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Short communication

Determination of epimedin C in rat plasma by reversed-phase high-performance chromatography after oral administration of Herba Epimedii extract

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Abstract

A simple high-performance liquid chromatographic (HPLC) method has been developed for the determination of epimedin C in rat plasma and applied to a pharmacokinetic study in rats after administration of Herba Epimedii extract. After addition of carbamazepine as an internal standard plasma samples were extracted with ethyl acetate. HPLC analysis of the extracts was performed on a Hypersil ODS2 analytical column using acetonitrile −0.4% acetic acid (25:75, v/v) as the mobile phase. The UV detector was set at 260 nm. The standard curve was linear over the range 0.05–4.0 μg/mL. The lower limit of quantification was 0.05 μg/mL. The HPLC method developed could be easily applied to the determination and pharmacokinetic study of epimedin C in rat plasma after giving the animals Herba Epimedii extract.

Keywords: Epimedin C; Herba Epimedii; HPLC

1. Introduction

Herba Epimedii, the dried aerial parts of Epimedium brevicornum Maxim., Epimedium sagittat Maxim., Epimedium pubescens Maxim., Epimedium wushanense T.S. Ying., and Epimedium koreanum Nakai is a well known traditional Chinese medicine (TCM) [1]. It has been widely used in the treatment of cardiovascular diseases and other chronic illnesses (infertility, amnesia and asthenia, impotence and declining senile function) in China for over 2000 years. Recently, it has been applied to the treatment of osteoporosis [2]. Flavones are the main constituents and epimedin C (Fig. 1A) is one of the active ingredients of Herba Epimedii [3,4]. It is reported that epimedin C promotes the phagocytic activity of the reticuloendothelial system and has a stimulant effect on macrophages [4]. Herba Epimedii extract promotes the proliferation of osteoblasts cells [5]. Epimedin C significantly enhances the immune response of spleen antibody-forming cells (SAFC) in mice following treatment with hydrocortisone acetate(HCA), and it also enhances lymphocyte proliferation and restores interleukin-2 (IL-2) production [6]. Therefore, epimedin C is used as one of the marker compounds for characterizing the Herba Epimedii. The mechanisms of action of its active constituents are not clear yet and, therefore, a method for pre-clinical studies is required.

As a knowledge of pharmacokinetics can help us explain and predict a variety of events related to the efficacy and toxicity of herbal preparations, it is important to investigate the pharmacokinetics of their active constituents. A number of published papers have reported the analysis and pharmacokinetics of active constituents from a number of herbal medicines. GC–MS methods are more suitable for determining compounds with small molecular weights with low boiling points [7]. while LC–MS methods are particularly useful for determining compounds at low levels in biological fluids [8]. Compared with LC–MS methods, HPLC-UV methods have several inherent limitations—lower sensitivity and lack of specificity. Nevertheless HPLC-UV methods are very popular and sensitive enough for the determination of

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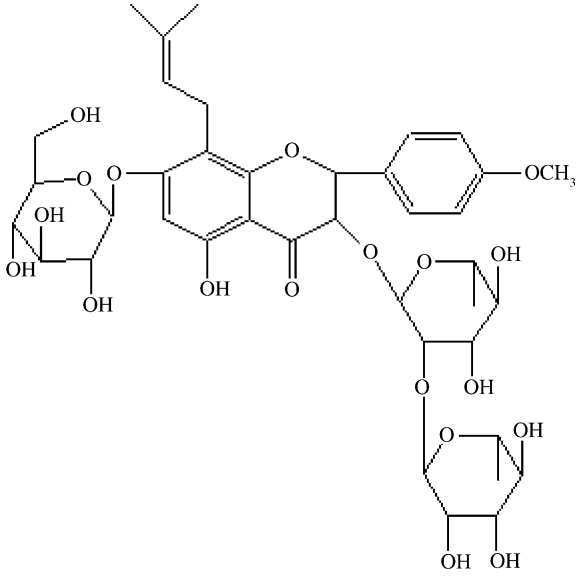
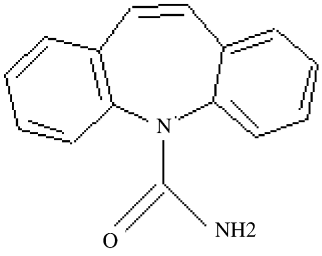


(A) Chemical structure of epimedin C



(B) Chemical structure of carbamazepine

Fig. 1. (A) Chemical structure of epimedin C, (B) chemical structure of carbamazepine.

a number of active compounds in some preclinical studies [9–11].

Gradient elution liquid chromatography methods have been used to study the content of flavonoids including epimedin C, in plants [12–13]. To date, there have been no published reports of the assay of epimedin C in rat plasma after oral administration of Herba Epimedii extract. The present study reports for the first time, the development and validation of an HPLC method for the determination of epimedin C concentrations in rat plasma and for its pharmacokinetic study following oral administration of Herba Epimedii extract.

2. Experimental

2.1. Materials and reagents

Aerial parts of *Epimedium brevicornum* Maxim. (including branches and leaves) were collected in a valley, located in Hanzhong, Shannxi province, China. The raw material was identified by Professor Qishi Sun (Department of Medicinal Plant, Shenyang Pharmaceutical University, Shenyang, China). Acetonitrile was of HPLC grade and provided by Shandong Yuwang Chemical Factory (Shandong, China), while all other reagents were of analytical grade. Healthy Wistar rats were obtained from the Experimental Animals Centers of Shenyang Pharmaceutical University. The TGL-16C centrifuge used in this investigation was from Shanghai Anting Science Instrument Factory (Shanghai, China). Epimedin C was isolated and purified in our laboratory. The purity was 99% determined by HPLC. Carbamazepine (Fig. 1B) used as internal standard (IS) was supplied by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

2.2. Chromatographic system

The analysis was carried out on an HPLC system (Elite, Dalian, China) equipped with a P200 II pump, UV detector and Echrom workstation (Elite, Dalian, China). The analyte was determined at room temperature on an analytical column (Hypersil ODS2, 250 mm × 4.6 mm, i.d., 5 μm). The mobile phase consisted of a mixture of acetonitrile–0.4% acetic acid (25:75, v/v). The mobile phase was filtered under vacuum through a 0.45 μm membrane filter, and degassed before use. The analysis was carried out at a flow rate of 1.0 mL/min with the detection wavelength set at 260 nm.

2.3. Preparation of standards and quality control samples

Stock standard solutions of epimedin C and the internal standard, carbamazepine, were prepared with methanol. Six calibrators of epimedin C with internal standard were prepared by dilution of stock solutions followed by spiking drug-free plasma. The calibration range was 0.05–4.0 μg epimedin C per mL plasma. Quality control (QC) samples were prepared at low (0.05 μg/mL), medium (1 μg/mL), and high (4 μg/mL) concentrations in the same way as the plasma samples for calibration.

2.4. Preparation of Herba Epimedii extract

One hundred grams of Herba Epimedii was extracted with 75% ethanol (1200 mL) by refluxing for 1.5 h in a water-bath at 100 °C, and then filtered. The extraction was repeated twice. The extraction solutions were combined, ethanol was removed under reduced pressure, and the residue was dissolved in water, to give an extract with a concentration of 1 g/mL (expressed as the weight of raw Herba Epimedii).

2.5. Plasma sample preparation

Five Wistar rats (body weight 220 ± 20 g) were not fed for 12 h prior to administration of the drug extract. The rats were then given the extract with an oral dose of 20 g Herba Epimedii (containing 364 mg of epimedin C)/kg body weight.

Animals had free access to water during the experiment. A blood sample (0.4 mL) was collected from the suborbital vein into heparinized tubes at 0, 0.5, 1, 2, 4, 6, 9, 12 and 24 h following drug administration.

All blood samples were immediately centrifuged for 10 min at 11000 × *g*, and the plasma was transferred into clean tubes and stored at −20 °C prior to HPLC analysis. To 200 μL of plasma, 50 μL of internal standard, carbamazepine, (3.9 μg/mL) and 1000 μL of ethyl acetate was added, followed by vortex mixing for 1 min and centrifuging at 11000 × *g* for 10 min, The extraction was repeated with 1000 μL ethyl acetate. The supernatant was combined and evaporated to dryness under nitrogen at 50 °C. The residue was reconstituted with 100 μL mobile phase and an aliquot (20 μL) was injected into the HPLC system.

3. Results and discussion

3.1. Specificity

Typical chromatogams of blank and spiked plasma with epimedin C and carbamazepine are given in Fig. 2A and B. There were no coeluting peaks in the vicinity of the epimedin C and carbamazepine peaks in the chromatogram of blank plasma. A chromatogram of a plasma extract from a rat at 4 h after oral administration of Herba Epimedii extract (20 g/kg body weight) is shown in Fig. 2C.

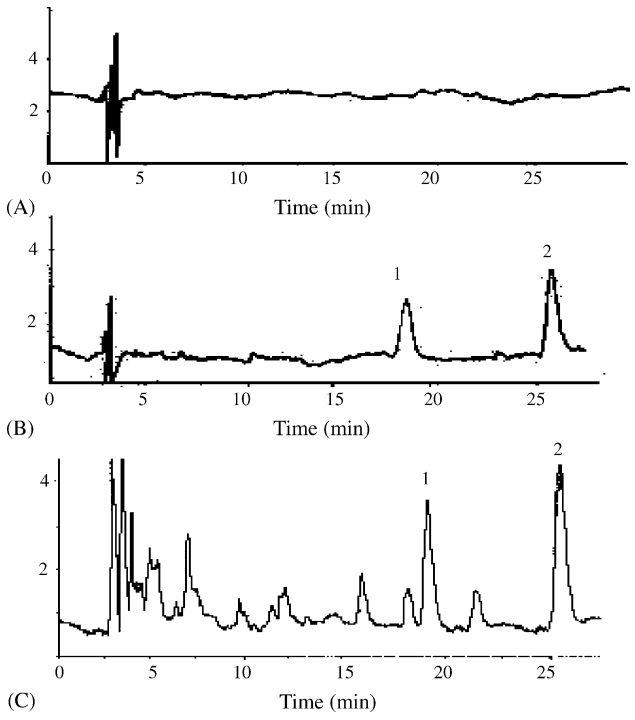


Fig. 2. Representative chromatogram of blank plasma (A); plasma spiked with IS (carbamazepine, 3.9 μg/mL) and epimedin C (1.0 μg/mL) (B); a plasma sample 4 h after oral administration of Herba Epimedin C extract (C). Peak 1, epimedin C; Peak 2, carbamazepine.

3.2. Method development

There are three absorption maxima at 202, 242 and 260 nm in the UV spectrum of epimedin C. However, interferences from endogenous substances and constituents of Herba Epimedii were observed with detection at 202 and 242 nm. A detection wavelength of 260 nm proved to be the most suitable and was therefore selected for the assay.

Ethyl acetate was employed as the extraction solvent in our procedure and provided a clean supernatant with a high extraction recovery for epimedin C without any significant interference. During development of the method, acetonitrile and methanol were tested as agents to deproteinize rat plasma, however, this method cause a high back pressure and shortened the column life. Liquid-liquid extraction was simple and clean. Other solvents besides ethyl acetate, such as diethyl ether and chloroform, were also tried as the extraction solvent. However, only samples extracted with ethyl acetate gave a good resolution and high recovery.

Internal standard are generally incorporated into assays in order to improve the precision, especially when biological samples are involved. Initially many compounds were investigated and it was found that carbamazepine was the most suitable internal standard, since it exhibited a similar behavior to the analyte during the sample preparation procedure.

Some methods for the determination of epimedin C in plants have already been reported [12,13], in which no baseline resolution was obtained within 40 min. In our study the total run time was less than 30 min, and a good separation of carbamazepine and epimedin C was achieved under the specified chromatographic conditions. No interference was found at the retention times of carbamazepine and epimedin C.

3.3. Calibration and validation

Evaluation of the assay was performed with a six-point calibration curve over the concentration range 0.05–4.0 μg/mL. Blank plasma was spiked with stock solutions of standard epimedin C to construct the calibration curve. The slope and intercept of the calibration graphs were calculated by weighted least squares linear regression. During the method validation, three sets of calibration standards were prepared and analyzed on three separate days. The regression equation of three standard curves was:

$$y = (0.5832 \pm 0.008429)x - (0.08412 \pm 0.003048) \quad (1)$$

Where y is the peak area ratio of epimedin C to the internal standard, and x is the plasma concentration of epimedin C. The calibration curve was linear over the concentration range 0.05–4.0 μg/mL in plasma with a mean correlation coefficient of 0.9990.

The lower limits of quantification (LLOQ) was defined as the lowest concentration on the calibration curve that can be measured with acceptable precision. The LLOQ was found to be 0.05 μg/mL for epimedin C in rat plasma with an accuracy

Table 1
Precision and accuracy of the HPLC-UV method to determine epimedin C in rat plasma ($n = 15$)

Concentration ($\mu g\,mL^{-1}$)		Relative error (%)	Intraday R.S.D. (%)	Interday R.S.D. (%)
Added	Found			
0.05	0.0548	9.6	8.8	7.7
1.0	1.003	2.5	1.0	3.4
4.0	3.83	−4.3	2.2	1.3

(relative error, R.E.) and precision (relative standard deviation, R.S.D.) not exceeding 20%. The accuracy and precision of the method were evaluated with QC samples at concentrations of 0.05, 1.0 and 4.0 μg/mL. The results are shown in Table 1. The intra-day and inter-day precision of the QC samples were satisfactory with R.S.D.s less than 8.8%. The determined values deviated from the nominal concentration with an R.E. of less than 9.6%.

The extraction recovery was determined by standard addition at three different concentrations (0.05, 1.0 and 4.0 μg/mL) and was calculated by comparing the peak areas of the prepared standard samples with those of the standard solutions. The mean extraction recoveries of epimedin C at the three concentrations were 79.3, 86.8 and 84.6%, with S.D.s of 5.9, 2.1 and 4.6%, respectively. The extraction recovery of carbamazepine (IS) was 77.2%.

The stability of prepared samples at room temperature was examined by comparing the data from samples analyzed immediately with those at 4, 10 and 24 h after sample preparation. The relative errors at the three different concentrations studied were 3.2, −5.9 and 2.8% for epimedin C indicating a stability of at least 24 h at room temperature. The stability of epimedin C in plasma was investigated using spiked QC samples at three different concentrations prepared in duplicate. The deviation of spiked QC samples stored at −20 °C for a week from fresh QC samples were 7.3, −6.5 and 4.2%. Epimedin C was stable in rat plasma under these storage condition.

Recently much effort has been put into the identification and determination of the active constituents of TCM and other herbal extracts in biological samples in order to understand their mechanisms of action and pharmacokinetic behavior. For example, Li et al [9] have described an HPLC method for the determination of spinosin in rat plasma after oral administration of a Suanzaoren decoction, with a lower limit of quantification of 18.07 ng/mL. Another HPLC-UV method has been reported for the pharmacokinetic study of cinnamic acid in rats given a TCM Ling-Gui-Zhu-Gan decoction [10]. The LLOQ for cinnamic acid in rat plasma was 0.84 μg/mL. In our laboratory, an HPLC method has been developed for the determination of chorogenic acid in rabbit plasma after an oral dose of Flos Lonicerae extract [11]. The present paper investigates for the first time the quantification and pharmacokinetics of epimedin C in rat plasma after oral administration of Herba Epimedii extract. Although all these HPLC-UV methods are applicable to preclinical studies using high doses, methods with a higher sensitivity are required for clinical studies. Another disadvantage of UV detection is its poor specificity. GC–MS [7] or LC–MS [8] methods provide a high sensitivity of at the ng/mL level and short analysis times of a few minutes combined with excellent selectivity. These methods should be more reliable and suitable for the determination of low concentrations of constituents in biological samples.

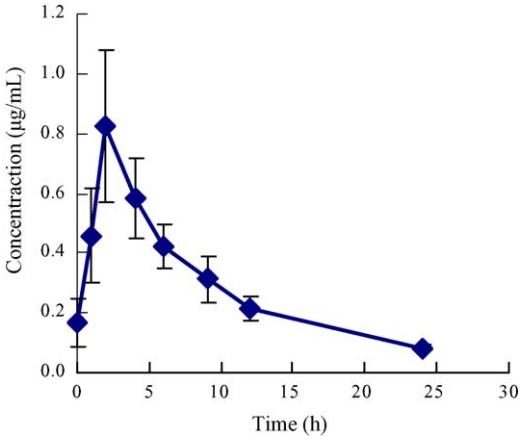


Fig. 3. Plot of the mean concentration of epimedin C in the plasma of rats vs. administration of Herba Epimedii extract ($n = 5$).

3.4. Pharmacokinetic applicability

This validated method was applied to monitoring the plasma concentrations of epimedin C in rats after a single oral administration of Herba Epimedii extract at a dose of 20 g/kg body weight. The pharmacokinetic parameters were estimated using the 3P97 computer program (The Chinese Society of Mathematical Pharmacology). The plasma epimedin C concentration–time curve was fitted to a two-compartment open model. The mean plasma concentration–time profile is illustrated in Fig. 3. The pharmacokinetic parameters are presented in Table 2.

The C_{max} of epimedin C was reached after approximately 2.5 h. The $T_{1/2}$ was calculated to be 8.3 h, which indicates that this herbal decoction may be administered 3 times a day. This result explain the suitability of the traditional administration method for Herba Epimedii and its compound TCM,

Table 2
Pharmacokinetic parameters of epimedin C in rats after oral administration of Herba Epimedii extract ($n = 5$)

C_{max} (μg/mL)	0.862 ± 0.020
T_{max} (h)	2.5 ± 1.0
$T_{1/2}$ (h)	8.3 ± 0.8
K_e (h^{-1})	0.0842 ± 0.00843
$AUC_{0-\infty}$ (μg h/mL)	7.90 ± 1.31

C_{max}, the maximum plasma concentration; T_{max}, the time to reach peak concentration; $T_{1/2}$, the apparent elimination half-life; K_e, the apparent elimination rate constant; $AUC_{0-\infty}$, the area under curve concentration–time.

namely, 3 times a day orally. The pharmacokinetic parameters of epimedin C suggest that it may be used as a maker compound to characterize some profiles of the Herba extract.

4. Conclusion

This paper describes a specific and simple HPLC method with UV detection for the determination of epimedin C in rat plasma after oral administration of Herba Epimedii extract. It has a potential application in preclinical studies of traditional Chinese medicines which contain Herba Epimedii. The study enable the successful determination of pharmacokinetic parameters broadening our knowledge about the therapeutic applications of such herbal extracts. A pharmacokinetic study of the active constituents in TCM will play an important role in identifying their mechanisms of action and investigating their synergetic effects.

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