HPLC Determination and Pharmacokinetics of Chlorogenic Acid in Rabbit Plasma after an Oral Dose of Flos Lonicerae Extract

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Abstract

A simple and sensitive high-performance liquid chromatography (HPLC) method has been developed for the determination of chlorogenic acid (3-*O*-caffeoyl-D-quinic acid) in plasma and applied to its pharmacokinetic study in rabbits after administration of Flos Lonicerae extract. Plasma samples are extracted with methanol. HPLC analysis of the extracts is performed on a C_{18} reversed-phase column using acetonitrile–0.2% phosphate buffer (11:89, v/v) as the mobile phase. The UV detector is set at 327 nm. The standard curves are linear in the range 0.0500–1.00 µg/mL (r = 0.9987). The mean extraction recovery of 85.1% is obtained for chlorogenic acid. The interday precision (relative standard deviation) ranges from 5.0% to 7.5%, and the intraday precision is better than 9.0%. The limit of quantitation is 0.0500 µg/mL. The plasma concentration of chlorogenic acid shows a C_{max} of 0.839 ± 0.35 µg/mL at 34.7 ± 1.1 min and a second one of 0.367 ± 0.16 µg/mL at 273.4 ± 39.6min.

Introduction

Flos Lonicerae is a famous herb of traditional Chinese medicines (TCMs) with the effects of bacteriostasis, antivirus, anti-inflammation, antioxidant, and hematischesis (1). It has been officially listed in the Chinese Pharmacopoeia (2). Flos Lonicerae contains a series of water soluble phenolic components. Chlorogenic acid is the main constituent of Flos Loniceae, which has been reported to have activity of suppressing the *N*-nitrosating reaction (3) and inhibiting hepatic glucose 6-phosphatase. Moreover, preventive effects of chlorogenic acid in the lipid peroxidation (4) and elimination of hydroxyl free radical (5) have been demonstrated.

Pharmacokinetic studies of active constituents in Chinese material medica would have a considerably important impact on illustrating their action mechanism and on promoting the development of TCM. However, it is a big challenge to investigate the pharmacokinetics of active constituents from Chinese material medicas because both the constituents of TCM and their actions in the body are very complicated and their contents in biological samples are usually low. In the case of Flos Lonicerae, the pharmacokinetics of its main active compound, chlorogenic acid, has been studied with high-performance liquid chromatography (HPLC) in rats after injection of the compound (6). A microbore liquid chromatography coupled to microdialysis was reported for the determination of unbound chlorogenic acid in rat blood after injection administration and for the pharmacokinetic study (7). The absorption of chlorogenic acid and caffeic acid in rats after oral administration was investigated using an HPLC method with UV detection (8). However, in TCM, Flos Lonicerae is usually administered orally in the form of a decoction (water extract), alone, or with other herbal medicines. It is therefore of importance to study the pharmacokinetics of active constituents of Flos Loniceae after oral adminiatration of this herbal medicine. In this study, an HPLC method was developed and validated for the determination of chlorogenic acid concentration in rabbit plasma and for its pharmacokinetic study after oral administration of Flos Lonicerae extract.

Experimental

Materials and reagents

Chlorogenic acid was provided by the China National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Flos Lonicerae was purchased from Tian yitang Drug Store (Shenyang, China) and identified as *Lonicera japonica* Thunb. by professor Qishi Sun (Department of Pharmacognosy, Shenyang Pharmaceutical University, Shenyang, China). Acetonitrile was of HPLC grade, and all other reagents were of analytical grade.

Chromatographic system

The HPLC system was constructed from an LC-10ATVP pump (Shimadzu, Japan) model 7125 injector with a 20-µL loop

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(Rheodyne, Rohnert Park, CA), SPD-10A vp (Shimadzu, Kyoto, Japan) UV detector set at 327 nm, and Ckchrom Star workstation (Scientific Development, Tianjin, China). The analyte was determined at room temperature on an analytical column (MTPERSIL BDS C_{18}, 250- × 4.6-mm i.d., 5 µm). The mobile phase consisted of a mixture of acetonitrile–0.2% H_3PO_4 (11:89, v/v) adjusted to pH 3.0 with sodium hydroxide (0.2 g/mL). The mobile phase was prepared daily, filtered under vacuum through a 0.45-µm membrane filter, and degassed before use. The flow rate was set at 0.8 mL/min.

Standard solutions and quality control samples

A stock solution of chlorogenic acid (25.0 µg/mL) was prepared in methanol. Working solutions (0.0250–0.500 µg/mL) were prepared by diluting the stock solutions. Calibration standards of concentrations 0.0500–1.00 µg/mL were independently prepared by spiking 100 µL blank plasma with 200 µL of appropriate working solution. The extraction recovery was determined in plasma (n = 9) spiked with chlorogenic acid at concentrations of 0.0500, 0.400, and 1.00 µg/mL. Quality control (QC) samples for the determination of accuracy and precision of the method were prepared also at low (0.0500 µg/mL), medium (0.400 µg/mL), and high (1.00 µg/mL) concentrations. All QCs were stored at –20°C until analysis.

Preparation of Flos Lonicerae extract

One hundred grams of Flos Lonicerae was refluxed three times with 1000 mL of boiling distilled water for 1 h, and the extract was concentrated to 100 mL under reduced pressure, yielding an extract at a concentration of 1 g/mL (expressed in the weight of raw material of Flos Lonicerae in water).

Blood sample preparation

Four rabbits (body weight 2.0–2.5 kg) were not fed for 12 h prior to the administration of the drug extract. A single oral dose of 10 g (raw material of Flos Lonicerae)/1 kg body weight was administered to the rabbits. Water was freely accessed during the experiment. A blood sample (2 mL) was collected from the marginal vein of the ear at 20, 30, 50, 70, 90, 120, 150, 180, 210, 240, 360, and 540 min following administration of the drug into heparinized tubes and gently mixed. All blood samples were immediately centrifuged for 10 min at 3000 rpm, and the plasma was transferred into clean tubes and stored at –20°C prior to HPLC analysis. To 100 µL of plasma, 200 µL of methanol was added. The mixture was vortexed for 30 s, centrifuged at 3000 rpm for 10 min, and then 20 µL of the supernatant was injected onto the HPLC system.

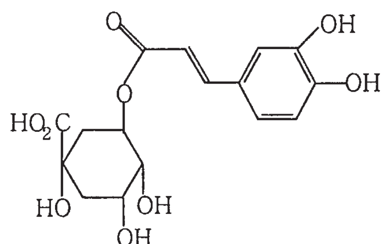

Figure 1. Chemical structure of chlorogenic acid.

Results and Discussion

Chlorogenic acid exhibits acidity because it contains one carboxyl group and two phenolic hydroxyls in its molecule (Figure 1). Therefore, an acidic mobile phase was employed to enhance its retention in reversed-phase chromatography and to separate the analyte from the interference of polar compounds of the plasma. The optimal mobile phase consisted of a mixture of acetonitrile–0.2% H_3PO_4 (11:89, v/v) adjusted to pH 3.0. Under the described conditions, the retention time of chlorogenic acid was 10 min (Figure 2). Typical chromatograms of the blank and spiked plasma are given in Figures 2A and 2B. There were no coeluting peaks in the vicinity of the chlorogenic acid peak on

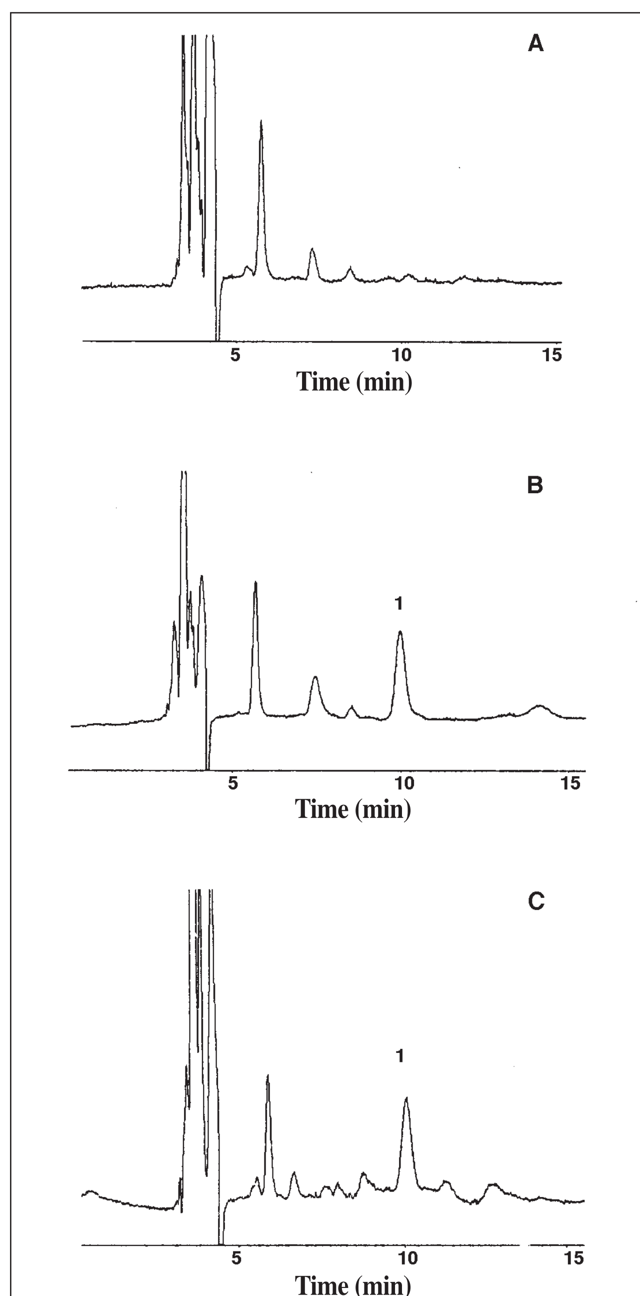

Figure 2. Typical chromatograms of chlorogenic acid (1): (A) blank plasma, (B) blank plasma spiked with chlorogenic acid (0.5 µg/mL), and (C) plasma sample 180 min after oral administration of Flos Lonicerae extract.

the chromatogram of blank plasma. A chromatogram of the plasma sample from a rabbit at 180 min after an oral administration of Flos Lonicerae extract (10 g/kg body weight) is shown in Figure 2C.

In the literature assay (6), acetonitrile was used as the protein precipitant, which produced a distorted chromatographic peak for chlorogenic acid in our assay. Methanol was employed in our procedure and provided a clean supernatant with high extraction recovery for chlorogenic acid without any significant interference. The mean extraction recovery of chlorogenic acid at three concentrations was 85.1%.

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:

$$y = (9486 \pm 4.2)x + (77.81 \pm 11.00) \qquad \text{Eq. 1}$$

where y is the peak area of chlorogenic acid and x is its plasma concentration. The calibration curve was linear over the concentration range 0.0500–1.00 μg/mL in rabbit plasma with a mean correlation coefficient of $r = 0.998\,7$.

The limit of quantitation (LOQ) was defined as the lowest drug concentration in the plasma that can be determined with an accuracy (relative error, RE) and precision (relative standard deviation, RSD) not exceeding 20% (9). The LOQ of this HPLC method was 0.0500 μg/mL chlorogenic acid. The accuracy and precision of the method were evaluated with QC samples at concentrations of 0.0500, 0.400, and 1.00 μg/mL. The results are shown in Table I. The intraday RSD determined by assaying four replicates at each concentration on the same day ranged from 3.5% to 8.5%, and the interday RSD determined on three successive days was from 5.0% to 7.5%. The intraday RE (n = 4) ranged from –5.4% to 4.0%, and the interday RE ranged from –3.6% to 11.2%. The intra- and interday accuracy and precision fell well within predefined limits of acceptability (9).

The validated method was used to monitor the plasma levels of chlorogenic acid in rabbit plasma after single administration of Flos Lonicerae extract at a dose of 10 g (containing 220 mg of chlorogenic acid)/1 kg of body weight. After an oral dose of Flos Lonicerae extract, chlorogenic acid was detected in rabbit plasma for up to 9 h, which demonstrated a rapid absorption of chlorogenic acid from the gastrointestinal tract. This result is contrary to that in previous literature (8) in which chlorogenic acid was reported not to have been absorbed into the blood from the alimentary tract when it was orally administered to the rats. This difference might be attributable to different dosage forms in the two assays. In the previous study, chlrogenic acid was provided in a form of pure compound to the animals, but in the present study it was administered in a form of herbal extract with complicated constituents. Those coexisting constituents may improve the absorption of chlorogenic acid from the gastrointestinal tract. A similar result has been described for a compound TCM containing Flos Lonicerae (10). In that report, chlorogenic acid was determined in the plasma, and its pharmacokinetics were studied after oral administration of the compound medicine.

The profile of mean plasma concentration of chlorogenic acid versus time in four rabbits after oral administration of Flos Lonicerae extract is presented in Figure 3. The plasma chlorogenic acid level reached a C_{max} of 0.839 ± 0.35 μg/mL at 34.7 ± 1.1 min. It is interesting to observe a second peak in the plasma concentration of chlorogenic acid of 0.367 μg/mL at 273.4 ± 39.6 min. This phenomenon may be relevant to hepatoenteral circulation. Further investigations are needed to clarify this pharmacokinetic behavior of chlorogenic acid from the administration of Flos Lonicerae extract. Pharmacokinetic data were analyzed based on the statistical moment theory. The pharmacokinetic parameters are presented in Table II.

Table I. The Accuracy and Precision of the HPLC Method Determining Chlorogenic Acid in Rabbit Plasma

Concentration (μg/mL)			
Added	Found	RSD (%)	RE (%)
Intra-assay (*n* = 4)			
0.0500	0.0473 ± 0.0046	8.5	–5.4
0.400	0.416 ± 0.030	7.2	4.0
1.00	1.01 ± 0.036	3.5	1.0
Interassay (*n* = 12)			
0.0500	0.0493 ± 0.013	6.5	–1.5
0.400	0.445 ± 0.033	7.5	11.2
1.00	0.964 ± 0.048	5.0	–3.6

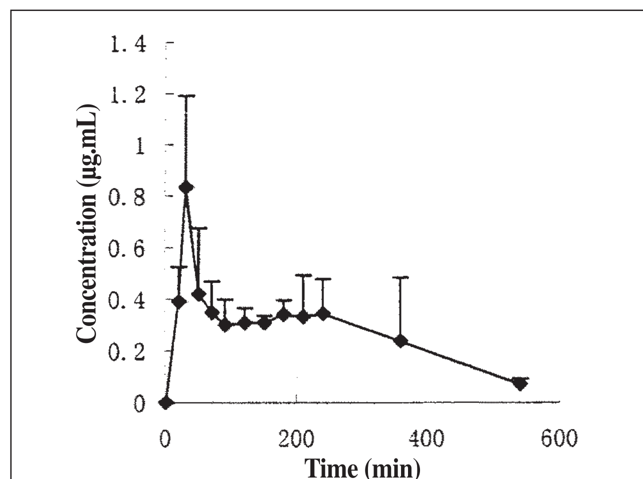

Figure 3. Plot of the mean concentration of chlorogenic acid in the plasma of rabbits versus time after oral administration of Flos Lonicerae extract.

Table II. Pharmacokinetic Parameters Obtained by Analyzing Plasma Samples after Administration of 10 mg/kg Flos Lonicerae Extract to Rabbits (*n* = 4)

Parameter	Value
AUC (μg min/mL)	140 ± 65.9
AUMC (μg min²/mL)	$2.80 \times 10^4 \pm 1.73 \times 10^4$
MRT (min)	190 ± 38.3
VRT (min·min)	$1.28 \times 10^4 \pm 2.66 \times 10^3$
Kel(1/min)	0.0130 ± 0.0023
T_{max}(min)	34.7 ± 1.09
C_{max}(μg /mL)	0.839 ± 0.350

* Abbreviations: AUC, area under the curve; AUMC, area under the first moment curve; MRT, mean residence time; VRT, variance of the mean residence time; and Kel, the elimination rate constant.

Conclusion

An HPLC method with UV detection was developed for the determination of chlorogenic acid in plasma from rabbits having taken Flos Lonicerae extract. The method is sensitive, precise, accurate, and linear in the concentration range of 0.0500–1.00 µg/mL. It was applied to the pharmacokinetic study of chlorogenic acid from Flos Lonicerae. Two maximum concentrations of chlorogenic acid were observed in rabbit plasma after a single dose of Flos Lonicerae extract.

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