

HPLC Determination and Pharmacokinetics of Osthole in Rat Plasma after Oral Administration of Fructus Cnidii Extract

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Abstract

A simple and sensitive high-performance liquid chromatographic (HPLC) method is developed for the determination of osthole in rat plasma and applied to a pharmacokinetic study in rats after administration of Fructus Cnidii extract. After addition of fluocinonide as an internal standard, plasma samples are extracted with diethyl ether. HPLC analysis of the extracts is performed on a Hypersil ODS2 analytical column, using methanol–0.4% acetic acid (65:35, v/v) as the mobile phase. The UV detector is set at 322 nm. The standard curve is linear over the range 0.0520–5.20 µg/mL ($r = 0.9979$). The mean extraction recoveries of osthole at three concentrations were 81.0%, 91.2%, and 90.7%, respectively. The intra- and interday precisions have relative standard deviations from 1.9% to 4.9%. The limit of quantitation is 0.0520 µg/mL. The HPLC method developed can easily be applied to the determination and pharmacokinetic study of osthole in rat plasma after the animals are given the Fructus Cnidii extract. The plasma concentration of osthole from six rats showed a C_{\max} of 0.776 ± 0.069 µg/mL at T_{\max} of 1.0 ± 0.3 h.

Introduction

Fructus Cnidii is the fruit of *Cnidium monnieri* (L.) Cuss (umbelifera), and it is a well-known traditional Chinese medicine (TCM) for the treatment of itchy skin, rashes, eczema, and ringworm, typically in the genital area (1). Coumarins are the main constituents, and osthole (Figure 1) is one of the active compounds in Fructus Cnidii (2). Recently, pharmacological studies also showed that Fructus Cnidii had potential activity against osteoporosis (3). Fructus Cnidii extract and osthole promote the proliferation of osteoblasts-like UMR106 cells (4). Osthole has been found to inhibit the secretion of hepatitis B virus surface antigens in vitro (5). It inhibits platelet aggregation and release reaction through suppression of thromboxane formation and phospho-

inositide breakdown (6). Osthole also exhibits vasorelaxant action by elevation of cGMP level of vascular smooth muscle and inhibition of calcium influx (7). It is reported that osthole exerts a nonspecific relaxant effect on the trachealis by inhibiting the cyclic adenosine monophosphate and cyclic guanosine monophosphate (cGMP) phosphodiesterases (8). Therefore, osthole is used as one of the marker compounds for the characterization of Fructus Cnidii.

Pharmacokinetic studies of active constituents in TCM would have a considerable impact on illustrating their action mechanism and on the development of TCM. However, it is a great challenge to investigate the pharmacokinetics of active constituents from TCM because both the constituents of TCM and their actions in the body are very complicated, and the contents of constituents in biological samples are usually low. There are some methods for the determination of osthole in the plasma of rats and rabbits after intravenous administration of osthole (9,10). However, Fructus Cnidii is usually administered orally in the form of a decoction, sometimes with other herbal medicines. The coexisting constituents may produce effects on the pharmacokinetic behaviors of osthole. To date, there have been no published reports of the assay of osthole in rat plasma after oral administration of Fructus Cnidii extract. In order to support preclinical pharmacokinetic studies requiring the quantitation of osthole in biological matrices, the present study reports, for the first time,

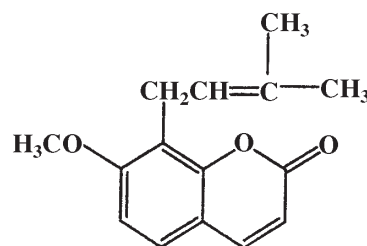


Figure 1. Chemical structure of osthole.

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the development and validation of a method for the determination of osthole concentrations in rat plasma and its pharmacokinetic study following oral administration of Fructus Cnidii extract.

Experimental

Materials and reagents

Osthole was provided by the China National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Fructus Cnidii was purchased from Tianyitang drug store (Shenyang, China) and identified as the fruits of *Cnidium monnieri* (L.) Cuss (umbelifera) by Professor Qishi Sun (Department of Medicinal Plant, Shenyang Pharmaceutical University, Shenyang, China). Methanol was of high-performance liquid chromatography (HPLC) grade and provided by Shandong Yuwang Chemical Factory (Shandong, China), and all other reagents were of analytical grade (Yuwang Chemical Factory, Shandong, China). Healthy Wistar rats were obtained from the Experimental Animals Centers of Shenyang Pharmaceutical University (Shenyang, China). The TGL-16C centrifuge used in this investigation was from Shanghai Anting Science Instrument Factory (Shanghai, China).

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). The analyte was determined at room temperature on an analytical column (Hypersil ODS2, 250- × 4.6-mm i.d., 5 μm). The mobile phase consisted of a mixture of methanol-0.4% acetic acid (65:35, 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 0.7 mL/min with the detection wavelength set at 322 nm.

Preparation of standards and quality control samples

Stock standard solutions of osthole and the internal standard, fluocinonide, were prepared with methanol. Seven calibrators of osthole with internal standard were prepared by dilution of stock solutions followed by spiking with drug-free plasma. The calibration range was 0.0520–5.20 μg osthole/mL plasma. Quality control (QC) samples were prepared at low (0.104 μg/mL), medium (0.520 μg/mL), and high (5.20 μg/mL) concentrations in the same way as the plasma samples for calibration.

Preparation of Fructus Cnidii extract

One hundred grams of Fructus Cnidii was extracted with 75% ethanol (1200 mL) by refluxing 1.5 h on 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 the raw material of Fructus Cnidii).

Plasma sample preparation

Six 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 10 g (containing 131 mg of osthole)/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, 0.17, 0.5, 1, 1.5, 2, 4, 6, 9, and 12 h following drug administration.

All blood samples were immediately centrifuged for 10 min at 12,000 rpm, and the plasma was transferred into clean tubes and stored at -20°C prior to HPLC analysis. To 200 μL of plasma, 100 μL of internal standard, fluocinonide (7.1 μg/mL), and 400 μL of diethyl ether were added, followed by vortex mixing for 1 min and centrifuging at 12,000 rpm for 10 min. The extraction was repeated twice with 400 μL diethyl ether. 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.

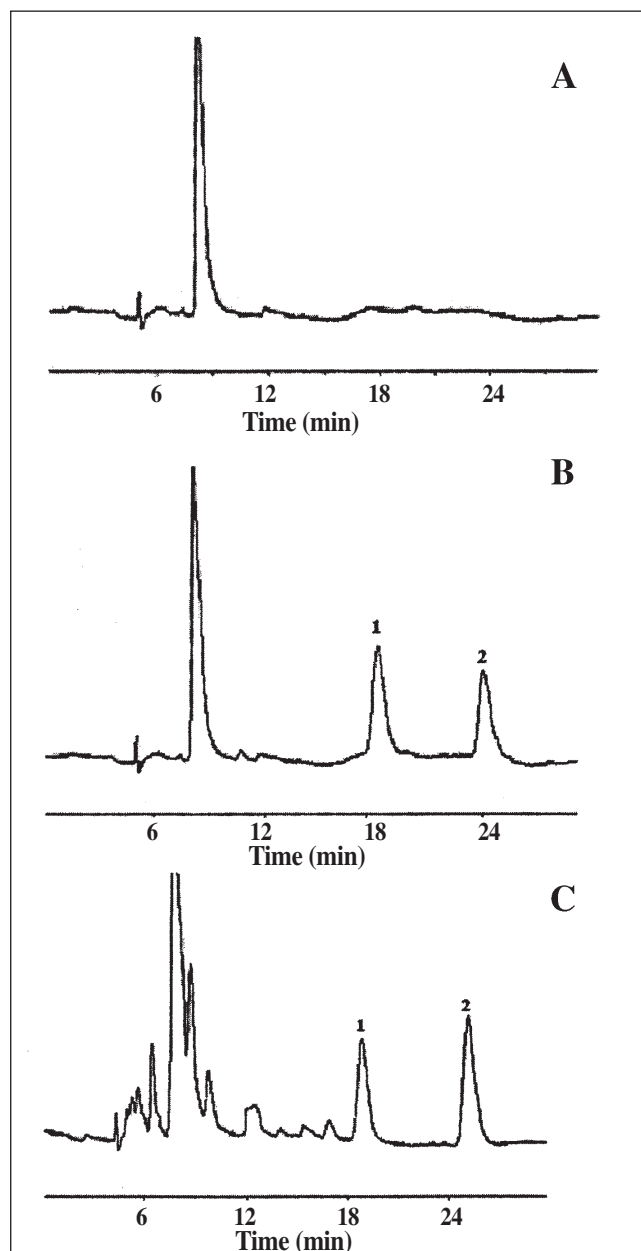


Figure 2. Representative chromatogram of blank plasma (A), plasma spiked with internal standard (1) and osthole (2) (B), and plasma sample 1 h after an oral administration of Fructus Cnidii extract (C).

Results and Discussion

Typical chromatograms of blank and spiked plasma with osthole and fluocinonide are given in Figure 2A and 2B, respectively. There were no coeluting peaks in the vicinity of the osthole and fluocinonide peaks on the chromatogram of blank plasma. A chromatogram of a plasma samples from a rat at 3 h after oral administration of Fructus Cnidii extract (10 g/kg body weight) is shown in Figure 2C.

There are two absorption maxima at 202 and 322 nm in the UV spectrum of osthole. However, interferences from endogenous substances and constituents of Fructus Cnidii were observed with detection at 202 nm. A detection wavelength of 322 nm proved to be the most suitable and was, therefore, selected for the assay.

Diethyl ether was employed as the extraction solvent in the procedure and provided a clean supernatant with a high extraction recovery for osthole without any significant interference. During development of the method, acetonitrile and methanol were tested as agents to deproteinize rat plasma; however, the recovery of osthole was unsatisfactory (< 60%). Other solvents besides diethyl ether, such as ethyl acetate and chloroform, were also tried as the extraction solvent. However, only samples extracted with diethyl ether gave a good resolution and high recovery.

The extraction recovery was determined by standard addition at three different concentrations (0.104, 0.520, and 5.20 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 osthole at the three concentrations were 81.0%, 91.2%, and 90.7%, respectively. The extraction recovery of fluocinonide (internal standard) was 82.3%.

Evaluation of the assay was performed with a seven-point calibration curve over the concentration range 0.0520 to 5.20 µg/mL. Blank plasma was spiked with stock solutions of standard osthole 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:

$$y = (1.559 \pm 0.0909)x - (0.01033 \pm 0.00479) \quad \text{Eq.1}$$

where y is the peak area ratio of osthole to the internal standard, and x is the plasma concentration of osthole. The calibration curve was linear over the concentration range of 0.0520–5.20

µg/mL in plasma with a mean correlation coefficient of 0.9979.

The limit of quantitation (LOQ) was defined as the lowest drug concentration on the plasma and determined at a signal-to-noise ratio of 10:1. The LOQ was found to be 0.0520 µg/mL for osthole in rat plasma with an accuracy [relative error (RE)] and precision [relative standard deviation (RSD)] not exceeding 20%. The LOQ of osthole is lower than that reported in the literature (0.1 µg/mL) (9,10). The accuracy and precision of the method were evaluated with QC samples at concentrations of 0.104, 0.520, and 5.20 µg/mL. The results are shown in Table I. The intra- and interday precision of the QC samples were satisfactory with RSDs less than 7.5%. The determined values deviated from the nominal concentration with an RE of less than 6.7%.

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 stability of osthole in plasma was investigated by using spiked QC samples at three different concentrations prepared in duplicate. The REs at the three different concentrations studied were less than 5.8% for osthole indicating a stability of at least 24 h at room temperature. The deviation of spiked QC samples stored at –20°C for a week from fresh QC samples were within 6.5%, –3.2%, and –5.9%. Osthole was stable in rat plasma under these storage conditions.

This validated method was applied to monitor the plasma concentrations of osthole in rats after a single oral administration of extract at a dose of 10 g (containing 131 mg of osthole)/kg body weight. The pharmacokinetic parameters were estimated using

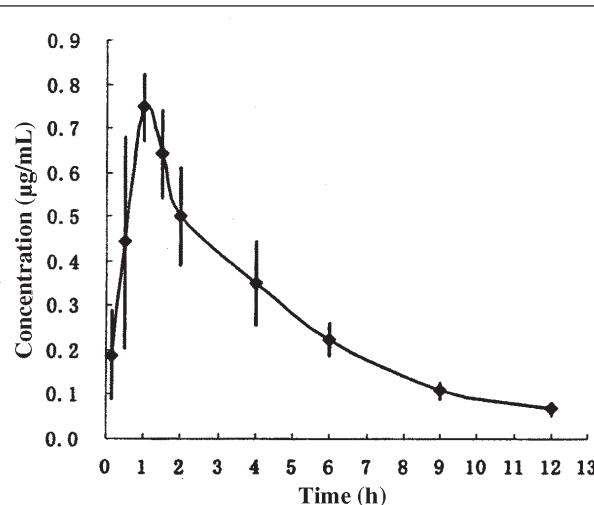


Figure 3. Plot of the mean concentration of osthole in the plasma of rats versus time after an oral administration of Fructus Cnidii extract.

Table I. Precision and Accuracy of the HPLC–UV Method to Determine Osthole in Rat Plasma

	Added concentration (µg/mL)		
	0.104	0.520	5.20
n	15	15	15
Mean (µg/mL)	0.095	0.532	5.078
Standard deviation	0.005	0.021	0.150
RE (%)	–6.7	2.2	–2.3
Intraday RSD (%)	5.7	7.5	5.9
Interday RSD (%)	4.9	2.6	1.9

Table II. Pharmacokinetic Parameters of Osthole in Rats after Administration of Fructus Cnidii Extract ($n = 6$)

C_{max} (µg/mL)	0.776 ± 0.069
T_{max} (h)	1.0 ± 0.3
$T_{1/2}$ (h)	3.6 ± 0.6
K_e (h^{-1})	0.192 ± 0.0282
AUC_{0-t} (µg·h/mL)	3.20 ± 0.386
$AUC_{0-\infty}$ (µg·h/mL)	3.55 ± 0.385

the 3P97 computer program (The Chinese Society of Mathematical Pharmacology, Beijing, China). The plasma osthole concentration-time curve was fitted to a two-compartment open model. The mean plasma concentration-time profile is illustrated in Figure 3. The plasma osthole level reached a C_{\max} of $0.776 \pm 0.069 \mu\text{g/mL}$ at T_{\max} of $1.0 \pm 0.3 \text{ h}$ with a $T_{1/2}$ of $3.6 \pm 0.6 \text{ h}$. The pharmacokinetic parameters are presented in Table II.

This is the first report on osthole in rat plasma after oral administration of Fructus Cnidii Extract. The coexisting constituents in the extract may improve the absorption of osthole from the gastrointestinal tract.

Conclusion

This paper describes a sensitive, specific, and rapid HPLC method with UV detection for the determination of osthole in rat plasma. It was applied to the pharmacokinetic study of ostholes from Fructus Cnidii extract. It has a potential application in pharmacokinetic studies of TCM, which contain Fructus Cnidii. 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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