao et al., 2012). Moreover, the selection of markers for the pharmacokinetic studies should not only based on the abundance and biological activities of the constituents in HM, but also should take into consideration that they are major compounds that get into the circulation to exhibit the therapeutic effects. As a conclusion, both of the metabolism and pharmacokinetic studies of HM are interdependent.

However, most current reports separate the metabolism and pharmacokinetics in the in vivo studies of HM. They just follow the procedures for chemical drugs, and use one or a few biological-effective marker compounds for metabolism or pharmacokinetic assessments after administration of the pure compounds obtained from HM (Qiu, 2010; Cai et al., 2003; Yang et al., 2005; Xia et al., 2007), or not taking the integrity of metabolism and pharmacokinetics of HM into consideration (Song, 2010; Wen et al., 2012; Wang et al., 2008; Bao et al., 2009). Since the therapeutic effect of HM is achieved by an array of phytochemicals, the co-existence of multiple compounds may lead to metabolism and pharmacokinetics of HM drastically different from those of pure compounds. The individual results thus could not represent the holistic metabolic profiles and comprehensively reveal the pharmacokinetic behaviors of HM. Moreover, the separately conducted studies for metabolism or pharmacokinetics requires a variety of expensive equipment, significantly increase the cost of study and also time-consuming.

Therefore in the present work, we propose a strategy to logically integrate the metabolism and pharmacokinetics for the in vivo study of multi-component HM, based on ultrahigh performance liquid chromatography coupled with diode array detection and quadrupole time-of-flight mass spectrometry (UPLC-DAD-QTOF-MS) technique. A flowchart illustrating the strategy is shown in Fig. 1. The strategy consists of three steps: 1) Firstly, characterize the absorbed constituents and their metabolites in plasma, urine and feces by UPLC-DAD-QTOF-MS after HM oral administration; 2) Secondly, the
marker components for pharmacokinetic study are generated based on global metabolic profiles and combining with the abundance and biological activities of the candidate constituents; 3) Thirdly, the corresponding markers were quantified in plasma for pharmacokinetic study by UPLC-DAD-QTOF-MS, and also the biotransformation pathway was provided to clarify the potential bioactive mechanisms of HM. This strategy was applied by using Tibetan “Snow Lotus” herb (*Saussurea laniceps*) as a model herb.

**Insert Fig. 1 here**

Tibetan “Snow Lotus” herb (*Saussurea laniceps*, SL) was selected as the test herb because it is a well-known HM for the treatment of rheumatoid arthritis and stomachache in China (Commission of Chinese Ethnomedicine, 1984; Commission of Chinese Materia Medica, 1999). However, the lack of metabolism study hinders its further study and wider clinical use. In our previous study, we have already elucidated the chemical composition of SL by using high performance liquid chromatography (Yi et al., 2009a; Yi et al., 2009b), investigated the anti-inflammatory effects by using carrageenan-induced paw edema in rats and xylene-induced ear edema in mice, and observed the anti-nociceptive effects by using acetic acid-induced writhing and hot-plate test in mice (Yi et al., 2010; Yi et al., 2012), which lay a solid foundation for the further metabolism and pharmacokinetic studies.

In the present study, to verify the feasibility of our proposed strategy, SL herb was selected as a test case and administered to rats, then, the blood plasma, urine and feces was analyzed to determine the metabolic profiles. Using our strategy, umbelliferone and scopoletin were evaluated to be the key bioactive components. Their pharmacokinetic parameters were measured and biotransformation pathways were elucidated. Our study of SL demonstrates that the proposed strategy is efficient, precise and accurate, and can be used for integrated metabolism and pharmacokinetic studies of HM.

**2. Materials and methods**

**2.1. Chemicals and reagents**

The standard compounds of chlorogenic acid, 3,4-dicaffeoylquinic acid, 1,5-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, apigenin 7-<sup>O</sup>-β-D-glucoside and 4,5-dicaffeoylquinic acid were purchased from the Biopurity Phytochemicals Ltd. (Chengdu, China). Syringoside and scopoletin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).
Umbelliferone were purchased from Fluka (Buchs, Switzerland). The internal standard (I.S.) of coumarin 120 was purchased from Phytomarker Ltd. (Tianjin, China). Stock solutions of all standard compounds were prepared in methanol at a concentration of 0.1 mg/mL and stored at 4 °C until use. For the chemical identification, the solutions were prepared by appropriate dilution of the stock solutions with methanol. Their chemical structures are shown in Fig. 2.

Ethanol and formic acid of analytical grade were purchased from Merck (Darmstadt, Germany). Acetonitrile of chromatography grade was purchased from Lab-scan (Bangkok, Thailand). Methanol of analytical grade was purchased from Lab-scan (Bangkok, Thailand). The water used in the experiments was collected from a Mili-Q ultrapure water system (Millipore; Bedford, MA, USA). Other chemicals (analytical grade) were purchased from Sigma (St. Louis, MO).

2.2. Herb materials and extraction

Samples of *Saussurea laniceps* (SL) were collected from Tibet in 2008. The dried herbal materials (0.2 kg) were cut into small pieces, and then soaked in 50% ethanol (5 L) at room temperature for 2 days with occasional shaking. The supernatant was filtered. This procedure was repeated once, and then the combined filtrates were evaporated to remove ethanol in a rotary evaporator (40 °C). The wet residues were lyophilized with a Labconco freeze-dry system to obtain the SL extract. Quantitative analysis of the main constituents was performed as previously described (Yi et al., 2009a), and the contents of main constituents are labeled in Fig. 3 and shown in Table S1 in the Supplementary materials. For administration to animals, the dried extracts were suspended in 1% (w/v) aqueous carboxy methylcellulose.

2.3. Instrumentation and conditions

An ultrahigh performance liquid chromatography coupled with diode array detector and quadrupole time-of-flight mass spectrometry (UPLC-DAD-QTOF-MS, Agilent Technologies, G6540A) system was used for metabolism and pharmacokinetic analyses. A Waters HSS C₁₈ column (1.8 μm, 2.1 × 100 mm, Waters Corp.) with a VanGuardTM pre-column (HSS C₁₈, 1.7 μm, 2.1 × 5 mm) was used for chromatographic separation. The column was eluted with a gradient mixture of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) at the flow rate of 0.3 mL/min. A gradient elution was adopted by water of 0.1% formic acid aqueous (A) and acetonitrile (B) was used as 2% B at 0-2
min, 2-10% B at 2-14 min, 10-18% B at 14-22 min, 18-25% B at 22-30 min. The column temperature was held at 40 °C and the detection wavelength was set to 280 nm. For the mass spectrometer conditions, an electrospray ionization (ESI) source was used, and the desolvation temperature was set at 300 °C, desolvation gas (N\textsubscript{2}) 8 L/min, fragmentor voltage 150 V, capillary voltage 5.0 kV for positive ion mode and 4.5 kV for negative ion mode. Sheath gas was set at 400 °C and 10 L/min for positive ion mode; 350 °C and 9 L/min for negative ion mode. MS\textsuperscript{2} data were acquired in the automatic data-dependant mode, and the collision energy was simultaneously set at 10, 20, 30 and 40 eV. All the results were analyzed by Agilent MassHunter Workstation with Quantitative Analysis (Q-TOF) B.04.00 software.

2.4. Metabolism study in rat plasma, urine and feces

2.4.1. Animals

Male Sprague–Dawley rats weighing 180-230 g were purchased from the Laboratory Animal Services Center, the Chinese University of Hong Kong, Hong Kong. The rats were housed individually in metabolic cages with temperature of 23 ± 1 °C, humidity of 60 ± 5%, and 12 h dark – light cycle. They were given tap water and fed normal food *ad libitum*. All the experiment animals were housed under the above conditions for 7 days’ acclimation, and were fasted overnight before the experiments. All experimental protocols were approved by the Committee on the Use of Human & Animal Subjects in Teaching and Research of Hong Kong Baptist University, in accordance with the Animals Ordinance (Department of Health, Hong Kong).

2.4.2. Drug administration and collection of plasma, urine and feces samples

A single oral administration dose (1.0 g/kg) of SL extract was given to each of two rats. The retro-orbital blood samples (c.a. 300 μL) were withdrawn via the cannular prior to dosing (*t* = 0) and at 20, 40, 60 min post-dosing of SL extract. Plasma was obtained by centrifuging the blood at 4000 rpm for 10 min, and then combined together. Methanol (1.1 mL) was added into 100 μL combined plasma and well mixed, then centrifuged at 12000 rpm for 10 min to precipitate the protein. The supernatant was dried using a vacuum oven at 37 °C, and reconstituted with 100 μL methanol to obtain the plasma sample. Rat urine and feces samples were collected from 0 to 24 h after the oral administration of SL extract. The urine sample (0.5 mL) was well mixed with 10-fold volume of methanol, and then centrifuged at 12000 rpm for 10 min to obtain the supernatant for analysis. The feces sample were dried in air and then ground into powder. The powder (0.5 g) was extracted by 20-fold of methanol in an ultrasonic bath for 30 min, and then centrifuged at 12000 rpm for 10 min to obtain the supernatant for analysis. An aliquot of 2 μL for the sample solution was analyzed by UPLC-DAD-QTOF-MS.
2.5. Pharmacokinetic study of umbelliferone (6) and scopoletin (7) in plasma

2.5.1. Preparation of standard solutions

Mixed calibration standards of umbelliferone (6) and scopoletin (7) were prepared by serial dilution with blank rat plasma yielding final concentrations of 0.05, 0.1, 0.2, 0.5, 1, 5 and 10 μg/mL. The working internal standard solution was prepared by diluting the primary stock solution with methanol giving a concentration of 50 μg/mL. Quality control (QC) samples were prepared at low (0.1 μg/mL), medium (1 μg/mL) and high (5 μg/mL) concentrations of umbelliferone (6) and scopoletin (7) in the same way as the plasma samples. Aliquots of 1 mL of methanol and 10 μL of I.S. solution were added to 100 μL of standard spiked plasma samples. The mixture was then vortex-mixed for 5 min and centrifuged at 12000 rpm for 10 min, and the supernatant was evaporated to dryness using a vacuum oven at 37 °C. The residue was then reconstituted with 100 μL of methanol, and an aliquot of 2 μL for the sample solution was injected into the UPLC-DAD-QTOF-MS system.

2.5.2. Drug administration and blood sampling

A single oral administration dose (1.0 g/kg) of SL extract was given to a group of six rats. The retro-orbital blood samples (c.a. 300 μL) were withdrawn via the cannula prior to dosing (t = 0) and at 10, 20, 30, 40, 60, 80, 100, 120 and 180 min post-dosing of SL extract. Plasma was obtained by centrifuging the blood at 4000 rpm for 10 min. Aliquots of 1.0 mL of methanol and 10 μL of I.S. solution were added to 100 μL of plasma samples. The mixture was then vortex-mixed for 5 min and centrifuged at 12000 rpm for 10 min, and the supernatant was evaporated to dryness using a vacuum oven at 37 °C. The residue was then reconstituted with 100 μL of methanol, and an aliquot of 2 μL for the sample solution was injected into the UPLC-DAD-QTOF-MS system.

2.5.3. Method validation for the pharmacokinetic study

The method developed in the present study has been validated for selectivity, linearity, sensitivity, precision, accuracy, matrix effects, recovery and stability according to the US Food and Drug Administration (FDA) bioanalytical method validation guidelines (Food and drug Administration, 2013). The acceptance criteria of precision and accuracy should be within 15% of the actual value except at lower limit of quantitation, where it should not deviate by more than 20%.
2.5.4. Data analysis

The pharmacokinetic parameters, including the maximal plasma concentration (C_max), the time for maximal concentration (T_max) and half-life (T_1/2), the area under the plasma concentration (AUC)–time curve and clearance (Cl), were calculated using non-compartmental methods with Kenetica 2000 software (Version 3.0, Thermo Fisher Scientific, Philadelphia, PA). All data are expressed as mean ± standard deviation (SD).

3. Results and discussion

3.1. Optimization of sample pretreatment

In order to remove endogenous interferences in plasma and to obtain satisfactory separation of the analytes, deproteinization procedures including precipitation with organic solvents and solid-phase extraction (SPE) were tested. The results from this investigation demonstrated that serum protein precipitation caused by adding organic solvent followed by centrifugation was preferable because of its efficacy and ease of handling. The choice of organic solvent was based on a comparison of ethanol, methanol and acetonitrile. Overlapped peaks of analytes were observed in sample precipitated with acetonitrile, and the use of ethanol to precipitate serum proteins resulted in relatively lower values for extracting the analytes. Therefore, methanol was chosen to remove serum proteins and extract analytes. Solvent consumption was further tested, and the optimal conditions were presented in detail above, in the section of “2.4.2. Drug administration and blood, urine and feces sample collection”.

3.2. Optimization of the chromatographic and mass conditions

The conditions for liquid chromatography were optimized based on our previous study (Yi et al., 2010; Yi et al., 2012). Various linear gradients of water and acetonitrile at a flow rate of 0.3 mL/min were further optimized to distinctly separate all of the characteristic peaks within 25 min.

Regarding the optimization of MS conditions, the factors affecting ionization, including formic acid concentration (0.05-0.2%), desolvation gas temperature (250-350 °C), desolvation gas flow (6-9 L/min), sheath gas temperature (300-400 °C), sheath gas flow (7-12 L/min), capillary voltage (positive ion mode 3.5-5.0 kV; negative ion mode 3.0-4.5 kV), nozzle voltage (0.5-2.0 kV), fragmentor (0.1-0.3 kV) were adjusted, and the response of the analytes in the mass spectrum was taken as criteria for optimization. The optimized conditions were presented in detail above, in the section of “2.3 Instrumentation and conditions”.
3.3. Metabolism study in rat plasma, urine and feces

3.3.1. Characterization of the main constituents in SL extracts

HM is a complex system with multiple components. When an HM is ingested, these multiple components then generate metabolites, further complicating the metabolic picture. For this reason, identification of the natural constituents in herbal products remains the primary task for further in vivo research.

Insert Fig. 3 here

Before the study on metabolic profiles of SL extract administered to rats, the constituents in extract were separated by UPLC-DAD-QTOF-MS (Fig. 3). The identified compounds and their data of (±) ESI-MS, (±) ESI-MS$^2$ and UV scan spectra are shown in Table 1. For most of the constituents, $[M+H]^+$, $[M+Na]^+$, $[M-H]$ and $[M+HCOO]$ were observed to identify their quasi-molecular ion. By comparing the molecular weight and retention time with standard compounds, peaks 2, 4, 6, 7, 9, 10, 11, 13 and 14 were attributed to chlorogenic acid, syringoside, umbelliferone, scopolin, 3,4-dicaffeoylquinic acid, 1,5-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, apigenin 7-$\beta$-D-glucoside and 4,5-dicaffeoylquinic acid, respectively (Table 1). Other peaks (1, 3, 8 and 12) were identified to be skimmin, scopolin, isoquercitroside and apigenin 7-$\alpha$-L-rhamnosyl-(1→2)-$\beta$-D-glucoside by comparing their UV spectra, molecular weight and characteristic mass fragments with data reported [26, 27]. For the unknown peak 5 and peak 15, the identification is in progress. Overall, the results of SL extract characterization show that the main constituents of SL extract are coumarins (1, 3, 6 and 7), phenol acids (2, 9, 10, 11 and 14), flavonoids (8, 12 and 13) and lignanoids (4).

Insert Table 1 here

3.3.2. Metabolic profiling of plasma sample

Typical chromatograms of plasma sample are shown in Fig. 4. The constituents in rat plasma after oral administration of SL were separated and identified by comparing the chromatograms of SL extract and control plasma. Seventeen peaks relevant to SL extract were identified. Their UV, MS and MS$^2$ data as well as the identification results are shown in Table 1.

Insert Fig. 4 here
In the rat plasma samples, most of the original constituents from SL extract were detected, with umbelliferone (6) and scopoletin (7) being the most absorbed components. In terms of the relative abundance of the constituents, the coumarins especially 6 and 7 are absorbed more easily than other constituents as phenol acids (2 and 10) which possessing comparable abundance with 6 and 7 in the original SL extract. The existence of umbelliferone (6) and scopoletin (7) in plasma were assumed to be come from two pathways: the original constituents of SL, or the products of skimmin (1, the glycoside of 6) and scopolin (3, the glycoside of 7) through a hydrolysis reaction of phase I metabolism.

Insert Fig. 5 here

Moreover, four metabolites (M1, M2, M3 and M4) relevant to 6 and 7 possessing significant abundance in the plasma were elucidated by their UV absorption, high resolution MS (HRMS) spectrum and comparison with their original compounds. Take M1 for example. The UV absorptions of M1 at $\lambda_{max}$ 198, 215, and 320 nm (Fig. 5a), meeting the UV features of umbelliferone (6) (Table 1). The exhibition of [$M+H]^+$ ion at accurate mass of $m/z$ 339.0806 and [$M+Na]^+$ ion at accurate mass of $m/z$ 361.0531 in the positive MS spectra (Fig. 5b) coming with the [$M-H]^-$ ion at accurate mass of $m/z$ 337.0691 in the negative MS spectra (Fig. 5c) irrefutably elucidated the molecular weight of M1 to be 338, with the molecular formula estimated to be C$_{15}$H$_{14}$O$_9$. The characteristic mass fragments were further confirmed by conducting the MS$^2$ fragmentation of the quasi-molecular ion [$M+H]^+$ of M1 at $m/z$ 339.0806, as illustrated in Fig. 5d. All the elemental composition of the fragments was calculated by the accurate mass determined by HRMS. To simplify, all MS data below were demonstrated as the nominal mass. The quasi-molecular ion at $m/z$ 339 lose a glucuronic acid moiety (176 Dalton) to generate the fragment ion at $m/z$ 163, which was further spitted into C$_7$H$_7$O at $m/z$ 107 and C$_8$H$_7$O at $m/z$ 119 and subsequently into C$_7$H$_7$ at $m/z$ 91. Therefore, M1 was elucidated as the glucuronide of umbelliferone (6) and named as umbelliferone glucuronide. By the similar way, M3 was elucidated to be the sulfate conjugated umbelliferone (6) and named as umbelliferone sulfate. M2 and M4 were elucidated to be glucuronide and sulfate of scopoletin (7), named as scopoletin glucuronide and scopoletin sulfate, respectively (details of the spectrums for elucidation were shown in Supplementary materials Fig. S1, Fig. S2 and Fig. S3). The metabolism of umbelliferone (6) and scopoletin (7) to M1, M3 and M2, M4 was further confirmed by the analysis of plasma samples obtained by oral administration of pure umbelliferone (6) and scopoletin (7) to rats (for detailed data, see the Supplementary materials Fig. S4 and Fig. S5). This finding reveals that glucuronidation and sulfation are the main phase II metabolism of umbelliferone (6) and scopoletin (7) after their absorption into the
blood stream. The original and the metabolites of umbelliferone (6) and scopoletin (7) co-existed in the blood.

3.3.3. Metabolic profiling of urine sample

Ten components relevant to SL extract were identified in urine samples by the same method as plasma sample (Fig. 6.), namely, six original components (1, 3, 4, 6, 7 and 12) and four metabolites (M1, M2, M3 and M4).

The metabolic profiles of rat urine sample demonstrated that: firstly, a few compounds (1, 3, 4 and 12), which were absorbed into blood from SL extract, were mainly excreted to urine in their original form. Secondly, umbelliferone (6) and scopoletin (7) were excreted to urine mainly in the form of metabolites (M1, M2, M3 and M4) and auxiliary to the original form. Thirdly, phenol acids (9, 10, 11 and 14) and flavonoids (8 and 13), which existed in plasma samples, have not been detected in urine samples. Their absence may be due to their low concentration in plasma, and/or the ease with which they are degraded to little unknown molecules for excretion.

Insert Fig. 6 here

3.3.4. Metabolic profiling of feces sample

The composition of rat feces is complex, and the constituents derived from SL are rare. Thus, the potential compounds relevant to SL extract were screened by an extracted ion chromatogram (EIC) analysis of all the components listed in Table 1, but only two components (6 and 7) were found (Fig. 7.).

Insert Fig. 7 here

Metabolic profiling showed traces of umbelliferone (6) and scopoletin (7) in the feces sample. They may have come from two sources. Firstly, they may be residual substances from SL extracts after gastrointestinal digestion and absorption. Secondly, a portion of metabolites of umbelliferone (6) and scopoletin (7) in the plasma may have been excreted from the rat liver to the bile, and then flowed into the small intestine where they were hydrolyzed by the intestinal flora. Most of the liberated umbelliferone (6) and scopoletin (7) were reabsorbed into the bloodstream, and a small amount of the two components remained in the feces.

In summary, the present method exhibited a satisfactory ability to precisely identify and comprehensively characterize the original constituents and metabolites after SL oral administration.
From the results of metabolism study, umbelliferone (6) and scopoletin (7) were found to be the two key components involved in the absorption, distribution and elimination process.

3.4. Pharmacokinetic study in plasma

3.4.1. Selection of representative markers

In our above completed study, umbelliferone (6) and scopoletin (7) exhibited an important role in the metabolic process after SL administration. The two components are also reported to be responsible for the therapeutic effect of SL for the treatment of rheumatoid arthritis and stomachache (Tanaka et al., 1977; Meotti et al., 2006; Bansal et al., 2013; Ou et al., 2000). Umbelliferone (6) and scopoletin (7) showed cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) dual inhibitory activity (Kim et al., 2006), which suggests that the two compounds produce anti-inflammatory activity in part via the inhibition of the generation of eicosanoids. It has also been reported that umbelliferone (6) and scopoletin (7) exhibit significant and dose-related anti-nociceptive effects against acetic acid-induced visceral pain in mice (Meotti et al., 2006; Bansal et al., 2013; Ou et al., 2000). Therefore, umbelliferone (6) and scopoletin (7) were selected as the representative markers for the pharmacokinetic study of SL.

3.4.2. Quantitation method

QTOF mass was operated in positive ion mode under full-scan conditions. Quantification was carried out by using extracted ion chromatograms (EICs) of 163.0390 for the \([M+H]^+\) ion of umbelliferone (6), m/z 193.0495 for the \([M+H]^+\) ion of scopoletin (7) and m/z 176.0706 for the \([M+H]^+\) ion of coumarin 120 (I.S.) with a 20 ppm window. Their peak areas were integrated for quantification. From the typical chromatograms of Fig. 7, it was shown that the present method was selective and the endogenous constituents did not affect the analysis.

\textit{Insert Fig. 8 here}

3.4.3. Linearity and sensitivity

The calibration curve was constructed based on the peak-area ratios of analyte to I.S. (x) vs. concentrations of spiked analyte (y). The results are shown in Table 2. The limit of detection (LOD) and limit of quantitation (LOQ) of the analyte were visually evaluated with a signal-to-noise ratio of about 3:1 and 10:1, respectively. Meanwhile, the precisions of the analyte at LOQ were less than 16.1%, and the accuracies were found to be within ± 14.5 % from their nominal values.
3.4.4. Precision and accuracy

The precision and accuracy of the present method were determined by analyzing 5 replicates of the QC samples at 3 concentration levels (0.1, 1.0 and 5.0 μg/mL) for a successive 5 days, and the results are shown in Table 3. The results show that the relative standard deviation (RSD) values of intra- and inter-day precision were less than 13.9%, and the accuracies ranged between 91.8 and 112.5%. Thus, the present UPLC-DAD-QTOF-MS method exhibited satisfactory precision and accuracy.

3.4.5. Matrix effects

Matrix effects were assessed by comparing the peak areas of analytes at three levels of QC concentrations in (A) reconstitution methanol or (B) the supernatant of extracted blank plasma. The peak area ratio of B/A was used to measure the matrix effect. Results in Table 4 showed that no significant ion suppression or enhancement was observed for any of the analytes under the present experimental conditions.

3.4.6. Recovery and stability

Recovery of the present method was determined by comparing the mean peak areas of the QC samples to those of the equivalent standard solution in methanol using five replicates. The recovery of internal standard was determined by the same method. The recovery ranged from 87.6 to 93.0%.

The stability of umbelliferone (6) and scopoletin (7) in rat plasma under different storage conditions is summarized in Table 5. The recovery was found to range from 92.1 to 113.0% for three QC concentrations. The results indicate that the plasma samples withstand three freeze-thaw cycles, and umbelliferone and scopoletin were stable in plasma kept at room temperature for at least 24 h and stored at -80 °C for at least 30 days.
3.4.7. Pharmacokinetic study

The above fully validated method was applied for a pharmacokinetic study of SL orally administered to rats. Fig. 8 shows a typical chromatogram of a plasma sample obtained from a rat at 20 min after oral administration of SL extract. The mean plasma concentration-time curves are shown in Fig. 9, and the main pharmacokinetic parameters are listed in Table 6.

Insert Fig. 9 here
Insert Table 6 here

Of note, two peak concentrations of umbelliferone (6) and scopoletin (7) after SL oral administration were observed at 20 and 60 min in the Fig. 9, respectively. Several reasons could explain this. Firstly, for reasons of timing of gastric emptying, the SL extract may have reached the small intestine in two portions. Alternatively, there may have been sustained release into the blood, thereby causing two peaks. Secondly, there may have been an enterohepatic circulation effect. Umbelliferone (6) and scopoletin (7) are coumarins, and there have been several reports on the enterohepatic circulation of coumarins (Kim et al., 2006; Ye et al., 1999; Feng et al., 2010). Our detection of small amounts of umbelliferone (6) and scopoletin (7) in the metabolic profile of feces also suggests an enterohepatic circulation (Fig. 7). Thirdly, umbelliferone (6) and scopoletin (7) are lipophilic drugs. They are likely to be rapidly distributed to the tissues after absorption, with a second release into the blood when the drugs are metabolized. There is a report that, after oral administration of scopoletin (7), it can be distributed to the rat heart, liver and lung and kidney (Xia et al., 2012). Lastly, skimmin (1, the glycoside of 6) and scopolin (3, the glycoside of 7) were detected in the metabolic profiling of plasma (Fig. 4). Thus they may be hydrolyzed into umbelliferone (6) and scopoletin (7) in the blood. Overall, the double-peak curves of umbelliferone (6) and scopoletin (7) in the present study can be explained by the above-described four scenarios, and ultimately probably represent a combination effect.

From the results of pharmacokinetic study, the established method exhibited a good linearity, precision and accuracy with high sensitivity. The proposed UPLC-DAD-QTOF-MS strategy is suitable for quantitative pharmacokinetics study of multi-component herbal medicines.

3.5. Elucidation of the potential metabolic pathways

Insert Fig. 10 here
Based on the completed metabolism and pharmacokinetic studies, the potential metabolic pathways of SL emerged as shown in Fig. 10.

Although SL is a HM with multiple components, it was found that umbelliferone (6) and scopoletin (7), play a pivotal role after SL administration. As two main components of SL (Fig. 3), umbelliferone (6) and scopoletin (7) were rapidly absorbed into bloodstream after SL oral administration to rats (Fig. 9, T_{max} = 20 min). The hydroxyl groups in umbelliferone (6) and scopoletin (7) could be glucuronidated into M1 and M2 by the UDP-glucuronosyltransferase (UGT), and be sulfated into M3 and M4 by the tyrosylprotein sulfotransferases (TPST) in blood (Fig. 4 and Fig. 10). Skimmin (1) and scopolin (3) may be respectively biotransformed to umbelliferone (6) and scopoletin (7) by the hydrolysis reaction, and then the products are glucuronidated and sulfated (Fig. 10).

It appears that most of the glucuronic acid conjugates and sulfate conjugates were readily secreted in the urine (Fig. 6 and Fig. 10). A small amount of conjugates were eliminated in the bile, and then hydrolyzed by the β-glucuronidase in the intestinal flora, and the liberated umbelliferone (6) and scopoletin (7) were reabsorbed into the bloodstream to form the double-peak curves in the plasma pharmacokinetics (Fig. 9). Only a trace of the umbelliferone (6) and scopoletin (7) were excreted to the feces (Fig. 7).

4. Conclusions

An integrated strategy based on UPLC-DAD-QTOF-MS technique for metabolism and pharmacokinetic studies of HM was proposed, and validated using Tibetan “Snow Lotus” (Saussurea laniceps, SL) as a test herb for the first time. Using this strategy, the metabolism and pharmacokinetic studies of SL were logically and successfully conducted.

The present strategy combines the precision of UPLC-DAD-QTOF-MS in qualitative analysis with the accuracy of quantitative analysis, which is suitable for analyzing multiple components in a biochemically complex system. Therefore, the strategy could be generally used for integrated metabolism and pharmacokinetic studies of HM, thereby facilitating valuable pharmaceutical research and clinical advances.

Acknowledgements

This research was funded by the Faculty Research Grant of Hong Kong Baptist University (FRG/08-09/II-52) and General Research Fund of Hong Kong (HKBU-260111).
Abbreviations: HM, Herbal medicine; SL, Saussurea laniceps; UPLC, Ultra performance liquid chromatography; DAD, Diode array detection; QTOF-MS, Quadrupole time-of-flight mass spectrometry; ESI, Electrospray ionization; BPC, Base peak chromatogram; EIC, Extracted ion chromatogram; QC, Quality control; RSD, Relative standard deviation; COX-2, Cyclooxygenase-2; 5-LOX, 5-Lipoxygenase; HRMS, High resolution mass spectra.

References


Legends for Tables

Table 1
Identified components from SL extract, rat plasma, urine and feces after oral administration of SL extract by UPLC-DAD-(±)MSⁿ.

Table 2
Regression data and limits of detection and quantification.

Table 3
Intra- and inter-day precision and accuracy (n = 5).

Table 4
Recovery and matrix effect of two analytes and I.S. (n = 5).

Table 5
Stability of two analytes in rat plasma (n = 5).

Table 6
Pharmacokinetic parameters for umbelliferone (equivalent to 23.4 mg/kg) and scopoletin (equivalent to 35.8 mg/kg) in rats plasma after oral administration of SL extract (1.0 g/kg) (Mean ± SD, n = 6).
<table>
<thead>
<tr>
<th>Peak</th>
<th>RT (min)</th>
<th>Compounds</th>
<th>Formula</th>
<th>Positive MS fragments (m/z)</th>
<th>Positive MS² fragments (m/z and % base peak)</th>
<th>Negative MS fragments (m/z)</th>
<th>Negative MS² fragments (m/z and % base peak)</th>
<th>UVₘₚₜ (nm)</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>8.1</td>
<td>Umbelliferone glucuronide</td>
<td>C₁₂H₁₀O₅</td>
<td>361 [M+Na]⁺</td>
<td>[339, 40 ev]: 163(100), 113(82), 175(25)</td>
<td>[337, 10 ev]: 161(100), 198</td>
<td>113(82), 175(25), 337(20), 85(19), 59(15)</td>
<td>198</td>
<td>Plasma</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>339 [M+H]⁺</td>
<td>107(60), 119(25), 91(25), 85(19)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>163 [M-gluA+H]⁺</td>
<td>77(5)</td>
<td></td>
<td></td>
<td>215</td>
<td>Urine</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>347 [M+Na]⁺</td>
<td>[325, 40 ev]: 163(100), 113(82), 175(25)</td>
<td></td>
<td></td>
<td></td>
<td>Extract</td>
</tr>
<tr>
<td></td>
<td>8.9</td>
<td>Skimmin</td>
<td>C₁₂H₁₀O₅</td>
<td>325 [M+H]⁺</td>
<td>107(71), 119(31), 91(30), 85(19)</td>
<td></td>
<td></td>
<td>203</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>163 [M-glu+H]⁺</td>
<td>77(7)</td>
<td></td>
<td></td>
<td>204</td>
<td>Extract</td>
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<tr>
<td>M2</td>
<td>10.4</td>
<td>Scopoletin glucuronide</td>
<td>C₁₂H₁₀O₁₀</td>
<td>391 [M+Na]⁺</td>
<td>[369, 40 ev]: 193(100), 135(100), 85(50)</td>
<td></td>
<td></td>
<td>318</td>
<td>Urine</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>369 [M+H]⁺</td>
<td>133(85), 178(65), 137(28), 77(7)</td>
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<td></td>
<td></td>
<td>193 [M-gluA+H]⁺</td>
<td>122(10)</td>
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<td>10.5</td>
<td>Chlorogenic acid</td>
<td>C₁₂H₁₀O₃</td>
<td>377 [M+Na]⁺</td>
<td>[355, 40 ev]: 135(100), 113(82), 77(7)</td>
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<td>355 [M+H]⁺</td>
<td>163(95), 117(88), 145(70), 93(15)</td>
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<td>M3</td>
<td>10.7</td>
<td>Umbelliferone sulfate</td>
<td>C₆H₈O₆S</td>
<td>243 [M+H]⁺</td>
<td>[243, 30 ev]: 107(100), 133(75), 93(15)</td>
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<td>Plasma</td>
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<td></td>
<td></td>
<td>163 [M-sulf+H]⁺</td>
<td>163(70), 91(35), 119(25)</td>
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</tr>
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<td></td>
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<td>377 [M+Na]⁺</td>
<td>[355, 30 ev]: 193(100), 133(75), 93(15)</td>
<td></td>
<td></td>
<td>312</td>
<td>Urine</td>
</tr>
<tr>
<td></td>
<td>11.1</td>
<td>Scopolin</td>
<td>C₁₂H₁₀O₃</td>
<td>355 [M+H]⁺</td>
<td>[355, 30 ev]: 193(100), 133(75), 93(15)</td>
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<td>Plasma</td>
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<td></td>
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<td></td>
<td>193 [M-glu+H]⁺</td>
<td>165(5)</td>
<td></td>
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<td>Urine</td>
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<td>3</td>
<td>11.1</td>
<td>Scopolin</td>
<td>C₁₂H₁₀O₃</td>
<td>395 [M+Na]⁺</td>
<td>[373, 20 ev]: 161(100), 93(20), 113(6)</td>
<td></td>
<td></td>
<td>205</td>
<td>Plasma</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>373 [M+H]⁺</td>
<td>[373, 20 ev]: 161(100), 93(20), 113(6)</td>
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<td>4</td>
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<td>Syringoside</td>
<td>C₁₇H₁₂O₃</td>
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<td>[373, 20 ev]: 161(100), 93(20), 113(6)</td>
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<td>Plasma</td>
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<td></td>
<td>373 [M+H]⁺</td>
<td>[373, 20 ev]: 161(100), 93(20), 113(6)</td>
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<td>M4</td>
<td>12.5</td>
<td>Scopoletin sulfate</td>
<td>C₁₀H₉O₄S</td>
<td>273 [M+H]⁺</td>
<td>[273, 30 ev]: 133(100), 113(82), 91(25)</td>
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<td>205</td>
<td>Plasma</td>
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<td>193 [M-sulf+H]⁺</td>
<td>[273, 30 ev]: 133(100), 113(82), 91(25)</td>
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<td>6</td>
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<td>Isoquercitrone</td>
<td>C₁₃H₁₂O₇</td>
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<td>9</td>
<td>22.3</td>
<td>3,4-Dicaffeoylquinic acid</td>
<td>C₂₅H₂₃O₁₂</td>
<td>539</td>
<td>517</td>
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<td>10</td>
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<td>1,5-Dicaffeoylquinic acid</td>
<td>C₂₅H₂₃O₁₂</td>
<td>539</td>
<td>517</td>
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<td>11</td>
<td>24.2</td>
<td>3,5-Dicaffeoylquinic acid</td>
<td>C₂₅H₂₃O₁₂</td>
<td>539</td>
<td>517</td>
<td>515</td>
<td>515</td>
<td>515</td>
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<tr>
<td>13</td>
<td>23.2</td>
<td>Apigenin</td>
<td>C₁₇H₁₉O₄</td>
<td>455</td>
<td>433</td>
<td>431</td>
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*Extracted from** Plasma, Urine, Feces
<table>
<thead>
<tr>
<th>14&quot;</th>
<th>24.3</th>
<th>4,5-Dicaffeoyl-quinic acid</th>
<th>C$<em>{25}$H$</em>{24}$O$_{12}$</th>
<th>539 [M+Na]$^+$</th>
<th>[517, 40 ev]: 163(100), 515 [M-H]$^-$</th>
<th>[515, 20 ev]: 353(100), 173(85), 179(50), 191(25), 218 Extract, 328 Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>517 [M+H]$^+$</td>
<td>145(28), 135(21), 117(9), 353 [C$<em>{16}$H$</em>{17}$O$_9$]$^-$</td>
<td>173(85), 179(50), 191(25), 135(13), 93(5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>499 [M-H$_2$O+H]$^+$</td>
<td>89(9)</td>
<td>161 [C$_3$H$_5$O$_3$]$^-$</td>
</tr>
</tbody>
</table>

* identified with standard compounds;
gluA: glucuronic acid; sulf: sulfoacid; glu: glucose; rha: rhamnose.
Table 2

Regression data and limits of detection and quantification.

<table>
<thead>
<tr>
<th>Components</th>
<th>Linear equations</th>
<th>Range (μg/mL)</th>
<th>( R^2 )</th>
<th>LOD (μg/mL)</th>
<th>LOQ (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Umbelliferone (6)</td>
<td>( y = 0.0476x + 0.0017 )</td>
<td>0.05-10.0</td>
<td>0.9982</td>
<td>0.012</td>
<td>0.03</td>
</tr>
<tr>
<td>Scopoletin (7)</td>
<td>( y = 0.2394x + 0.0038 )</td>
<td>0.05-10.0</td>
<td>0.9963</td>
<td>0.003</td>
<td>0.01</td>
</tr>
</tbody>
</table>
Table 3

Intra- and inter-day precision and accuracy (*n* = 5).

<table>
<thead>
<tr>
<th>Components</th>
<th>Added (μg/mL)</th>
<th>Found (μg/mL) (Mean ± SD)</th>
<th>Precision (%) (RSD)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Umbelliferone (6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intra-day</td>
<td>0.1</td>
<td>0.099 ± 0.011</td>
<td>11.1</td>
<td>98.6</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>1.103 ± 0.071</td>
<td>6.5</td>
<td>110.3</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>4.649 ± 0.269</td>
<td>5.8</td>
<td>93.0</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.102 ± 0.014</td>
<td>13.9</td>
<td>102.2</td>
</tr>
<tr>
<td>Inter-day</td>
<td>1.0</td>
<td>0.918 ± 0.068</td>
<td>7.4</td>
<td>91.8</td>
</tr>
<tr>
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<td>5.0</td>
<td>4.943 ± 0.523</td>
<td>10.6</td>
<td>98.9</td>
</tr>
<tr>
<td>Scopoletin (7)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intra-day</td>
<td>0.1</td>
<td>0.104 ± 0.011</td>
<td>10.2</td>
<td>103.5</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.921 ± 0.062</td>
<td>6.7</td>
<td>92.1</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>4.839 ± 0.411</td>
<td>8.5</td>
<td>96.8</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.098 ± 0.013</td>
<td>12.8</td>
<td>98.0</td>
</tr>
<tr>
<td>Inter-day</td>
<td>1.0</td>
<td>1.097 ± 0.130</td>
<td>11.9</td>
<td>109.7</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>5.627 ± 0.465</td>
<td>8.3</td>
<td>112.5</td>
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</table>
Table 4

Recovery and matrix effect of two analytes and I.S. (n = 5).

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration (μg/mL)</th>
<th>Mean recovery yield (Mean ± RSD) (%)</th>
<th>Matrix effect (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Umbelliferone (6)</td>
<td>0.1</td>
<td>87.6 ± 10.2</td>
<td>97.6</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>89.8 ± 6.5</td>
<td>102.2</td>
</tr>
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<td></td>
<td>5.0</td>
<td>92.9 ± 5.3</td>
<td>99.8</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>92.3 ± 7.7</td>
<td>101.9</td>
</tr>
<tr>
<td>Scopoletin (7)</td>
<td>1.0</td>
<td>91.6 ± 4.9</td>
<td>93.1</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>93.0 ± 6.8</td>
<td>96.4</td>
</tr>
<tr>
<td>Coumarin 120 (I.S.)</td>
<td>5.0</td>
<td>88.1 ± 5.2</td>
<td>92.6</td>
</tr>
</tbody>
</table>
Table 5

Stability of two analytes in rat plasma ($n = 5$).

<table>
<thead>
<tr>
<th>Components</th>
<th>Added (μg/mL)</th>
<th>Room temperature (24 h)</th>
<th>Three freeze–thaw cycles</th>
<th>-80 °C (30 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Found (μg/mL, Mean ± SD) (%)</td>
<td>Accuracy</td>
<td>Found (μg/mL, Mean ± SD) (%)</td>
</tr>
<tr>
<td>Umbelliferone (6)</td>
<td>0.1</td>
<td>0.099 ± 0.011 (98.6)</td>
<td>0.103 ± 0.009 (103.5)</td>
<td>0.113 ± 0.015 (113.0)</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>1.103 ± 0.071 (110.3)</td>
<td>0.979 ± 0.107 (97.9)</td>
<td>1.086 ± 0.124 (108.6)</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>4.649 ± 0.269 (93.0)</td>
<td>5.012 ± 0.345 (100.2)</td>
<td>5.148 ± 0.408 (103.0)</td>
</tr>
<tr>
<td>Scopoletin (7)</td>
<td>0.1</td>
<td>0.104 ± 0.011 (103.5)</td>
<td>0.099 ± 0.011 (98.9)</td>
<td>0.109 ± 0.010 (109.0)</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.921 ± 0.062 (92.1)</td>
<td>1.023 ± 0.085 (102.3)</td>
<td>1.129 ± 0.115 (112.9)</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>4.839 ± 0.411 (96.8)</td>
<td>4.825 ± 0.297 (96.5)</td>
<td>5.334 ± 0.441 (106.7)</td>
</tr>
</tbody>
</table>
Pharmacokinetic parameters for umbelliferone (equivalent to 23.4 mg/kg) and scopoletin (equivalent to 35.8 mg/kg) in rats plasma after oral administration of SL extract (1.0 g/kg) (Mean ± SD, n = 6).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Umbelliferone (6)</th>
<th>Scopoletin (7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_{max}$ (min)</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>$C_{max}$ (μg /mL)</td>
<td>7.59 ± 0.93</td>
<td>5.55 ± 0.69</td>
</tr>
<tr>
<td>$AUC_{0-t}$ (μg min /mL)</td>
<td>100.94 ± 10.49</td>
<td>89.22 ± 8.64</td>
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<tr>
<td>$AUC_{0-\infty}$ (μg min /mL)</td>
<td>102.00 ± 10.72</td>
<td>90.53 ± 8.97</td>
</tr>
<tr>
<td>$V_d$ (L/kg)</td>
<td>10.05 ± 1.21</td>
<td>19.32 ± 2.15</td>
</tr>
<tr>
<td>$T_{1/2}$ (min)</td>
<td>30.13 ± 2.75</td>
<td>33.51 ± 3.71</td>
</tr>
<tr>
<td>MRT (min)</td>
<td>21.31 ± 0.86</td>
<td>30.20 ± 2.78</td>
</tr>
<tr>
<td>$K_e$ ($10^2$*min$^{-1}$)</td>
<td>2.32 ± 0.22</td>
<td>2.09 ± 0.21</td>
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<tr>
<td>CL (mL/min /kg)</td>
<td>231.62 ± 25.34</td>
<td>398.97 ± 42.64</td>
</tr>
</tbody>
</table>

$^a$ $T_{max}$, time to reach maximum plasma concentration; $C_{max}$, maximum plasma concentration; $AUC_{0-t}$, area under the concentration-time curve from zero up to a definite time t; $AUC_{0-\infty}$, area under the concentration-time curve from zero up to infinite time; $V_d$, apparent volume of distribution; $T_{1/2}$, half-life of elimination; MRT, mean retention time; $K_e$, elimination rate constant and CL, total clearance.
Legends for Figures

Fig. 1. Flowchart of the integrated strategy for metabolism and pharmacokinetic studies of multi-component herbal medicine.

Fig. 2. Chemical structures of the identified components from SL extract, rat plasma, urine and feces.

Fig. 3. Typical chromatograms of SL extract. (a) DAD chromatogram at 280 nm, (b) base peak chromatogram (BPC) in positive ion mode and (c) BPC in negative ion mode.

Fig. 4. Typical chromatograms of rat plasma sample. (a) DAD chromatogram at 280 nm, (b) BPC in positive ion mode and (c) BPC in negative ion mode.

Fig. 5. (a) UV, (b) +MS, (c) -MS and (d) +MS² spectra and the proposed fragmentation pathways of umbelliferone glucuronide (M1).

Fig. 6. Typical chromatograms of rat urine sample. (a) DAD chromatogram at 280 nm, (b) BPC in positive ion mode and (c) BPC in negative ion mode.

Fig. 7. Typical chromatograms of rat feces sample. (a) UPLC–DAD chromatogram at 280 nm, (b) BPC and (c) EIC of umbelliferone (6) and scopoletin (7) in positive ion mode, and (d) BPC and (e) EIC of umbelliferone (6) and scopoletin (7) in negative ion mode.

Fig. 8. Typical chromatograms of plasma sample in positive ion mode. (a) BPC, (b) EIC of umbelliferone, (c) EIC of scopoletin and (d) EIC of internal standard.

Fig. 9. Mean plasma concentration-time curves of umbelliferone (6) and scopoletin (7) after oral administration of SL extract (1.0 g/kg) in rats (Mean ± SD, n = 6).

Fig. 10. The potential metabolic pathways of the main constituents in SL.
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**Supplementary Materials**

**Table S1**

The contents of five main constituents in the SL extracts.

**Fig. S1.** UV (a), +MS (b), +MS$^2$ (c), -MS (d) and -MS$^2$ (e) spectra of scopoletin glucuronide (M2).

**Fig. S2.** UV (a), +MS (b), +MS$^2$ (c), -MS (d) and -MS$^2$ (e) spectra of umbelliferone sulfate (M3).

**Fig. S3.** UV (a), +MS (b), +MS$^2$ (c), -MS (d) and -MS$^2$ (e) spectra of scopoletin sulfate (M4).

**Fig. S4.** Extracted ion chromatograms (EICs) at m/z 163, m/z 243 and m/z 339 of plasma samples after oral ministration of pure umbelliferone (6) to rats (10 mg/kg).

**Fig. S5.** Extracted ion chromatograms (EICs) at m/z 193, m/z 273 and m/z 369 of plasma samples after oral ministration of pure scopoletin (7) to rats (10 mg/kg).
Table S1

The contents of five main constituents in the SL extracts.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Chlorogenic acid (2)</th>
<th>Syringoside (4)</th>
<th>Umbelliferone (6)</th>
<th>Scopoletin (7)</th>
<th>1,5-Dicaffeoyl quinic acid (9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanolic extract</td>
<td>24.6 ± 0.7</td>
<td>6.4 ± 0.1</td>
<td>23.4 ± 0.5</td>
<td>35.8 ± 0.3</td>
<td>8.5 ± 0.2</td>
</tr>
</tbody>
</table>

Values shown are mean ± S.D.
Fig. S1. UV (a), +MS (b), +MS² (c), -MS (d) and -MS² (e) spectra of scopoletin glucuronide (M2).
Fig. S2. UV (a), +MS (b), +MS² (c), -MS (d) and -MS² (e) spectra of umbelliferone sulfate (M3).
Fig. S3. UV (a), +MS (b), +MS$^2$ (c), -MS (d) and -MS$^2$ (e) spectra of scopoletin sulfate (M4).
**Fig. S4.** Extracted ion chromatograms (EICs) at m/z 163 (umbelliferone, 6), m/z 243 (M3) and m/z 339 (M1) of plasma samples after oral ministration of pure umbelliferone (6) to rats (10 mg/kg).
Fig. S5. Extracted ion chromatograms (EICs) at m/z 193 (scopoletin, 7), m/z 273 (M4) and m/z 369 (M2) of plasma samples after oral ministration of pure scopoletin (7) to rats (10 mg/kg).