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Determination of echinacoside in rat serum by reversed-phase high-performance liquid chromatography with ultraviolet detection and its application to pharmacokinetics and bioavailability

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Abstract

A rapid and simple high-performance liquid chromatographic (HPLC) method has been developed and validated for the determination of echinacoside (ECH) in rat serum. After protein precipitation of serum sample with trichloroacetic acid, the supernatant was directly injected and analyzed on a C_{18} CapcellACR analytical column (150 mm × 4.6 mm I.D. 5 μm) with a mobile phase consisting of acetonitrile–0.5% acetic acid (15.5:84.5, v/v). The UV detector was set at 330 nm. The lower limit of detection and quantification were 9 and 29.2 ng/mL, respectively, and the calibration curves were linear over the concentration range of 29.2–18250 ng/mL. The assay method was successfully applied to the study of the pharmacokinetics and bioavailability of ECH in rat.

Keywords: Echinacoside; Pharmacokinetics; Bioavailability; HPLC; Phenylethanoid glycoside

1. Introduction

Echinacoside (ECH, chemical structure is shown in Fig. 1) is one of the major phenylethanoid glycosides (PhGs) in Herb Cistanchis [1], which is an important traditional Chinese medicine and used for the treatment of kidney deficiency and neurasthenia. It is also the main phenolic component in *Echinacea angustifolia* and *E. pallida* roots [2], which are widely used in Europe, North America and Australia for their immunostimulating activities. As a natural product, ECH exhibited good antioxidant activities [3–6]. Recently, scientists found that ECH displayed neuroprotective activities [7,8]. In addition, ECH possessed radical scavenging [9,10], NO radical-scavenging [11] and anti-hepatotoxic activities [12] and so on.

There are many analysis methods including high performance-liquid chromatography (HPLC) [1,9,13–15], capillary electrophoresis (CE) [16] and high-performance thin layer chromatography (HPTLC) [17], which have been developed for ECH determination. However, all of these methods were developed for the quantitative determination of ECH in the plants, foods and medical preparations. Though there were many studies about the activities of ECH and PhGs, only three studies have investigated the potential bioavailability of Echinacea Constituents using a Caco-2 cell monolayer model and the actual bioavailability in a Phase I clinical trial [18–20]. The reported method was mainly developed for the analysis of alkylamides.

In the present paper, we first reported a simple and rapid HPLC method with ultraviolet detection for the quantification of ECH in rat serum. The method was highly sensitive, selective and fully validated. The assay was successfully applied to the pharmacokinetic study of ECH in rat and the absolute bioavailability assessing.

2. Experimental

2.1. Chemicals and reagents

Echinacoside was separated and purified from an ethanol extract of *Cistanche tubulosa* (Schenk) R. Wight and its structure was confirmed by UV, IR, MS and NMR spectroscopy.

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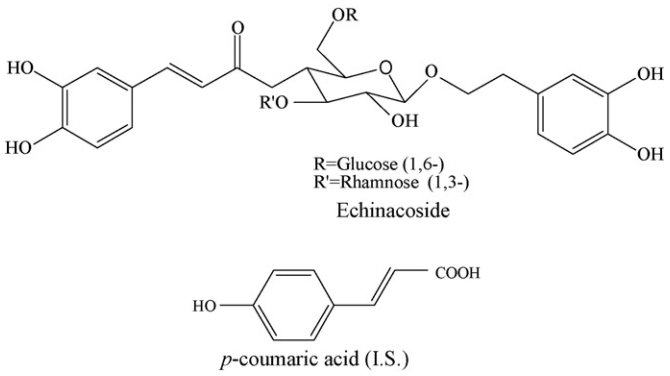

Fig. 1. Structures of Echinacoside and *p*-coumaric acid (I.S.).

The purity was 96.4% as determined by HPLC. *p*-Coumaric acid used as internal standard was supplied by the National Institute for the Control of Pharmaceutical and biological Products (Beijing, China). HPLC grade acetonitrile was obtained from Caledon Laboratories Ltd. (Georgetown, Ont., Canada). HPLC grade methanol was obtained from Concord Tech (Tianjin, China). Trichloroacetic acid was analytical grade. Water was glass-double distilled and filtered with 0.2 μm membranes.

2.2. Instrument and chromatographic conditions

HPLC analysis was carried out on an Agilent 1100 Series HPLC (Palo Alto, CA, USA) with UV detector. A C_{18} Capcell-ACR analytical column (150 mm × 4.6 mm I.D. 5 μm, Shiseido, Tokyo, Japan) protected by a C_{18} Securityguard (4 mm × 3.0 mm I.D. 5 μm, Phenomenex) was used. The column was maintained at 25 °C. A mobile phase composed of acetonitrile–0.5% acetic acid (15.5:84.5, v/v) was used throughout the analysis at a flow rate of 1.0 mL/min. The ultraviolet (UV) detector was set at 330 nm.

2.3. Preparation of stock solutions, calibration samples and quality control samples

Stock solutions of ECH and internal standard were prepared in methanol–water (60:40, v/v) at concentrations of 730.0 μg/mL and 67.2 μg/mL, respectively. The ECH stock solution was diluted with methanol–water (60:40, v/v) to working solutions ranging from 0.1168 to 73.0 μg/mL. Internal standard working solution (8.1 μg/mL) was also prepared by diluting the stock solution with methanol–water (60:40, v/v). All described solutions were protected from light, stored at 4 °C and were stable for at least 4 weeks. Calibration samples were obtained by diluting standard working solutions (40 μL) with drug-free rat serum (120 μL), to span a calibration standard range of 29.2–18250 ng/mL (29.2, 58.4, 116.8, 584, 1168, 5840, and 18250 ng/mL). Quality control (QC) samples (14600, 1168, 58.4 ng/mL) were prepared in a similar way.

2.4. Sample preparation

Blood samples were collected in polypropylenes tube and centrifuged at 860 × *g* for 10 min at 0 °C (Anting Sci. instr., Shanghai, China). The serum samples were stored at −80 °C until analysis. After thawing at room temperature, the serum samples were kept in ice–water bath. Aliquots (160 μL) of the blank, calibration standard, QC samples or aliquots (120 μL) of unknown rat serum samples (added with 40 μL of methanol–water (60:40, v/v) additionally) were spiked with internal standard working solution (20 μL), vortex mixing for 60 s, and with 10% trichloroacetic acid (80 μL), vortex mixing for 120 s. Then the tubes were centrifuged at 11,600 × *g* for 10 min at 0 °C. A volume of 80 μL supernatant was injected into HPLC system. All procedures were carried out under light-protected conditions.

2.5. Calibration curves

The calibration curves consisted of seven points covering the range from 29.2 to 18250 ng/mL. The regression equations were calculated by weighted ($1/x^2$) least-squares linear regression analysis of peak-area ratios (ECH/I.S.) versus the ECH concentrations.

2.6. Method validation

The method was fully validated for its specificity, linearity, lower limits of detection (LOD), lower limits of quantification (LOQ), accuracy and precision. The LOD was defined as the concentration that produced a signal-to-noise ratio of 3. The LOQ was determined as the lowest concentration of the analyte in serum that could be quantified with an inter-assay coefficient of variation (CV) lower than 20% and an accuracy between 80 and 120%. The intra- and inter-day accuracy and precision were evaluated by assaying the QC samples with high, mid, and low concentrations. The intra-day variation was determined by assaying six replicates on the same day and inter-day variation was assayed for six replicates on three days. The precision was expressed as the relative standard deviation (R.S.D.). The accuracy was determined by comparing the calculated concentration (obtained from the calibration curve) to the theoretical concentration. The extraction recoveries from serum was determined at three concentrations by comparing peak areas extracted from serum with those of the same amounts of standard solutions in 60% methanol. The recovery of I.S. from serum was determined at a concentration of 8.06 μg/mL by the same method. Stability of ECH in serum was assessed with QC samples ($n = 3$) stored at −80 °C for 4 weeks. Freeze–thaw stability of ECH in serum was investigated with QC samples ($n = 6$) subjected to three freeze/thaw cycles. Stability of ECH in serum in ice–water bath was assessed by analyzing QC samples ($n = 3$) every 4 h for 8 h. Stability of ECH in protein-precipitated QC samples ($n = 3$) was analyzed every 4 h by storing processed samples at 4 °C for 12 h.

2.7. Pharmacokinetic study

Male Sprague–Dawley rats (200–220 g) were obtained from the Laboratory Animal Center of Peking University

Health Science Center (Beijing, PR China). They were kept in an environmentally controlled breeding room (temperature: 20 ± 2 °C, humidity: 60 ± 5%, 12 h dark/light cycle) for three days before starting the experiments and fed with standard laboratory food and water ad libitum and fasted overnight before the test. After an intragastric (i.g.) administration of ECH (100 mg/kg), 0.5 mL blood samples via orbital veins were collected at 0.08, 0.25, 0.5 h, at 0.67, 1, 1.5 h, and at 2, 4 and 6 h from the first, the second and the third groups, respectively. And after an intravenous (i.v.) administration of ECH (5 mg/kg) via vena caudalis, 0.5 mL blood samples via orbital veins were collected at 0.03, 0.25, 0.5 h, at 0.67, 1, 1.5 h and at 2 and 4 h from the first, the second and the third groups, respectively. The blood samples were placed on ice–water bath for 30 min and centrifuged at 860 × *g* for 10 min at 0 °C. The serum samples were stored at −80 °C until analysis.

3. Results

3.1. Specificity, linearity and sensitivity

Representative chromatograms are shown in Fig. 2. Under the chromatographic conditions described, ECH and the I.S. were eluted with retention times of 4.3 and 10.0 min, respectively. Endogenous components in rat serum did not give any interfering peaks. Calibration curves established on three different days with ECH spiked to rat serum demonstrated good linearity over a range of 29.2–18250 ng/mL. The mean linear regression equation of the peak area ratios (*Y*) versus ECH concentration (*X*) was typically of the form $Y = (a \pm \text{S.D.}) + (b \pm \text{S.D.})X$ and it was $Y = (0.0021 \pm 0.0002) + (0.0045 \pm 0.0003)X$ with mean correlation of 0.998 ± 0.003. The LOD was approximately 9 ng/mL and LOQ was 29.2 ng/mL.

3.2. Precision and accuracy

The intra- and inter-day precision and accuracy of ECH are shown in Table 1. The precision (R.S.D.) were all less than 5%. The intra-day accuracy were 93.7–99.2% and the inter-day accuracy ranged from 92.8 to 100.0%. These results indicated that the present method had a good precision and accuracy.

Table 1
Intra- and inter-day precision and accuracy

Concentration (ng/mL)		R.S.D. (%)	Accuracy (%)
Added	Found (mean ± S.D.)		
Intra-day ($n = 6$)			
58.4	54.7 ± 3.5	6.3	93.7
1168	1158.9 ± 26.9	2.3	99.2
14600	14406.4 ± 222.6	1.5	98.7
Inter-day ($n = 3$)			
58.4	54.2 ± 1.1	2.0	92.8
1168	1139.8 ± 45.9	4.0	97.6
14600	14595.6 ± 246.7	1.7	100.0

Table 2
Recoveries of ECH and I.S. ($n = 6$)

Compound	Concentration (ng/mL)	R.S.D. (%)	Recovery (%) (mean ± S.D.)
ECH	58.4	1.3	114.2 ± 1.5
	1168	1.8	110.8 ± 2.0
	14600	1.4	107.7 ± 1.5
I.S.	8060	0.9	65.1 ± 0.5

3.3. Recovery

Table 2 shows the recoveries of ECH and I.S. from rat serum. The absolute recovery of ECH at three concentrations ranged from 107.7 to 114.2%. The recovery of I.S. was 65.1% at the concentration of 8.06 ug/mL.

3.4. Stability

The stability of ECH in rat serum during the sample storing and processing procedures was fully evaluated by analyzing QC samples at three concentrations. After three cycles of freezing and thawing, the measured concentrations were between 90.8 and 97.3%, indicating no significant substance loss during repeated freezing and thawing. Serum samples stored at −80 °C for 4 weeks showed good stabilities. And the accuracy ranged from 96.9 to 100.3%. During the assay, the QC samples placed in ice–water bath were stable for at least 8 h. The stability of processed QC samples indicated that ECH in rat serum kept at 4 °C is stable within 12 h. All the results are shown in Table 3.

3.5. Pharmacokinetic parameters and bioavailability

The method was applied to determine the serum concentration of ECH following i.g. administration (100 mg/kg) and i.v. administration (5 mg/kg) to rat, respectively. The pharmacoki-

Table 3
Stability of the samples

Concentration (ng/mL)		R.S.D. (%)	Accuracy (%)
Added	Found (mean ± S.D.)		
Three freeze and thaw cycles ($n = 6$)			
58.4	53.0 ± 1.6	2.9	90.8
1168	1071.0 ± 19.6	1.8	91.7
14600	14212.7 ± 251.4	1.8	97.3
4-weeks stability at −80 °C ($n = 3$)			
58.4	58.0 ± 3.1	5.3	99.3
1168	1131.5 ± 45.7	4.0	96.9
14600	14649.9 ± 57.5	0.4	100.3
In ice–water bath for 8 h ($n = 3$)			
58.4	53.0 ± 1.1	2.1	90.8
1168	1057.8 ± 13.9	1.3	90.6
14600	13804.7 ± 460.2	3.3	94.6
At 4 °C for 12 h ($n = 3$)			
58.4	56.8 ± 1.2	2.0	97.3
1168	1151.6 ± 51.7	4.5	98.6
14600	14528.4 ± 91.5	0.6	99.5

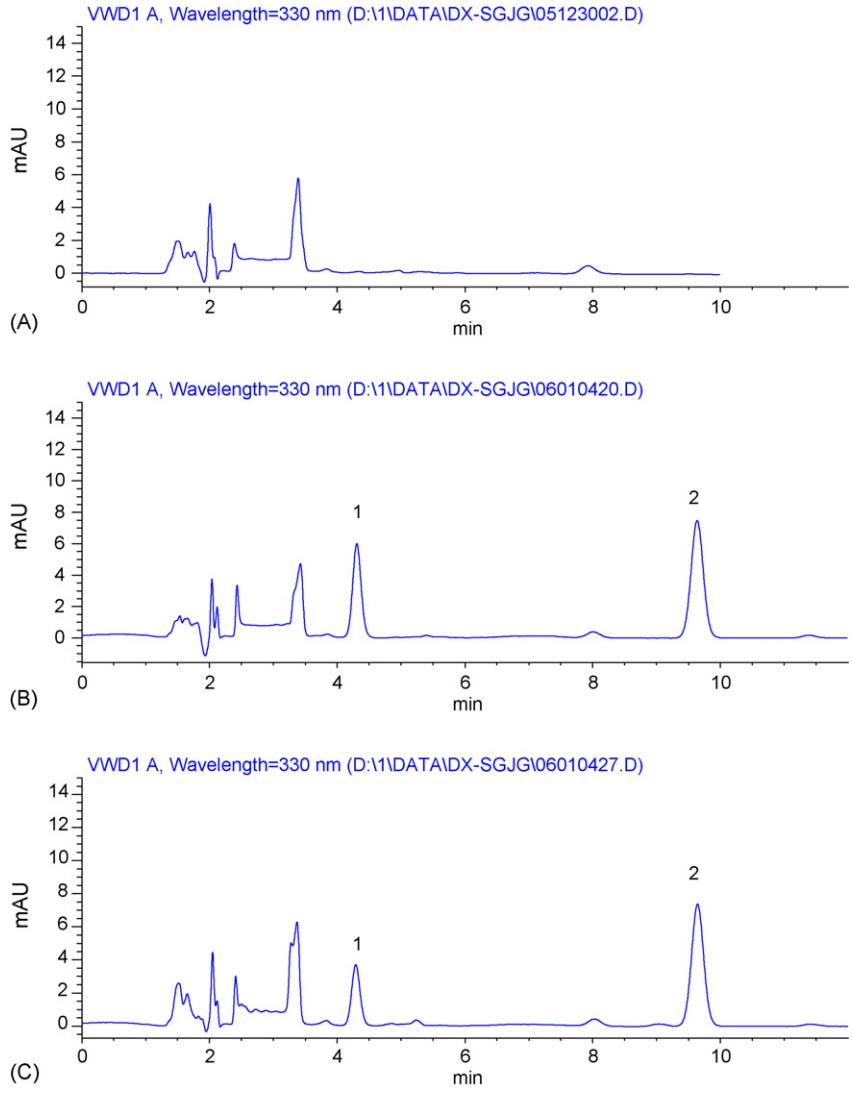

Fig. 2. Typical chromatograms of blank serum (A); serum spiked with ECH (1168 ng/mL) and I.S. (8.06 μg/mL) (B); a serum sample at 15 min after intragastric administration of ECH (C). Peak 1: ECH; peak 2: I.S.

netic parameters were estimated using 3P87 computer program (The Chinese Society of Mathematical Pharmacology). The serum ECH concentration–time curves for intragastric and intravenous administration were fitted to a one-compartment model and a two-compartment model, respectively. The mean serum concentration–time profiles of ECH are illustrated in Fig. 3A and B. The pharmacokinetic parameters are presented in Tables 4 and 5. The absolute bioavailability (F, %) of ECH was 0.83%.

Table 4
Mean pharmacokinetic parameters for ECH in rats ($n = 5$) after intragastric administration at 100 mg/kg dose level

Parameters	Unit	Value
C_{max}[a]	ng/mL	612.2 ± 320.4
T_{max}[a]	min	15.0
$T_{1/2}$	min	74.4
K_e	min^{-1}	0.01
$AUC_{0–360}$	ng min/mL	60704.9

C_{max}, the maximum serum concentration; T_{max}, the time to reach peak concentration; $T_{1/2}$, the apparent elimination half-life; K_e, the apparent elimination rate constant; AUC_{0-360}, the area under blood concentration–time curve.
[a] Calculated from the actual data.

4. Discussion

The chromatographic conditions were investigated to optimize for sensitivity, speed and peak shape. The UV spectrum of echinacoside has two absorption maxima at 218 and 330 nm. A wavelength of 330 nm was chosen to achieve the greatest sensitivity with minimal interference. Different chromatographic columns and mobile phase compositions were also screened. The CapcellACR-C_{18} column is a acid tolerant column. The analysis time was shorter and the sensitivity was higher. Acetic acid was used as a mobile phase modifier as it significantly restrained the peak tailing of echinacoside in our experiments. As a result, acetonitrile–0.5% acetic acid (15.5:84.5, v/v) was chosen as the eluting solvent system, as with it the desired separation and sensitivity was achieved.

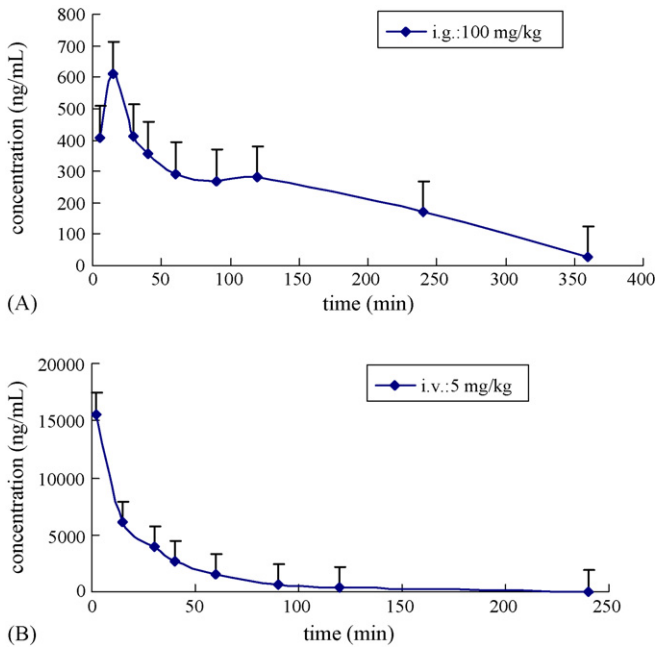

Fig. 3. Mean serum concentration time curves in rat after receiving 100 mg/kg oral dose (A) and 5 mg/kg intravenous dose (B).

The selection of the internal standard was an arduous and hard process. *p*-Coumaric acid was finally selected because it had a suitable retention time and was well resolved from the target analyte in our mobile phase.

The extraction of serum samples was optimized in our preliminary studies by comparing liquid–liquid extraction, solid-phase extraction and protein precipitation. Liquid–liquid extraction is not suitable for ECH extraction from serum because ECH is a hydrophilic compound. Solid-phase extraction was time-consuming and resulted in lower recovery and higher LOD. Other protein-precipitating reagents, such as methanol, acetonitrile and perchloric acid, were also investigated but produced endogenous interference and/or low recovery. However, the results were satisfactory when trichloroacetic acid was used in protein precipitation.

In a previous report, echinacoside was demonstrated to be highly susceptible to enzymic degradation and oxidation in hydroalcoholic solutions during extraction process [21]. In our preliminary studies, we also found that echinacoside in biosamples was susceptible to degradation at a higher temperature during the whole process. So the operation must be carried out carefully at lower temperature.

The absorption of echinacoside was extremely fast in rats (T_{max}, 15.0 min) after intragastric administration (100 mg/kg). However, the serum concentration maximum was very low (C_{max}, 612.2 ± 320.4 ng/mL). The elimination was fast after intragastric administration ($T_{1/2}$, 74.4 min). And at 6-h post-dosing, the concentration decreased to be only about 36.3 ng/mL. All the pharmcokinetic parameters are presented in Tables 4 and 5 and Fig. 3. The serum concentration–time curves for intragastric and intravenous administration were fitted to a one-compartment model and a two-compartment model, respectively. The distribution and elimination of echinacoside were extremely fast in rats ($t_{1/2\alpha}$, 12.4 min; $t_{1/2\beta}$, 41.0 min) after intraveneous administration (5 mg/kg), and the mean concentrations decreased from 15598.8 ng/mL (2 min) to 43.6 ng/mL (4 h).

The bioavailability of ECH in rat was very low in this study. This was consistent with the result that the caffeic acid conjugates (echinacoside, caftaric acid and cichoric acid) permeated poorly through the Caco-2 monolayers, in Matthias' report [20]. But it was very interesting that caffeic acid conjugates could not be identified in any human plasma sample at any time after echinacea tablet ingestion [18,19]. The metabolites of caffeic acid conjugates are likely to be absorbed and be active components. Further study needs to be carried out to elucidate the human absorption mechanism to ECH and caffeic acid conjugates.

Table 5
Mean pharmacokinetic parameters for ECH in rats ($n = 5$) after intravenous administration at 5 mg/kg dose level

Parameters	Unit	Value
A	ng/mL	12182.5
α	min^{-1}	0.06
B	ng/mL	2508.0
β	min^{-1}	0.02
V_c	mg/kg/(ng/mL)	0.0003
$t_{1/2\alpha}$	min	12.4
$t_{1/2\beta}$	min	41.0
K_{21}	min^{-1}	0.02
K_{10}	min^{-1}	0.04
K_{12}	min^{-1}	0.01
AUC	ng min/mL	367019.1
CL	mg/kg/min/(ng/Ml)	0.0001

V_c, the center compartment volume of distribution; $T_{1/2\alpha}$, the half life of distribution; $T_{1/2\beta}$, the half life of elimination; K_{21} and K_{12}, the transportation constants; K_{10}, the elimination constant; AUC, the area under blood concentration–time curve; CL, the clearance.

5. Conclusion

This paper firstly describes a sensitive, selective, simple and rapid method for the determination of ECH in rat serum using high-performance liquid chromatography with ultraviolet detection. The method was successfully applied to evaluate the pharmacokinetic parameters and the bioavailability of ECH in rat.

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