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Determination of acteoside in *Cistanche deserticola* and *Boschniakia rossica* and its pharmacokinetics in freely-moving rats using LC–MS/MS

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Abstract

A sensitive LC–MS/MS method with a simple solid-phase extraction for the determination of acteoside in rat plasma and tissue homogenates was established for the investigation of bioavailability and brain distribution in freely-moving rats. Acteoside in *Cistanche deserticola* and *Boschniakia rossica* was also determined. Acteoside and internal standard were separated on a RP-select B column (125 mm × 4.6 mm i.d., particle size 5 μm). The mobile phase consisted of 35% methanol and 65% acetic acid–water (1:100, v/v) at a flow-rate of 1 mL/min. Acteoside and the internal standard were monitored using the multiple-reaction monitoring (MRM) mode at *m/z* transitions of 623 → 161 and 609 → 301, respectively. The acteoside content was 38.4 ± 2.4 mg/kg ($n = 3$) for *B. rossica*, which is obviously lower than 21134.2 ± 805.5 mg/kg ($n = 3$) of *C. deserticola*. The protein binding in rat plasma was 75.5 ± 1.8%. The brain distribution result indicated that acteoside was evenly distributed in brain tissues (brain stem, cerebellum, the rest of the brain, cortex, hippocampus and striatum) which was about 0.45–0.68% of that in plasma (4.5 ± 0.5 μg/mL) after 15 min of acteoside administration (10 mg/kg, i.v.). After acteoside was given (3 mg/kg, i.v.; 100 mg/kg, p.o.), the oral bioavailability ($AUC_{p.o.}/dose_{p.o.}$)/($AUC_{i.v.}/dose_{i.v.}$) was only 0.12%.

Keywords: Acteoside; Automated blood sampling; Bioavailability; LC–MS/MS; Pharmacokinetics

1. Introduction

Oxidative stress resulting in reactive oxygen species (ROS) generation and inflammation plays an important role in clinical diseases such as arteriosclerosis, ischemia-reperfusion injury and neurodegenerative disorders. Numerous in vitro and in vivo studies have indicated that polyphenols are active in preventing or reducing the deleterious effects of oxygen-derived free radicals associated with these diseases [1]. Polyphenolic compounds are known to possess various pharmacological effects, especially antioxidant activity. Acteoside (Fig. 1) is a phenylethanoid glycoside belonging to water-soluble polyphenolic compounds, which are widely distributed in many medicinal plants, including *Verbascum sinuatum* [2], *Orobanche rapum* [3], *Cistanches salsa* (C.A. Mey) G. Beck [4] and *Plantago psyllium* L. [5]. According to recent pharmacological studies, acteoside can protect endothelial cells against Fenton's reagent-induced oxidative stress [6], and the inhibition of Fenton reaction by acteoside could be partially explained by the sequestration of Fe ions [7]. Acteoside can also inhibit apoptosis induced by 1-methyl-4-phenylpyridinium ion in cerebellar granule neurons [8].

Though acteoside has many important pharmacological actions, there is limited information related to the pharmacokinetic parameters of acteoside in the literature. Also, there are no studies reporting oral bioavailability and the brain distribution profile of acteoside. Oral bioavailability describes the rate and extent to which the compound is absorbed by the gastrointestinal tract and becomes available at the systemic circulation, while brain distribution profile can show if this compound could arrive at the brain to protect the cells from the oxidative stress.

In this study, we applied an automated blood sampling system to sample blood sample from conscious and freely moving rats to

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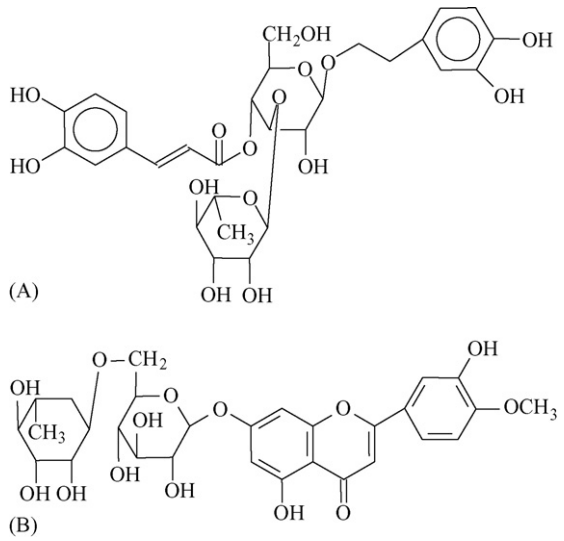

Fig. 1. Chemical structure of (A) acteoside and (B) hesperidin.

avoid anesthesia or restraining stress by conscious experimental animal for pharmacokinetic study [9,10]. This automated system can minimize the stress caused by the restraint or the effect of anesthesia on the experimental animals, which may delay gastric emptying and slow the absorption of drugs in the gastrointestinal tract [11], and it can also save manpower. Due to these advantages, we used this novel system in this study.

A number of analytical methods have been applied using liquid chromatography with UV detection and mass spectrometry for the identification and quantification of acteosdie from *Cistanches salsa* (C.A. Mey.) G. Beck [4], *Plantago psyllium* L. [5], brined olive drupes [12], olives [13] or cultured plant cell extracts [14,15]. However, none has been established for the determination of acteoside in biological fluids. Therefore, we developed a sensitive and reliable LC–MS/MS method with a simple solid-phase extraction (SPE) for the determination of acteoside in rat plasma and tissue homogenates. In this work, the method was validated to further investigate the pharmacokinetics of acteoside in conscious, freely-moving rats. In addition, the oral bioavailability of acteoside and its brain distribution in various brain regions will be examined.

2. Experimental

2.1. Purification of acteoside

Acteoside was isolated from the whole plant of *Cistanche deserticola*. Crushed herb was extracted with 95% EtOH. The concentrated ethanolic extract suspended in H_2O was then partitioned successively against EtOAc and *n*-BuOH. The *n*-BuOH extract was subjected to Diaion HP-20 column chromatography with gradient solvent systems of H_2O–MeOH, increasing the concentration of MeOH. In total, six fractions were collected. Fr. 3 was repeatedly rechromatographed on Sephadex LH-20 (MeOH/H_2O) to obtain acteoside. The structure of acteoside was determined by spectra data and compared with data from the literature [16].

To investigate the relative contents of acteoside in two plants, 0.5 g powdered *C. deserticola* was transferred to a 200 mL Erlenmeyer flask and immersed with 30 mL of methanol for 30 min. The mixture extraction was placed in an ultrasonic bath (Branson 2510, Danbury, CT, USA) for 10 min. The filtrate (filtered through Whatman #5 filter paper, Maidstone, UK) was collected and the residue was extracted repeatedly with methanol for two more times (30 mL × 2). All the filtrates were combined and diluted to 100 mL in a volumetric flask. One milliliter of extract was centrifuged at 10,000 rpm (Eppendorf Centrifuge 5402, Kent City, MI, USA) for 10 min. The supernatant was then analyzed by HPLC. The overall procedure was repeated three times ($n = 3$). Sample preparation of *Boschniakia rossica* was the same as the procedures of *C. deserticola*.

2.2. Chemicals and reagents

Hesperidin (97%, structure in Fig. 1) purchased from Aldrich Chemical Co. (Milwaukee, WI, USA) was used as the internal standard (IS). Methanol (HPLC grade) and acetic acid (96%, GR for analysis) were obtained from Merck (Darmstadt, Germany). Pure water for all preparations was prepared by the Milli-Q system (Millipore, Milford, MA, USA).

2.3. LC–MS/MS instrumentation

HPLC analysis was using a Waters 2690 Separations Module (Milford, MA, USA). Acteoside and IS was separated on a RP-select B column (125 mm × 4.6 mm i.d., particle size 5 μm, Merck). The mobile phase consisted of 35% methanol and 65% acetic acid-water (1:100, v/v) solution at a flow-rate of 1 mL/min, and 20 μL of the reconstituted sample was injected into the LC–MS/MS system. LC–MS/MS experiments were conducted using a Quattro Ultima triple-quadrupole mass spectrometer (Micromass, Manchester, UK). A pneumatically assisted electrospray ionization (ESI) ion source was used throughout the experiments. Multiple-reaction monitoring (MRM) conditions were established for acteoside and the internal standard by infusion analysis of each compound (1 μg/mL) dissolved in methanol at 20 μL/min. The optimized parameters are shown in Table 1. Acteoside and the internal standard were monitored at *m/z* transitions of 623 → 161 and 609 → 301, respectively. The MassLynx 3.5 (Micromass, Manchester, UK) software was used for data processing.

Table 1
Parameter setting of mass spectrometer

Parameters	Value
Ionization mode	ES
Capillary voltage (kV)	−2.5
Cone voltage (V)	−34 for acteoside; −51 for IS
Source temperature (°C)	85
Desolvation temperature (°C)	390
Cone gas flow (L/h)	122
Desolvation gas flow (L/h)	539
Collision energy (eV)	37 for acteoside; 25 for IS

2.4. Sample preparation

A 50 μL aliquot of the rat plasma was transferred into a 1.5 mL polypropylene vessel containing 10 μL of IS (1 μg/mL). The mixture was then mixed with pure water to 1 mL, and was loaded onto the Oasis HLB cartridge (Waters, Milford, MA, USA), which had been previously conditioned with 1 mL of methanol and equilibrated with 1 mL pure water, under a vacuum extraction manifold. The cartridge was washed with 1 mL of 20% methanol solution, and the analytes were then eluted with 1 mL of methanol into another 1.5 mL polypropylene tube containing 10 μL of 1% ascorbic acid solution. The eluate was evaporated to dryness under a gentle stream of nitrogen at room temperature. The residue was reconstituted with 200 μL of 50% methanol in water (v/v), and transferred to autosampler vials to be analyzed. Brain tissues were homogenized with 50% methanol (1 g: 5 mL). After centrifugation of the homogenate at 10,000 rpm at 4 °C for 10 min, 100 μL of the supernatant was mixed with 20 μL IS. The following preparation procedures were the same as that for plasma.

2.5. Preparation of calibration standard and quality control (QC) standard

Calibration standards were prepared by adding known volumes of acteoside (0.25, 0.5, 1, 2.5, 5, 10, 25 and 50 ng) to 50 μL of blank plasma and then adding 10 ng of hesperidin as the IS. The calibration standard samples were prepared in triplicate and the standard curves were obtained by least-square linear regression of the peak-area ratios versus the concentrations of acteoside. The QC samples for both low and high range calibration curves in six replicate were prepared in the same manner to determine the precision and accuracy of the analytical method. Calibration standards for brain homogenate were prepared by adding known volumes of acteoside (0.5, 1, 2, 5, 10, 20, 50 and 100 ng) to 100 μL brain tissue homogenate and then adding 20 ng of hesperidin as the IS, and the following steps were the same as that for plasma.

2.6. Validation

Linearity was evaluated in the concentration range of 0.005–1 μg/mL. The calibration curve was considered linear with a correlation coefficient of determination (r^2) greater than 0.99, and the LLOQ was defined as ±20% of nominal concentration. The within-run precision values were determined in six replicates at the concentrations of 0.01, 0.02, 0.2 and 0.5 μg/mL. The between-run precision was determined across four concentrations at six different days, and the mean concentrations and the coefficient of variation were calculated. The accuracy of the assay was determined by comparing the nominal concentrations with the corresponding calculated concentrations via linear regression. The recovery of acteoside was estimated by comparing the peak area of the extracted acteoside to that of the untreated acteoside at concentrations of 0.01, 0.1 and 1 μg/mL.

2.7. Animals

Male Sprague–Dawley rats (National Yang-Ming University Animal Center, Taipei, Taiwan), 300–350 g, were housed with a 12-h light:12-h dark cycle. Free access to food (Laboratory rodent diet 5P14, PMI Feeds, Richmond, IN, USA) and water was allowed at all times. Animal experimental protocols were reviewed and approved by the Institutional Animal Experimentation Committee of the National Yang-Ming University. For cannulation, rats were anesthetized with pentobarbital sodium (50 mg/kg i.p.). Surgical sites were shaved, cleaned with 70% ethanol solution, and polyethylene tubes were implanted in the right jugular and right femoral veins. For the oral administration group, only the right jugular was cannulted for sampling purpose. The cannulae were exteriorized, fixed in the dorsal neck region and connected to the sampling system. The patency of the tubing was maintained by flushing with heparinized saline (15 U/mL). Rats were allowed a minimum of 24 h to recover prior to experimentation.

2.8. Protein binding ratio and brain distribution evaluation

For protein-binding of acteoside assay, a blood sample (2 mL) was withdrawn by cardio-puncture at 15 min after acteoside administration (10 mg/kg, i.v.). The rat blood sample was centrifuged at 6000 rpm under 4 °C, for 10 min. The plasma was divided into two parts; 0.1 mL of plasma was used to measure the total form concentration of acteoside (Ct). The remaining plasma was transferred to an ultrafiltration tube (Centrifree, Millipore, Bedford, MA, USA). Further centrifugation was performed at 12,000 rpm for 10 min at 4 °C for free form measurement (Cf). Measurement of acteoside was the same as the above description. The protein-binding ratio (B) of acteoside was calculated by the following equation: $B = [(Ct - Cf)/Ct] \times 100\%$. For brain distribution study, the animal was sacrificed at 15 min by decapitation after acteoside administration (10 mg/kg, i.v.). The brain stem (Bs), cerebellum (Cb), cortex (Cx), hippocampus (Hp), striatum (St) and the rest of the brain (Rb) were further dissected and weighed for the determination of regional distribution of acteoside.

2.9. Pharmacokinetics

For the intravenous group, 3 mg/kg acteoside dissolved in normal saline was given via the femoral vein for intravenous injection, while acteoside at dose of 100 mg/kg was given via gastric gavage in the oral group. Blood sampling was accomplished by the automated blood sampling system (DR-II, Eicom Corp., Kyoto, Japan) in conscious and freely-moving rats. A 150 μL blood sample was withdrawn from the jugular vein into a heparin rinsed vial with fraction collector according to a predetermined schedule after acteoside administration, and finally made up with equal volume (150 μL) heparinized saline to avoid the loss of body fluid after each sampling. Plasma was separated by centrifugation at 6000 rpm for 10 min at 4 °C. The resulting plasma was then processed as described in sample preparation.

2.10. Pharmacokinetic data analysis

Pharmacokinetic calculations were performed on each individual set of data using the pharmacokinetic software WinNonlin Standard Edition Version 1.1 (Pharsight Corp., Mountain View, CA, USA) by noncompartmental method. The area under the concentration–time curve (AUC) was calculated according to the linear trapezoidal method. The oral bioavailability was calculated as $(AUC_{PO}/Dose_{PO})/(AUC_{IV}/Dose_{IV})$.

3. Results

3.1. Purification of acteoside from C. deserticola and relative acteoside contents in C. deserticola and B. rossica

The characteristics of acteoside describes following: amorphous powder; UV (MeOH) λ_{max} (log ε): 333 (4.28), 291 (4.10), 246 (3.98), 219 (4.23) nm; IR (KBr) ν_{max}: 3400, 1698, 1635, 1603, 1515, 1445, 1283, 1062, 1031 cm^{-1}; ^1H NMR (CD_3OD) δ: 1.10 (3H, d, J = 6.0 Hz, H-6‴), 2.80, (2H, m, H-7), 4.38 (1H, d, J = 8.0 Hz, H-1″), 4.93 (1H, t, J = 10.0 Hz, H-4″), 5.20 (1H, s, H-1‴), 6.28 (1H, d, J = 16.5 Hz, H-8′), 6.57 (1H, dd, J = 7.5, 1.5 Hz, H-6), 6.68 (1H, d, J = 7.5 Hz, H-5), 6.71 (1H, d, J = 1.5 Hz, H-2), 6.79 (1H, d, J = 8.5 Hz, H-5′), 6.97 (1H, d, J = 8.5 Hz, H-6′), 7.06 (1H, s, H-2′), 7.60 (1H, d, J = 16.5 Hz, H-7′); ^{13}C NMR (CD_3OD) δ: 18.5 (C-6‴), 36.6 (C-7), 62.4 (C-6″), 70.4 (C-5‴), 70.6 (C-4″), 72.0 (C-3‴), 72.2 (C-8), 72.3 (C-2‴), 73.8 (C-4‴), 76.0 (C-5″), 76.2 (C-2″), 81.6 (C-3″), 103.0 (C-1‴), 104.2 (C-1″), 114.7 (C-8′), 115.2 (C-2′), 116.3 (C-5), 116.5 (C-5′), 117.1 (C-2), 121.3 (C-6), 123.2 (C-6′), 127.7 (C-1′), 131.5 (C-1), 144.7 (C-4), 146.1 (C-3), 146.8 (C-3′), 148.0 (C-7′), 149.8 (C-4′), 168.3 (C-9′); FAB-MS m/z: 623 $[M-H]^-$. The mass spectrum revealed a base peak at m/z 623 corresponding to $[M-H]^-$ (Fig. 2A); and its fragmentation when collided with reagent gas, the selected product ion m/z 161 as shown in Fig. 2B, was consistent with the previous report [16].

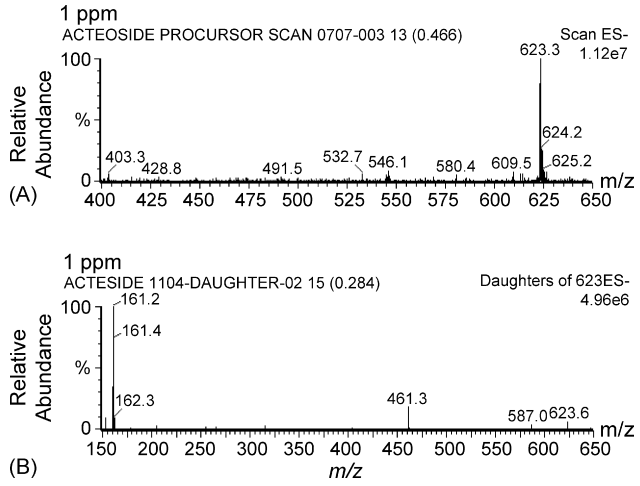

Fig. 2. Full scan mass spectrum of (A) acteoside (molecular weight 624) and (B) product ion scan spectrum with electrospray negative-ion mode.

The assay for herb extract analysis was validated. Standard stock solutions of acteoside and hesperidin were prepared in methanol at 1 mg/mL and diluted serially with 50% methanol to obtain desired concentrations of the working solutions. The peak area ratios between acteoside at different concentrations and internal standard were determined to establish the calibration curve. The assay was linear in the range of 0.01–2 μg/mL (r^2 = 0.9993). The intra-day (n = 3) and inter-day (n = 3) precision (% R.S.D. between 0.4 and 13.3%) and accuracy (90.1–110.5%) for acteoside were also acceptable. The

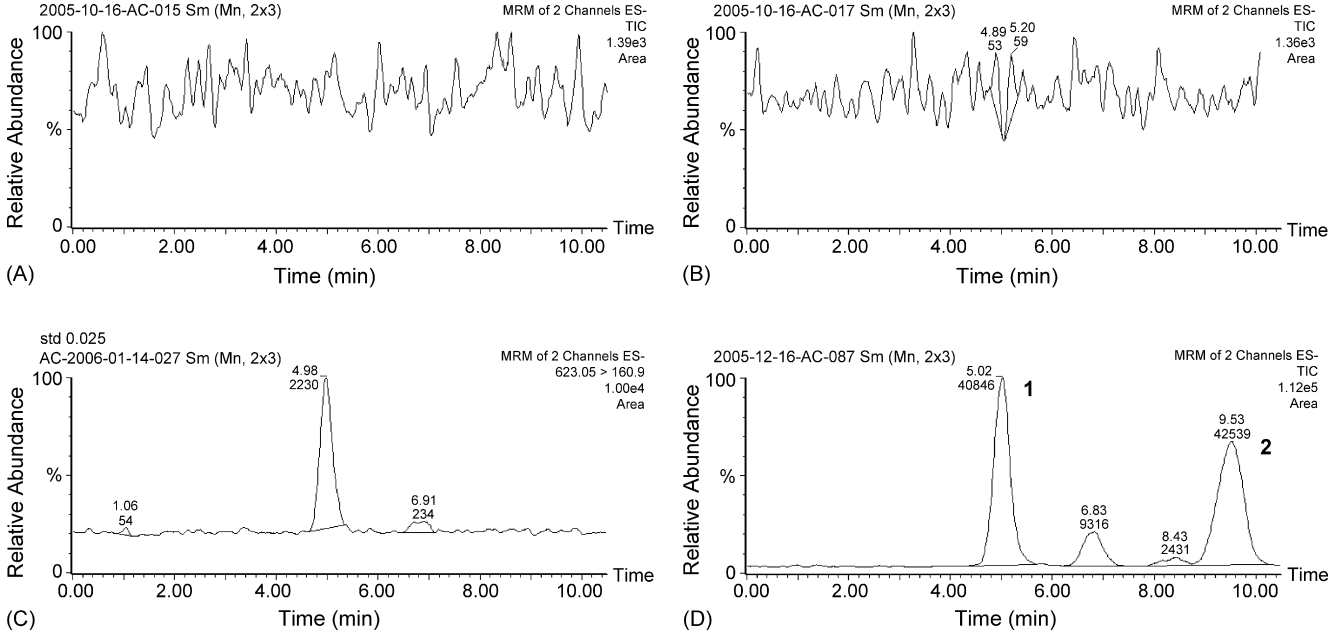

Fig. 3. Chromatograms of acteoside, (A) showing a chromatogram of an extracted blank plasma; (B) showing a chromatogram of an extracted blank brain homogenate; (C) showing a chromatogram of a standard of acteoside at LLOQ (0.005 μg/mL); (D) showing a chromatogram of a plasma sample containing acteoside (0.1 μg/mL) collected from rat plasma 30 min after acteoside administration (100 mg/kg, p.o.). (1) Acteoside; (2) internal standard (hesperidin).

Table 2
Precision and accuracy for acteoside QC samples in plasma

Nominal concentration (μg/mL)	Observed concentration (μg/mL)	Accuracy (%)	Precision (R.S.D.)
Between-run			
0.01	0.0105 ± 0.0009	94.8	12.8
0.02	0.0197 ± 0.0003	98.6	1.6
0.2	0.1908 ± 0.0056	95.4	2.9
0.5	0.4684 ± 0.0182	93.7	3.9
Within-run			
0.01	0.0101 ± 0.0012	100.6	12.1
0.02	0.0199 ± 0.0006	99.3	3.3
0.2	0.1889 ± 0.0117	94.4	6.2
0.5	0.4716 ± 0.0329	94.3	7

Data expressed as mean ± S.D.

acteoside content was 38.4 ± 2.4 mg/kg ($n = 3$) for *B. rossica*, which is clearly lower than the value of 21134.2 ± 805.5 mg/kg ($n = 3$) of *C. deserticola*.

3.2. Chromatography and assay validation

Using this select LC–MS/MS with SPE method, acteoside was detectable in rat plasma. Blank plasma showed no significant interfering peaks at the retention time of acteoside and the IS (Fig. 3A and B). The retention time was 5 min for acteoside and 9.5 min for the IS, respectively (Fig. 3C and D). The coefficient of determination (r^2) generated by linear least-square regression was used to evaluate the method linearity. Calibration curves for acteoside in plasma and brain tissue homogenate had r^2 greater than 0.99. The LLOQ of acteoside assay in plasma was determined to 0.005 μg/mL. Tables 2 and 3 provide the within- and between-run accuracy and precision profiles. The data indicates that the accuracy and precision are within ±15% deviation from the nominal levels. The extraction recovery was determined at low (0.01 μg/mL), mid (0.1 μg/mL) and high (1 μg/mL) concentrations of acteoside-spiked rat plasma and brain homogenate. As shown in Table 4, the mean recovery for plasma samples was about 80%, whereas the mean recovery for brain homogenate samples was about 55%.

Table 3
Precision and accuracy for acteoside QC samples in brain homogenate

Nominal concentration (μg/mL)	Observed concentration (μg/mL)	Accuracy (%)	Precision (R.S.D.)
Between-run			
0.01	0.0105 ± 0.0006	105	5.4
0.02	0.0193 ± 0.0009	96.4	4.7
0.2	0.1944 ± 0.0187	97.2	9.6
0.5	0.5020 ± 0.0564	100.4	11.2
Within-run			
0.01	0.0105 ± 0.0007	100.5	6.7
0.02	0.0189 ± 0.0009	94.3	5
0.2	0.2091 ± 0.0175	104.6	8.4
0.5	0.4897 ± 0.023	97.9	4.7

Data expressed as mean ± S.D.

Table 4
Extraction recovery (%) for plasma and brain homogenate

	0.01 (μg/mL)	0.1 (μg/mL)	1 (μg/mL)
Plasma	81.4 ± 7.9	79.2 ± 4.4	82.2 ± 8.7
Brain homogenate	55.2 ± 1.2	52.5 ± 4.1	55.4 ± 4.5

Data expressed as mean ± S.D. ($n = 3$).

Table 5
Protein binding ratio of acteoside in rat plasma.

Experimental no.	Protein binding ratio (%)
Rat 1	77.5
Rat 2	74.9
Rat 3	72.7
Rat 4	75.6
Rat 5	76.6
Average	75.5 ± 0.8

Values are expressed as mean ± S.E. ($n = 5$).

3.3. Protein binding ratio and brain distribution

The ultrafiltration method was used in this experiment and the protein-binding (B) of acteoside was calculated by $B = [(Ct - Cf)/Ct] \times 100\%$. The protein binding in rat plasma was 75.5 ± 1.8% (shown in Table 5). The acteoside level in the brain showed no significant difference among various regions (brain stem, cerebral cortex, cerebellum, hippocampus, striatum and the rest of brain) at 15 min after acteoside administration (10 mg/kg, i.v.) (Fig. 4). The concentration of acteoside in different brain region was approximately 0.45–0.68% of that in plasma (4.5 ± 0.5 μg/mL).

3.4. Pharmacokinetic study

Fig. 5 illustrates the concentration versus time profiles of acteoside with a single intravenous and oral dose administered to five individual rats for each group. The AUC were 379.9 ± 52.8 and 15.2 ± 2.3 min μg/mL for intravenous (3 mg/kg) and oral (100 mg/kg) doses, respectively (Table 6),

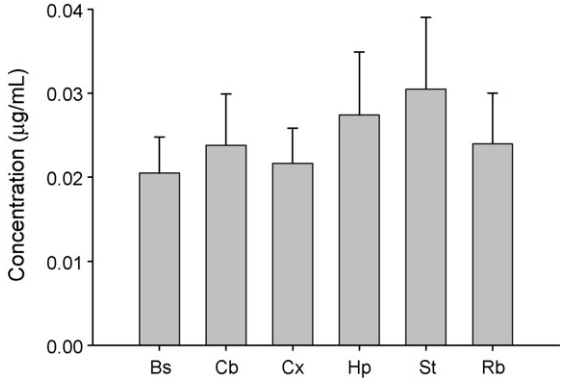

Fig. 4. Acteoside levels in various regions of rat brain at 15 min after administration of i.v. 10 mg/kg. Bs: brain stem; Cb: cerebellum; Cx: cortex; Hp: hippocampus; St: striatum; Rb: the rest of the brain. Data was expressed as mean ± S.E. ($n = 4$).

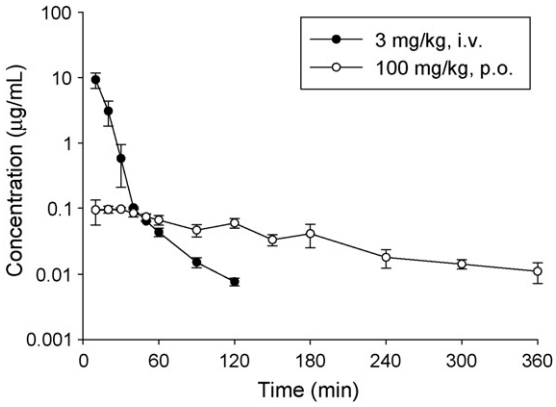

Fig. 5. Concentration vs. time curves of acteoside after drug administration (3 mg/kg, i.v. and 100 mg/kg, p.o.) in rats (data was expressed as mean ± S.E., $n = 5$ for each group).

Table 6
Pharmacokinetic data after acteoside administration (3 mg/kg, i.v. and 100 mg/kg, p.o.) in rats

	100 mg/kg, p.o.	3 mg/kg, i.v.
C_{max} (μg/mL)	0.13 ± 0.03	48.6 ± 12.8
$t_{1/2}$ (min)	92.1 ± 30.4	10.7 ± 0.9
AUC (min μg/mL)	15.2 ± 2.3	379.9 ± 52.8

Values are expressed as mean ± S.E. ($n = 5$ for each group).

and the oral bioavailability $(AUC_{p.o.}/dose)/(AUC_{i.v.}/dose)$ of acteoside was 0.12 ± 0.04%.

4. Discussion

In this study, we have compared the relative contents of acteoside from different sources of plants and also investigated the absorption and brain distribution of acteoside. The origins of herbal materials are complex, and using improper substitutions may sometimes harm the recipient's health. According to our results, *B. rossica* may not be a good source to obtain the benefits of acteoside, although *B. rossica* is sometimes used as a substitute for *C. deserticola* in traditional medicine.

In order to study the pharmacokinetics of acteoside in rats, we first had to develop an assay for biological samples. For plasma sample clean-up, our first attempt was to use liquid–liquid extraction (LLE). Three different solvents were used, namely ethylacetate, acetone and *n*-hexane. Using ethylacetate with repeated triplicate extraction gave the best recovery, but the value was below 50%. The effect of sample pH was also examined, but no significant improvement was observed. Due to the low recovery and greater variability of extraction efficiency using LLE, we finally chose the simple SPE method. The procedures were optimized to give maximal recovery of acteoside in samples, and no obvious interference was observed at the retention time of acteoside or the IS (Fig. 3A and B). All samples were analyzed immediately after their pretreatment to avoid any possible degradation of acteoside.

The stability of acteoside in plasma was evaluated from three aspects—during sampling, extraction processes, and in the autosampler. The phenolic compounds in human plasma have already been pinpointed, mainly how they are affected by temperature [17]. In order to minimize the degradation due to temperature, the collector of the automated blood sampling system equipped with a refrigeration device to keep the Eppendorf vials for receiving blood at 4 °C and minimize the effect of temperature. After blood colleted into the Eppendorf vial, the blood sample was immediately centrifuged to separate the plasma from cells and then prepared with solid phase extraction (SPE) as soon as possible. At the final step of SPE, the resulting methanolic eluent was mixed with 10 μL of 1% ascorbic acid in the vial.

In the course of prior experiments, an obvious decrease in acteoside stability was observed during drying process. Therefore, 10 μL of 1% ascorbic acid was added into the eluent before nitrogen drying. Ruiz-Gutiérrez et al. [18] had also indicated that the lack of stability was not noticed in the SPE extraction from plasma and the addition of ascorbic acid into the final methanolic extract of SPE stabilized hydroxytyrosol for at least one week. After drying, the residue was stored at −20 °C if they were not immediately analyzed within the same day. Under this condition, the dried residue was found to be stable at −20 °C for at least a week. Our extraction procedure was in accordance with previous report [18]. The residue was dissolved in 50% methanol solution and placed in the autosampler rack prior to analysis. The temperature of the samples in the autosampler was kept at 20 °C. According to the data of intra-day assay, the samples should be stable for at least one day. Furthermore, anticoagulants (such as heparin) should not play a significant role in the stability of polyphenolic compounds according to previous report [19]. Overall, the critical step for the stability of acteoside should be the drying process. With the addition of ascorbic acid, the stability of acteoside was preserved. The assay was validated for linearity, accuracy and precision. Validation data indicate this LC–MS/MS method had good reproducibility.

Since high drug protein binding ratio is important in clinical applications, we are interested in understanding any possible herb-drug interaction of acteoside due to protein binding displacement. Methods for evaluating drug protein binding ratio, including equilibrium dialysis [20] and ultrafiltration [21], all make use of a semipermeable membrane that separates the protein and protein-bound drug from the free or unbound drug. The protein-bound drug is a large complex that cannot easily transverse cell or possibly even capillary membranes and therefore has a restricted distribution. Our preliminary result showed the protein binding ratio of acteoside is about 75%.

The brain distribution result indicated that acteoside was evenly distributed in the brain tissue. The ethanol extract of *C. deserticola* has the sedative effects on hexobarbital-induced sleeping time in mice and spontaneous motor activity by using automated activity meter in rats [22], and acteoside existing mainly in the butanolic layer of the extract is the active ingredient of *C. deserticola* [23]. Our present results have shown the ethanolic extract of *C. deserticola* contains acteoside and acteoside can be detected in the brain tissue after intravenous administration, which could be evidence supporting the sedative effect of acteoside on the central nervous system.

The pharmacological effects of acteoside have been studied widely in vitro, but the in vivo absorption characteristics and related pharmacokinetic information are not yet clearly understood. The maximal concentration of acteoside in the plasma was reached within the first 30 min after oral administration (Fig. 5), which indicated acteoside was rapidly absorbed from the gastrointestinal tract and was detectable in the blood. However, oral bioavailability is quite low. The peak concentration of rosmarinic acid, a polyphenolic substance from culinary herbs, has been shown to reach 0.5 h after intra-gastric administration in rats [24]. The absorption of tea polyphenols – including (−)-epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin (EGC), and (−)-epicatechin (EC) – has also been investigated, and the results show a C_{max} within 1 h and the low bioavailability of EGCG (0.1%) [25]. The absorption characteristics of acteoside are in keeping with these polyphenolic substances.

The first-pass effect at the intestinal tract and liver might explain the low oral bioavailability of acteoside, but further studies are needed to understand the metabolic routes and main metabolites of acteoside. Acteoside has been shown to have protective effects against carbon tetrachloride-induced hepatotoxicity in mice, and the mechanisms related to its ability to block the P450-mediated carbon tetrachloride bioactivation and free radical scavenging effects have also been studied [26]. Whether the pharmacological activity results from the original compound or its metabolic forms is worthy of further investigation. A recent study on morin glucuronides/sulfates demonstrated that the metabolites were much more potent than the parent form for use in reducing inflammation [27]. Therefore, our future works will include characterizing the metabolic fate and identifying the metabolites of acteoside in rats to understand the bioactive differences between acteoside and its metabolite.

In summary, we have developed a LC–MS/MS with a simple SPE method to monitor the plasma levels of acteoside in freely moving rats, and an automated system is applied for blood sampling. This method has been used to investigate the oral bioavailability and the brain distribution of acteoside. The results indicate the oral bioavailability of acteoside is low, and acteoside can reach the brain. This method will be useful in determination of acteoside in biological fluids for pharmacokinetic studies.

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