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Liquid chromatographic method with amperometric detection to determine acteoside in rat blood and brain microdialysates and its application to pharmacokinetic study

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Abstract

A simple and sensitive liquid chromatography with amperometric detection was developed for the first time to monitor the protein-unbound acteoside in the rat blood and brain microdialysate by microdialysis technique. Microdialysis samples without further cleanup procedures were directly injected into the HPLC and separated using a reversed-phase C18 column (150 mm × 2 mm, i.d. 5 μm) maintained at ambient temperature and a mobile phase comprised of acetonitrile–50 mM monosodium phosphate (pH 2.8) (17:83, v/v) with a flow rate of 0.2 mL/min. Based on the experimental voltamogram, the applied potential was set at +0.9 V oxidative mode. The concentration–response relationship was linear ($r^2 > 0.99$) over a concentration range of 5–500 ng/mL; method precision and accuracy fell within predefined limits (less than 20%). The developed method was applied to assess the pharmacokinetics of acteoside, and the results suggested that acteoside was fitted better by the two-compartmental model following a single intravenous injection of acteoside. Acteoside was unable to be detected in the brain dialysate. The distribution and elimination half-lives of unbound acteoside in the blood were 5 and 28 min, respectively, which suggested the rapid distribution of acteoside.

Keywords: Acteoside; Herbal medicine; Microdialysis; Pharmacokinetics

1. Introduction

Numerous in vitro and in vivo studies have indicated that plant polyphenols are active in preventing or reducing the deleterious effects of oxygen-derived free radicals associated with these diseases [1]. Though many physiological benefits have been discovered, knowledge related to the in vivo absorption, distribution and elimination of these bioactive polyphenols were insufficient and rarely discussed. One possible reason for this lack of information is connected with the necessity of sensitive, convenient methods for measuring polyphenols in plasma and body fluids, compared with the number of methods devised for their investigation in food [2]. In this work, acteoside was chosen for investigation. Acteoside (Fig. 1) is a phenylethanoid glycoside belonging to water-soluble polyphenolic compounds, which are widely distributed in many medicinal plants, including *Verbascum sinuatum* [3], *Orobanche rapum* [4], *Cistanches salsa* (C.A. Mey) G. Beck [5] and *Plantago psyllium* L [6], *Olea europaea* [7]. Acteoside inhibits apoptosis induced by 1-methyl-4-phenylpyridinium ion in cerebellar granule neurons [8], and another report also indicates its in vitro neuroprotective activities [9]. Recently, it has been discovered that acteoside and its aglycones protect primary cultures of rat cortical cells from glutamate-induced excitotoxicity [10], and research has also found that acteoside attenuated scopolamine-induced memory impairments in mice [11].

A number of pharmacological effects of acteoside on neuron protection and central nervous system have been found, but the pharmacokinetic characteristics of acteoside, especially its kinetics in the brain, have not been clarified yet. To know the in

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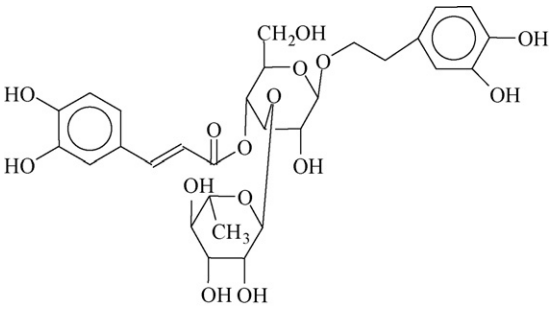

Fig. 1. Chemical structure of acteoside.

vivo distribution of acteoside, a suitable detection method has to be developed first. As mentioned above, most of the analytical methods using HPLC with UV and MS detectors were posed to identify and quantify acteoside from plants, such as *Cistanches salsa* (C.A. Mey.) G. Beck [5], *Plantago psyllium* L. [6], olives [7], or cultured plant cell extracts [12,13]. An HPTLC method was developed to measure acteoside in *Plantago palmata* Hook. f.s. [14]. Only one HPLC–MS method has been established for the determination of oral bioavailability of acteoside in rats [15]. Though mass spectrometer can provide excellent sensitivity for quantification, it is an expensive and not freely accessible instrument. Therefore, this study was to develop a new, sensitive and relatively inexpensive electrochemical detection method for analyzing acteoside in biological matrices. In addition, in vivo microdialysis sampling was applied in this work. The principles and features of microdialysis have been reviewed in detail in previous papers [16,17]. Briefly, microdialysis, a technique driven by the diffusion force caused by concentration gradient between the two borders of the semi-permeable membrane surface, samples continuously the extracellular fluids of target tissues in a living subject to monitor the drug concentrations without needing to remove any body fluids.

For pharmacokinetic study, microdialysis provides special advantages. First, the complex composed of drug molecules and proteins (e.g. albumin) was excluded by the semi-permeable membrane with a specific molecular weight cut-off value covered on the tip of the microdialysis probe, so microdialysis sampled the protein-unbound drug molecules at the target sites, which were the fraction available for absorption, distribution, metabolism and elimination and delivery to the target sites for pharmacodynamic actions. Second, this method did not drain the biological fluid from the subjects and cause the loss of the body fluids. It is useful to increase the data points from a relatively small number of animals, so continuous sampling over long periods of time and pharmacokinetic profiles of higher temporal resolution can be achieved. Finally, regulatory authorities, such as Food and Drug Administration (FDA) in the USA and the Committee for Proprietary Medicinal Products (CPMP) in the European Union, emphasize the value of human-tissue drug concentration data and support using clinical microdialysis to obtain this information [18], and then showing the potential and importance of microdialysis. Therefore, the aim of this work is to try to use the microdialysis coupled with a newly developed liquid chromatographic method with amperometric detection to study the pharmacokinetics of protein-unbound acteoside in blood and brain after a single intravenous administration in rats.

2. Experimental

2.1. Chemicals and standard solution

Acteoside was purified and identified as described in the previous paper [15]. Methanol and acetonitrile of HPLC-grade, sodium dihydrogen phosphate, and other chemicals were obtained from Merck (Darmstadt, Germany). Purified water prepared by the Millipore system (Millipore, Bedford, MA, USA) was used for all preparations. Stock solution of acteoside at 1 mg/mL was prepared in methanol and stored at −20 °C. Standard working solutions were prepared in 50% methanol every day immediately before use.

2.2. HPLC system

The HPLC system consisted of a chromatographic pump (Bioanalytical Systems, BAS PM-92E, West Lafayette, IN, USA), a CMA/200 refrigerated microsampler and a CMA/240 sample injector with a 20 μL loop (CMA, Stockholm, Sweden). Acteoside and dialysates were separated using a Phenomenex Gemini column (150 mm × 2.0 mm i.d.; particle size 5 μm) with its guard column (10 mm × 2.0 mm i.d.). The mobile phase comprised of acetonitrile–50 mM monosodium phosphate (pH 2.8, adjusting with 85% orthophosphoric acid) (17:83, v/v), and the flow rate of the mobile phase was 0.2 mL/min. The buffer was filtered through a Millipore membrane filter (Durapore, type: 0.22 μm) and degassed by sonication prior to use. The electrochemical detector consisted of an amperometric detector (BAS LC-4C), a glassy carbon electrode cell and an Ag/AgCl reference cell. The working electrode was set at an applied potential of +0.9 V relative to an Ag/AgCl reference electrode, filter setting was 0.1 Hz, and range setting was 20 nA. The EZChrom chromatographic data system (Scientific Software, San Ramon, CA, USA) was used for data processing.

2.3. Calibration curves and method validation

Stock solution of acteoside was diluted with 50% methanol to make the working standard solutions of serial concentrations (25, 50, 250, 500 and 2500 ng/mL). Calibration standards were prepared by mixing 20 μl of the working standard solutions with 80 μl of blank blood dialysate. The quality control samples with low, middle and high concentrations (5, 50 and 200 ng/mL) were prepared in the same manner. Calibration curves were derived from the peak area versus the concentrations of acteoside of the calibration standards ranging from 5 to 500 ng/mL by the least square method.

The method was validated to examine its selectivity, precision, accuracy, linearity, lower limit of detection (LOD) and lower limit of quantification (LOQ). Intra- and inter-day variation of the proposed method was evaluated by analyzing quality control samples with low, middle and high concentrations (5, 50 and 200 ng/mL) in a single and five different days, respectively.

The method precision was expressed as the relative standard deviation (%RSD), and the method accuracy was calculated by comparing observed concentration with nominal concentration. The concentration resulting in a signal-to-noise (S/N) ratio of 3 was defined as the LOD for this method. The LOQ was defined as the lowest concentration of acteoside in blood microdialysate that could be quantified with acceptable criteria (the inter-assay coefficient of variation ≤20% and accuracy within ±20%.

2.4. Animals

Six male Sprague–Dawley rats weighing 280–320 g (National Yang-Ming University Animal Center, Taipei, Taiwan) were housed with a 12-h light and 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. Rats were anesthetized with the mixture solution of urethane (1 g/mL) and α-chloralose (0.1 g/mL) at the dose of 1 mL/kg by intraperitoneal administration. A heating pad was used to maintain the rat's body temperature. Surgical sites were shaved, cleaned with 70% ethanol solution, and the femoral vein was canulated for drug administration.

2.5. Microdialysis experiments

The dialysis probes for blood (10 mm in length) and brain (3 mm in length) were made of silica glass capillary tubing arranged in a concentric design [19,20]. Their tips were covered by dialysis membrane (150 μm outer diameter with a nominal molecular weight cut-off of 13 000, Spectrum Co, Laguna Hills, CA, USA) and all unions were cemented with epoxy glue. To allow adequate time for the glue to dry, the probes were made at least 24 h prior to use.

The probe for blood sampling was implanted within the jugular vein/right atrium and then perfused with anticoagulant dextrose (ACD) solution (citric acid 3.5 mM; sodium citrate 7.5 mM; dextrose 13.6 mM) at a flow rate of 2 μL/min by the CMA/100 microinjection pump (CMA, Stockholm, Sweden). For brain microdialysis, the rat was mounted on a stereotaxic frame and perfused with Ringer's solution (containing 147 mM Na^+; 2.2 mM Ca^{2+}; 4 mM K^+; pH 7.0). After being washed with Ringer's solution at a flow-rate of 2 μL/min, the microdialysis probe was implanted in the right hippocampus (coordinates: 5.6 mm posterior to bregma, 5.0 mm lateral to midline and 7.0 mm lower to tip) according to the Paxinos and Watson atlas [21]. After a 2 h post-surgical stabilization period of probe implantation, acteoside (10 mg/kg, dissolved in normal saline) was administered by intravenous injection. The dialysates were collected every 15 min with a CMA/140 fraction collector.

To estimate the in vivo recovery, a retrodialysis calibration technique was utilized [22]. The blood microdialysis probe was inserted into the rat's jugular vein under anesthesia, and then the ACD solution (for blood microdialysis) containing acteoside was perfused through the probe at a constant flow rate (2 μL/min) by the microinjection pump after a stabilization period of 2 h post probe implantation. The perfusate (C_{perf}) and dialysate (C_{dial}) concentrations of acteoside were determined by the HPLC systems. The in vivo relative recovery (R_{dial}) of acteoside across the microdialysis probe was calculated by the following equation: $R_{dial} = (C_{perf} - C_{dial})/C_{perf}$. Acteoside microdialysate concentrations (C_m) were converted to unbound concentration (C_u) as follows: $C_u = C_m/R_{dial}$. To estimate brain probe recovery, the procedures were similar to that of blood microdialysis probe, but Ringer's solution containing acteoside was perfused through the probe implanted in the rat hippocampus. The drug concentrations in blood and brain dialysates were measured by the developed HPLC systems.

2.6. Pharmacokinetic data analysis

Pharmacokinetic calculations were performed on each individual animal's data utilizing the software WinNonlin Standard Edition Version 1.1 (Scientific Consulting, Apex, NC, USA) by a compartmental method. The results are represented as mean ± standard deviation.

3. Results and discussion

3.1. HPLC systems

In order to obtain the proper sensitivity, the applied potentials were examined by analyzing acteoside-spiked microdialysate at 500 ng/mL (Fig. 2). Generally, higher applied potential obtained better sensitivity, but the baseline noise also increased. To provide enough sensitivity and stable performance, +0.9 V was selected as the working potential. The effect of pH values (2.5, 3.0 and 4.0) for phosphate buffer on peak retention time and shape were investigated. One of the major purposes for the addition of sodium phosphate is to increase the conductivity of the mobile phase, which is beneficial for amperometric detection. In addition, the acidified mobile phase was to improve the peak shape. No obvious difference on retention time was observed by changing the pH values, but lowering pH value gave better peak shape. Therefore, pH 2.8 of the buffer was chosen. Different amounts (10–20%, v/v) of acetonitrile in the mobile phase were

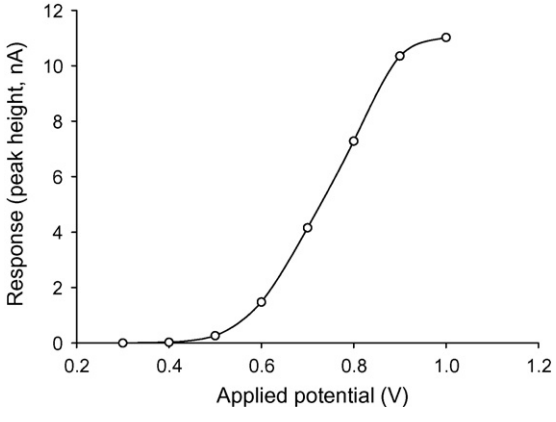

Fig. 2. Hydrodynamic voltammogram got by injection of acteoside standard (500 ng/mL) at different applied potentials (ranged from +0.3 to 1.0 V).

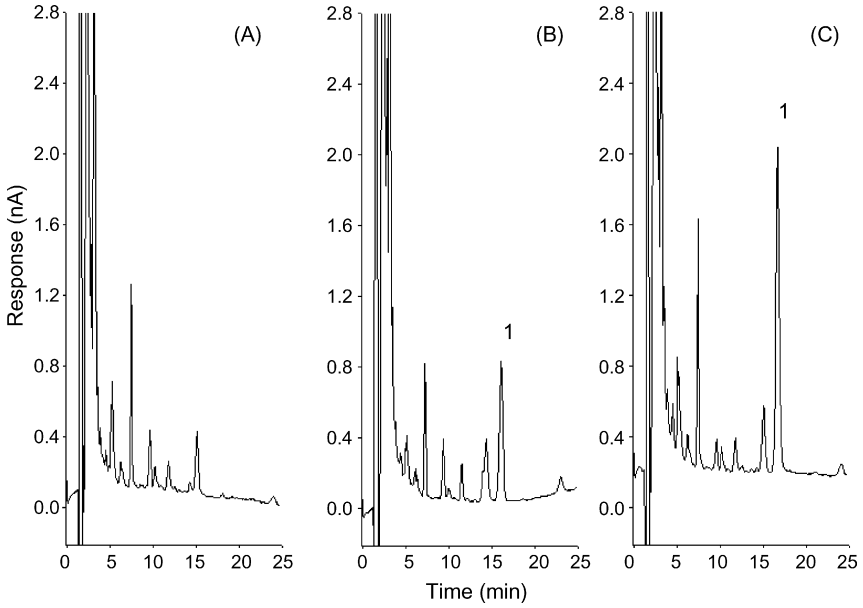

Fig. 3. Typical chromatograms of (A) blank blood dialysate from the microdialysis probe before drug administration; (B) blank blood dialysate spiked with acteoside (50 ng/mL); and (C) blood dialysate sample containing acteoside (101.5 ng/mL) collected from 135 to 150 min after acteoside administration (10 mg/kg). Peak 1: acteoside, 16.5 min. HPLC conditions: stationary phase, Gemini C18 (150 mm × 2.0 mm, 5 μm); mobile phase, acetonitrile: 50 mM monosodium phosphate (pH 2.8, adjusting with 85% orthophosphoric acid) (17:83, v/v); flow rate, 0.2 mL/min; detection, App $E = +0.9$ V.

tested to examine the retention behavior of acteoside. To achieve adequate separation from the peaks of endogenous interference and a shorter run-time, finally 17% of acetonitrile was selected. Under the optimized conditions described above, the retention time of acteoside was 16.5 min (Fig. 3). Fig. 3A–C show the chromatograms of a blank blood dialysate, a blank blood dialysate spiked with acteoside (50 ng/mL) and a blood dialysate sample collected from 135 to 150 min after drug administration (10 mg/kg, i.v.), respectively. Fig. 4A–C represent the chromatograms of a blank brain dialysate, a blank brain dialysate spiked with acteoside (100 ng/mL) and a brain dialysate sample collected from 0 to 15 min after drug administration (10 mg/kg, i.v.), respectively.

Knowledge related to the in vivo pharmacokinetics of the botanic polyphenols was insufficient and rarely discussed. The probable reason for this lack of information may be due to

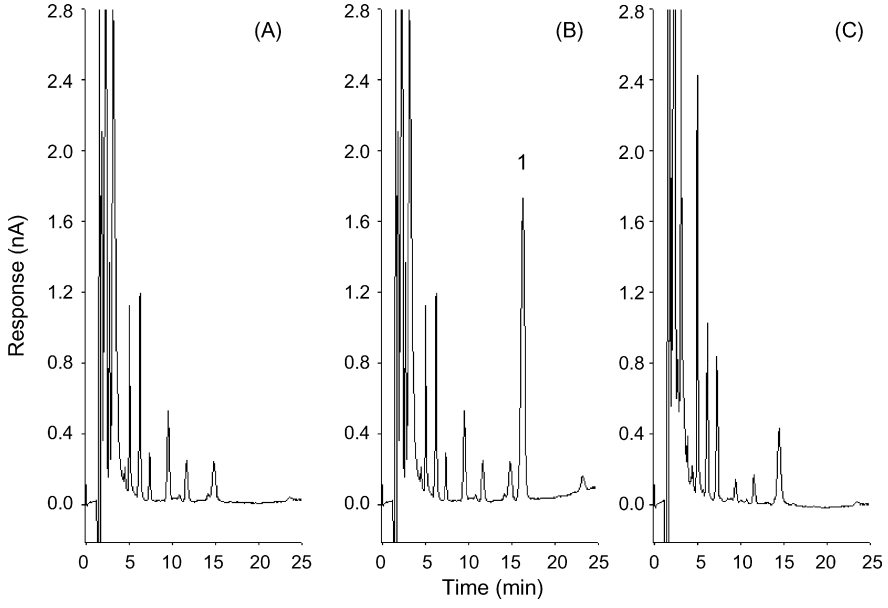

Fig. 4. Typical chromatograms of (A) blank brain dialysate from the microdialysis probe before drug administration; (B) blank brain dialysate spiked with acteoside (100 ng/mL); and (C) brain microdialysate sample collected from 0 to 15 min after acteoside administration (10 mg/kg, i.v.). HPLC conditions were the same as in Fig. 3.

the necessity of sensitive, convenient methods for measuring polyphenols in plasma and body fluids [2]. The proposed method using microdialysis and liquid chromatography with amperometric detection with direct injection in the chromatographic system seems to be simple but efficient. When it comes to sample handling, microdialysis provided a more straightforward option than extraction methods, such as liquid–liquid or solid-phase extraction. In addition, microdialysis samples without further preparations can be directly analyzed by the electrochemical detection system, and most of buffer salts or ion-pairing reagents used in the mobile phase were compatible with electrochemical detection. Furthermore, to maintain the sensitivity of electrochemical detection, the products of the electrochemical reaction attached to the electrode active surface of the working electrode (e.g. a glassy carbon electrode) can be cleaned by mechanical polishing using pure water or chromic acid solution [23]. On the contrary, mass spectrometric detection is incompatible with microdialysis samples in terms of signal stability, because the dialysis salts can clog the ionization source and create high background noise [24]. Besides, only volatile acids or salts can be used to prepare the mobile phase for LC–MS, such as trifluoroacetic acid or ammonium acetate [25]. Moreover, the maintenance for mass spectrometer was much more complicated than electrochemical detection. In addition to basic maintenance, such as ion source cleaning and periodical mass calibration, the upkeep of accessory instruments is expensive.

3.2. Method validation

3.2.1. Selectivity

The selectivity of this method was evaluated by comparing chromatograms from blank dialysate and spiked samples. As shown in Figs. 3A and 4A, no interference peaks appeared at the retention time (16.5 min) of acteoside, indicating the good selectivity of this method.

3.2.2. Limits of detection and quantitation

The LOD and LOQ for acteoside were found to be 2 ng/mL and 5 ng/mL, respectively. The sensitivity of this system is comparable to previous HPLC–MS method [15] for the determination of acteoside in the rat plasma, which possessed the limit of quantitation of 5 ng/mL.

3.2.3. Linearity, accuracy and precision

Five calibration standard samples of acteoside 5–500 ng/mL in blood dialysate were analyzed in triplicate in order to determine the linearity of this assay. This assay possessed linearity within the studied concentration range. The mean linear regression equation of the peak area (Y) versus acteoside concentration (X, ng/mL) was present as the form $Y = aX + b$, with a correlation ranging from 0.998 to 0.999. As shown in Table 1, the overall mean precision ranged from 1.6 to 11.8%, while the accuracy varied from 92 to 105.4%. Thus, the intra- and inter-day accuracy and precision were found to be acceptable for the analysis of a dialysis sample in support of pharmacokinetic studies.

Table 1
Precision and accuracy for acteoside quality control samples in blood dialysate

Nominal concentration (ng/mL)	Observed concentration (ng/mL)	Precision (%)	Accuracy (%)
Intra-day			
5	4.6 ± 0.3	6.5	92.0
50	52.7 ± 4.8	9.1	105.4
200	200.9 ± 3.2	1.6	100.5
Inter-day			
5	5.1 ± 0.6	11.8	102.0
50	51.8 ± 3.9	4.5	103.6
200	202.8 ± 5.7	2.8	101.4

Data expressed as mean ± SD

3.3. Application to pharmacokinetic study in rats

Compared to methods of biological fluids sampling and sample clean-up procedures, microdialysis provides a powerful sampling system for small animals, requiring no loss of biological fluids, and providing higher temporal resolution than traditional total blood sampling techniques. Instead of the discrete time point sampling method of traditional total blood sampling techniques, microdialysis makes continuous sampling possible. Furthermore, the dialysate can be injected directly without tedious sample pretreatments, and this technique increases the throughput.

Unbound drug concentration data obtained from the microdialysis technique are transferred by recovery. The average in vivo relative recovery of acteoside in blood probe and brain probe were at 20.9 ± 2.8% and 2.7 ± 0.2%, respectively. After drug administration to the rats, no obvious peak of acteoside could be detected in the brain microdialysate samples as shown in Fig. 4C. The poor permeability of acteoside across the blood–brain barrier might attribute to the low recovery of brain microdialysis probe, which possesses a shorter semi-permeable membrane size than blood microdialysis probe. Besides, the drug protein binding also influences the correction of dialysate from microdialysis probe. Since the semi-permeable membrane blocks molecules with a molecular weight greater than 13 000, the complex of acteoside and protein cannot diffuse into the perfusate, and only acteoside molecules (molecular weight: 624) can diffuse into the perfusate. These molecules are defined as protein-unbound acteoside. This might attribute to limited amount of acteoside reaching the brain after intravenous administration [15] as well as the low recovery for the brain probe.

The concentration versus time curve of acteoside in rat blood (Fig. 5) indicated that the disposition of acteoside in rat blood has a slower and longer elimination phase. The pharmacokinetic models (one- versus two-compartment) were decided according to the criteria of Akaike information criterion (AIC) [26], and the minimum AIC values indicate the suitable fitting model and representation of the blood concentration-time course data. Data from each rat was processed with the WinNonlin software by fitting two kinds of compartment models. This AIC value decreases from −4.08 for one-compartment model to −86.26 for the two-compartment model, indicating that the two-compartment model is more suitable than the one-compartment

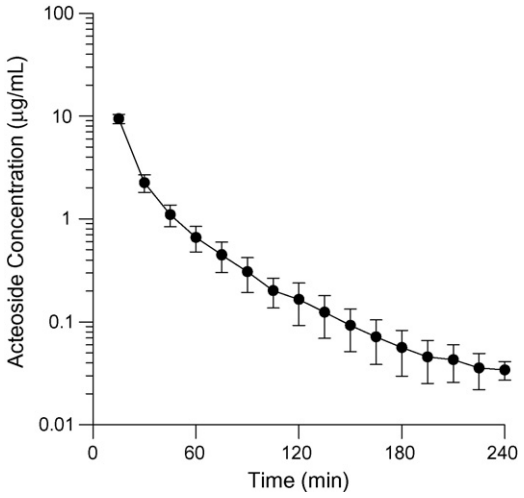

Fig. 5. Concentrations of acteoside in blood microdialysate vs. time curve after drug administration (10 mg/kg, i.v.) in rats.

Table 2
Pharmacokinetic parameters of acteoside in rats after 10 mg/kg administration

Parameters	Estimated
A (μg/mL)	71.9 ± 30.9
B (μg/mL)	2.9 ± 0.5
α (1/min)	0.14 ± 0.03
β (1/min)	0.02 ± 0.004
$t_{1/2,\alpha}$ (min)	5.0 ± 1.2
$t_{1/2,\beta}$ (min)	28.5 ± 4.7
AUC (min μg/mL)	592.3 ± 124.7
Vss (mL/kg)	277.7 ± 185.8
Cl (mL/min/kg)	17.7 ± 4.5

Data expressed as mean ± SD ($n = 6$); $t_{1/2,\alpha}$: distribution half-life; $t_{1/2,\beta}$: elimination half-life; AUC: area under the concentration vs. time curve; Vss: volume of distribution at steady-state; Cl: clearance.

model. The following equation applies to the two-compartment pharmacokinetic model: $C = Ae^{-\alpha t} + Be^{-\beta t}$; where A and B are the concentrations (C), of intercept for fast and slow disposition phases, respectively; and α and β are the disposition rate constants for fast and slow disposition phases, respectively. Data analysis after i.v. injection of acteoside at 10 mg/kg yields the following equation $C = 71.9e^{-0.14t} + 2.9e^{-0.02t}$. The pharmacokinetic parameters calculated by WinNonlin program are shown in Table 2. The pharmacokinetics of acteoside in total blood concentration has been previously characterized by the non-compartment model [15], and a rapid drop of the concentration and short half-life (10.7 min) was observed after intravenous administration. The rapid distribution characteristic for acteoside was very similar to other natural polyphenolic compounds, such as $t_{1/2,\alpha}$ of 6 min for (−)-epigallocatechin gallate (EGCG) [27] and $t_{1/2,\alpha}$ of 12.4 min for echinacoside [28].

4. Conclusions

In conclusion, we have developed a simple, sensitive and economical liquid chromatography with amperometric detection and microdialysis sampling method for the determination of protein-unbound acteoside in rat blood for the first time. Validation results had shown the good reproducibility of this method, and the sensitivity is comparable with HPLC–MS. This method was successfully applied to the pharmacokinetic study of acteoside in rat blood, and the data obtained suggested that two-compartmental pharmacokinetic model was more suitably fitted to describe the pharmacokinetic behavior of acteoside after single intravenous administration of acteoside.

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