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Determination of Icariin in Bushenqiangshen Capsule in High-Performance Liquid Chromatography with Fluorescence Detection by Precolumn Chelation with Aluminum

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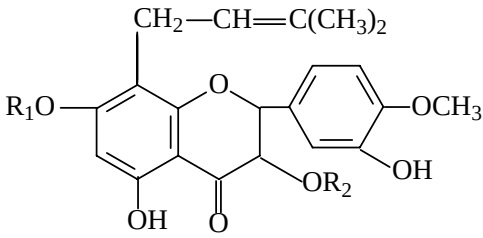
Summary. A simple and sensitive method of high-performance liquid chromatography with fluorescence detection (HPLC–FLD) was developed for the determination of icariin in capsules by precolumn chelation with aluminum. In order to obtain a stable fluorescence signal, the reaction conditions of the fluorescent chelation complex between icariin and aluminum were investigated in detail. Chromatography was carried out on an Agilent Zorbax Extend C18 column (150 mm × 4.6 mm, 5.0 μm) using methanol as mobile phase at a flow rate of 1.0 mL min^{-1}. The excitation and emission wavelengths were set at 430 and 480 nm, respectively. At optimum conditions, the calibration curve was linear in the concentration range from 0.010 to 100.0 μg mL^{-1} with the limit of detection of 3.5 ng mL^{-1} (S/N = 3). A comprehensive method was validated for precision and accuracy. The method described here has been successfully applied for the determination of the icariin content in a capsule with satisfactory results.

Key Words: high-performance liquid chromatography, fluorescence detection, icariin, $AlCl_3$, capsule

Introduction

Icariin *(Scheme 1)* is a flavonoid isolated from *Epimedii herba*, which belongs to a traditional Chinese herbal medicine. Previous studies indicated that icariin possesses many biological actions, such as liver and cardiovascular function improvement, hormone regulation, immunological function modulation, and antitumor activity [1, 2]. The recent methods dealing with the analysis of icariin in biological fluids mainly include high-performance liquid chromatography (HPLC) with ultraviolet (UV) or diode array detection (DAD) [2, 3], capillary electrophoresis [4], liquid chromatography–tandem mass spectrometry (LC–MS/MS) [5–8], and ultrahigh-performance liquid chromatography–mass spectrometry (UPLC–MS) [9].

The bushenqiangshen capsule belongs to the class of health protectors, and is made of five types of Chinese herbs, namely, *Herba Epimedii*, *Fructus*



R_1 = ¡[a] Glc R_2 = ¡[a] Rha

Scheme 1. Chemical structure of icariin

Rosae Laevigatae, *Semen Cuscutae*, *Fructus Ligustri Lucidi*, and *Rhizoma Cibotii*. This product is believed to tone up the kidney and enhance health. Its indications include soreness and weakness of the back and legs, dizziness and tinnitus, dim eyesight, and palmus. Icariin is one of the key bioactive compounds in the bushenqiangshen capsule. The purpose of the present study, therefore, was the development and validation of a simple, sensitive, and accurate HPLC–FLD method for the determination of icariin content in the bushenqiangshen capsule. The proposed method can simplify the separation conditions to effectively eliminate the interference in the matrix and be used for the quality control of bushenqiangshen capsules in future.

Experimental

Reagents, Chemicals, and Solutions

Icariin (>98%) was purchased from Nanjing ZeLang Medical Technology Co., China. Methanol (HPLC-grade) was purchased from Fisher (Nepean, ON, Canada). Analytical grade aluminum chloride was obtained from Sinopharm Chemical Reagent Co., China. Ultrapure water, of 18.3 MΩ resistance and used for HPLC mobile phase, was obtained from a Nanopure (New Haven, CT, USA) filtration system. The other chemicals were of analytical-reagent grade.

Bushenqiangshen capsules (Batch No. 090501) were manufactured by Jiangsu Pharmaceutical Industry Co., Ltd, China.

An amount of icariin was weighed accurately, dissolved in methanol, and then diluted to 1.0 mg mL^{-1}. Working solutions were prepared by diluting the stock solution with the mobile phase. All standard solutions were stored at −20°C.

Before HPLC–FLD analysis, the working solution of icariin was reacted with 9% $AlCl_3$ in 5.0 mL methanol for 40 min at the temperature of 70°C in a water bath, and was filtered through a 0.22-μm Econofilter prior to injection into the HPLC system.

Sample Preparation

One gram powdered sample, which was mixed with 20 bushenqiangshen capsules, was treated by ultrasonic extraction using 50 mL methanol for 30 min. The lost weight of the extracted solution was compensated prior to filtration. After filtration through a 0.22-μm Econofilter, 1.0 mL of the transferred sample solution was reacted with 9% $AlCl_3$ in 5.0 mL methanol for 40 min at 70°C. After filtration through a 0.22-μm Econofilter again, the chromatographic analysis was carried out.

HPLC

Chromatography was carried out using an Agilent Series 1200 (Agilent Technologies, USA) system, equipped with a vacuum degasser, a quaternary pump, an autosampler, a column compartment, and a fluorescence detector (G1312A), and controlled by the Agilent Software. The chromatographic column was an Agilent Zorbax Extend C18 column (5 μm, 150 mm × 4.6 mm i.d). Absorption spectra were recorded using a UV-2501PC UV–vis recording spectrophotometer (Shimadzu Corporation, Japan). A sonication bath (KQ3200B, Shanghai Branson Ultrasound Co. Ltd., China) working at 40 kHz frequency and 150-W input power was employed as the degassing device.

The HPLC mobile phase was methanol at a flow rate of 1.0 mL min^{-1}. Before delivering the mobile phase into the system, it was filtered through a 0.45-μm filter (Sartorius, Germany). The sample injection volume was 20 μL and the column temperature was 25°C. The fluorescence detector was set at an excitation wavelength of 430 nm and an emission wavelength of 480 nm.

Method Validation

A linear calibration curve was generated by plotting the peak area versus icariin concentrations. The slope, intercept, and correlation coefficient values were estimated by using a least squares regression analysis. The limit of detection (LOD) was defined as 3 times the signal-to-noise ratio (S/N). The limit of quantification (LOQ) was defined as the lowest concentration in the linear calibration curve.

Validation of HPLC method for icariin was then carried out. Quality control samples containing low, medium, and high concentrations (0.50, 5.0, and 25.0 μg mL^{-1}) were used to evaluate the precision and accuracy of the proposed method. Intraday variability and precision were determined by analyzing the quality control sample in duplicate on the same day. Interday variability and precision were evaluated by injecting duplicate processed samples at each control concentration for 5 days. The assay precision was reflected by the relative standard deviation (RSD).

The accuracy of the method was assessed by the use of the standard addition technique. Known amounts of pure icariin were added to the capsule sample in which the icariin concentration was determined. The amounts of icariin recovered were estimated by use of the regression equation of the calibration plots. The relative recovery in the capsule sample was evaluated at three concentrations, 0.50, 5.0, and 25.0 μg mL^{-1}. The accuracy study was performed three times.

Results and Discussion

Precolumn Chelation Between Icariin and Aluminum

Fig. 1 shows the chromatogram of icariin before and after reaction with $AlCl_3$. It can be seen that icariin cannot be detected in the eluent by the fluorescence detector because it has no native fluorescence (*Fig. 1* inset). After the reaction between icariin and $AlCl_3$, the signal in the fluorescence detector increased significantly *(Fig. 1)*. It resulted in the formation of the

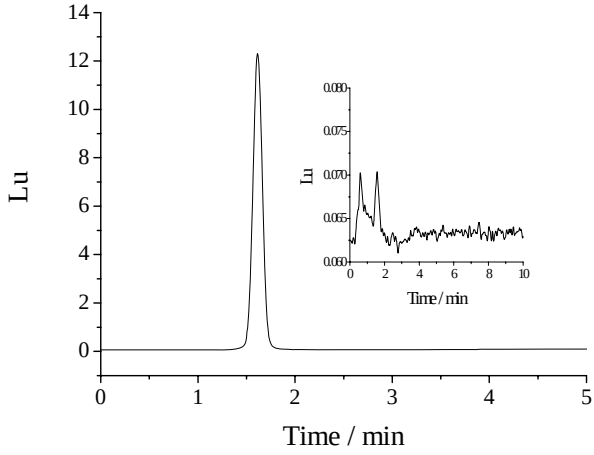
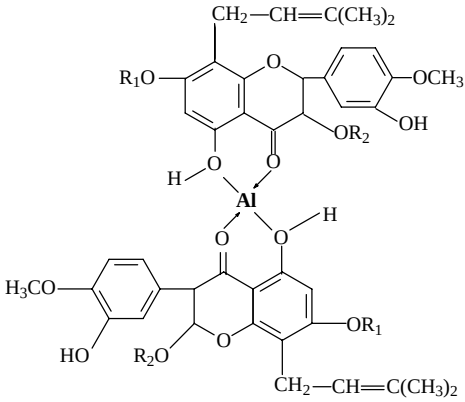


Fig. 1. HPLC–FLD chromatogram of the precolumn chelation complex between 10.0 μg mL^{-1} icariin and 9% $AlCl_3$ in methanol for 40 min at 70°C.
Inset: HPLC–FLD chromatogram of 10.0 μg mL^{-1} icariin

Al^{3+}–icariin chelation complex, which increased the number of more rigid and planar molecules [10]. In order to realize the chelation complex, some experiments were carried out. The results obtained by the molar ratio and Job methods showed that the molar ratio of Al^{3+}–icariin was 1:2. Based on previous reports [10, 11], the structure of chelation complex can be expressed as shown below:



It confers molecular rigidity and increases fluorescence efficiency. Therefore, HPLC–FLD by precolumn derivatization was an effective method for analyzing icariin.

Precolumn Reaction Conditions

In order to achieve a substantial enhancement of the detector response, some factors of the precolumn reaction between icariin and aluminum chloride were investigated as follows:

The effect of water content in the reaction solvent on the peak area of the chelation complex between icariin and $AlCl_3$ was investigated, and the results are shown in *Fig. 2*. It can be seen that the peak area decreases sharply with increasing water content in the reaction solvent. It may be due to the enhanced dissociation of the chelation complex between icariin and $AlCl_3$ as a result of the increasing dielectric constant [10]. Therefore, methanol as reaction solvent was selected in the precolumn reaction between icariin and aluminum chloride.

The effect of $AlCl_3$ concentration on the peak area of chelation complex is shown in *Fig. 3*. It can be seen that the peak area reaches a maximum at the 8% $AlCl_3$, and then hardly changes with the increase of $AlCl_3$ concentration. Therefore, 9% $AlCl_3$–methanol solution was used as the reagent to form the chelation complex with icariin in order to enhance the fluorescence intensity.

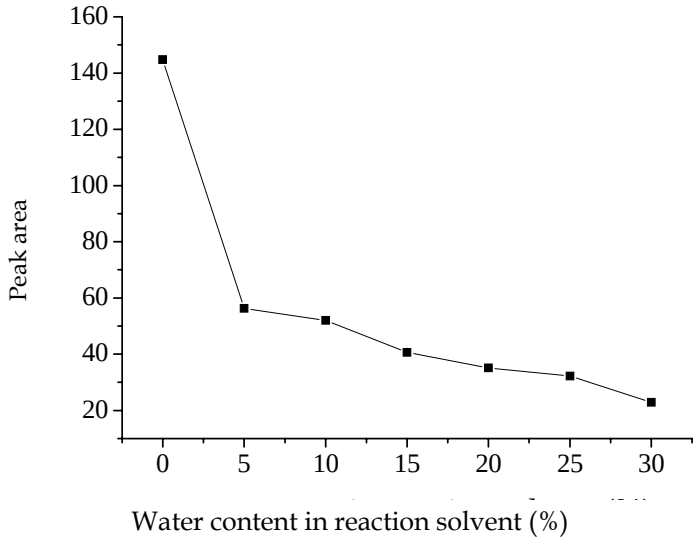


Fig. 2. The effect of water content in the reaction solvent on the peak area of the chelation complex between icariin (10.0 μg mL^{-1}) and 9% $AlCl_3$ after 40 min of reaction time at 70°C

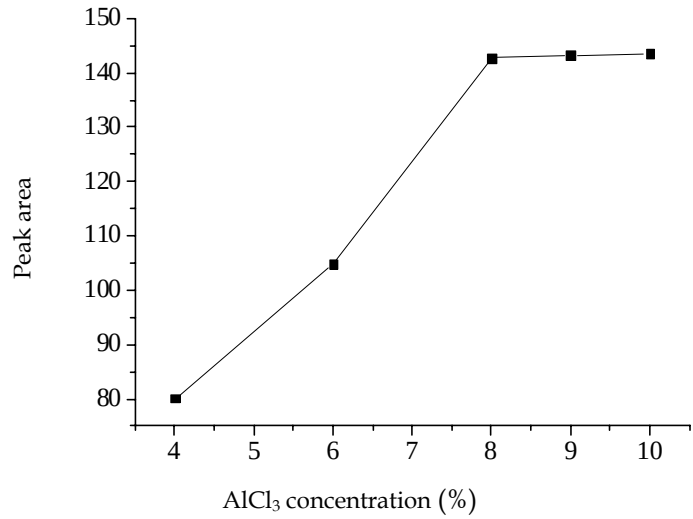


Fig. 3. The effect of different $AlCl_3$ concentrations on the peak area of the chelation complex between icariin (10.0 μg mL^{-1}) and $AlCl_3$ after 40 min of reaction time at 70°C

The effect of the reaction temperature on the peak area of the chelation complex was also investigated. *Fig. 4* depicts the effect of the reaction temperature on the peak area after 40 min of reaction time between icariin and 9% $AlCl_3$. It was found that the largest area of the chromatographic peak was obtained at the temperature of 70°C. Thus, 70°C was selected as the optimal reaction temperature.

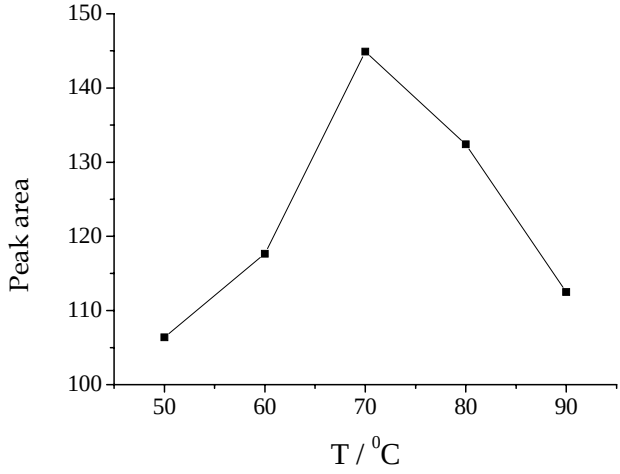


Fig. 4. The effect of different reaction temperatures on the peak area of the chelation complex between icariin (10.0 μg mL^{-1}) and 9% $AlCl_3$ at the reaction time of 40 min

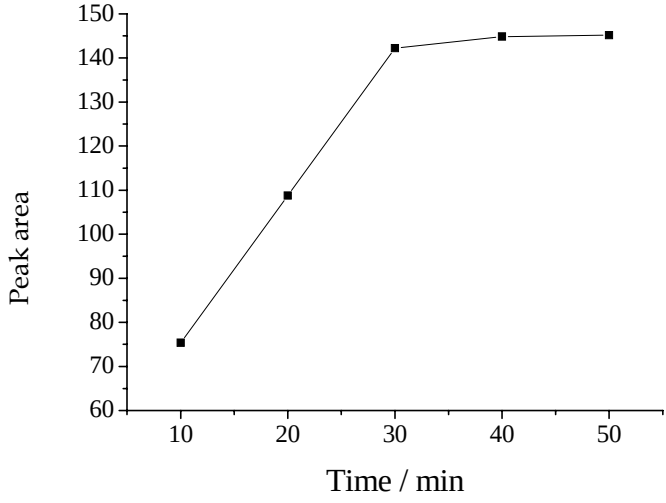


Fig. 5. The effect of different reaction times on the peak area of the chelation complex between icariin (10.0 μg mL^{-1}) and 9% $AlCl_3$ at the reaction temperature of 70°C

The study of the reaction time was also carried out at the temperature of 70°C. From *Fig. 5*, when the reaction time is less than 30 min, the peak area of the chelation complex increases gradually with increasing reaction time. However, with further increase in the reaction time, the peak area of the chelation complex hardly changes. So a reaction time of 40 min was chosen as the optimal reaction time.

Effect of Water Content in Mobile Phase

Methanol is commonly used as the organic modifier in reversed-phase HPLC. The effect of water content in the mobile phase on the HPLC detector response to the chelation complex, which was obtained under the optimized reaction conditions between icariin and $AlCl_3$, was also studied. The experimental results show that increasing the water content in mobile phase causes a dramatic drop in the detector response for methanol *(Fig. 6)*. In order to obtain a high detector response, the mobile phase was selected as methanol in this experiment.

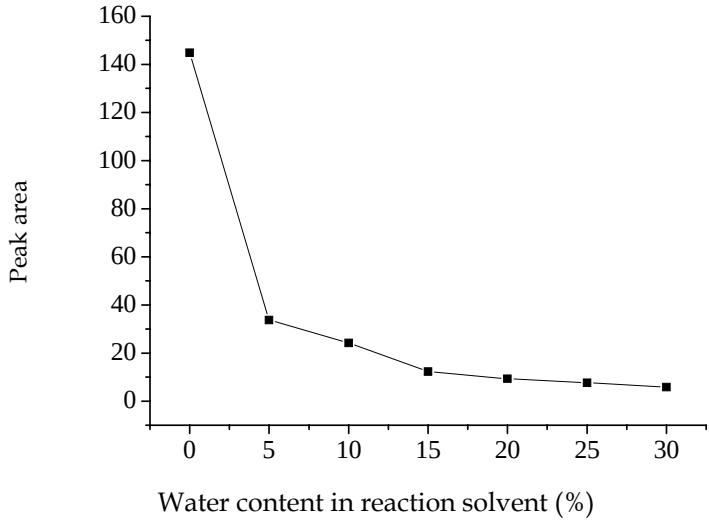


Fig. 6. HPLC detector response to the chelation complex as a function of the water content in the mobile phase with methanol as organic modifier

Method Validation

Under the optimized conditions, a calibration curve was established by linear regression analysis of icariin against the peak area of the chelation complex. The experimental results show the peak area is proportional to the concentration of icariin from 0.010 to 100.0 μg mL^{-1}, with a regression equation $y = 17.74 + 12.30x$ ($r = 0.9991$). Here, x means the concentration of icariin (in μg mL^{-1}) and y means the peak area of the complex. The limits of quantification (LOQ) and detection (LOD) were 0.010 and 3.5 μg mL^{-1} (S/N = 3), respectively.

The precision of the HPLC analysis was carried out in two ways: retention times and peak areas. In the present method, the RSD for the precision of the HPLC analysis was obtained through five injections of the chelation complex with different concentrations, and the results are listed in *Table I*. The RSD for the retention time of all peaks was below 1.9% and that for the peak area was less than 3.9%, indicating the acceptable precision of the present method.

Table I. The intraday and interday precisions ($n = 5$)

Concentration (μg mL^{-1})	RSD (%)			
	Retention time		Integrated area	
	Intraday	Interday	Intraday	Interday
0.50	0.16	0.86	1.7	2.4
5.0	0.22	1.5	2.3	3.7
25.0	0.14	1.9	2.8	3.9

Sample Analysis

The developed HPLC–FLD method was applied to analyze icariin in bushenqiangshen capsules. The chromatogram of a sample is shown in *Fig. 7*. Based on the peak area of *Fig. 7*, the content of icariin in bushenqiangshen capsules is about 2.55 mg g^{-1} with an RSD of 2.5% ($n = 5$) according to the previously established linear regression equation.

In order to verify the accuracy of determining the icariin content in the capsule by the proposed HPLC–FLD method, another method of HPLC–UV was also carried out. Under optimized conditions, the linear regression equation of $y = 2.05 \times 10^4 + 9.95 \times 10^4 x$ (μg mL^{-1}) with a correlation coefficient of 0.9999 was obtained in the range of 0.10–50.0 μg mL^{-1} icariin. The

content of icariin in the bushenqiangshen capsule was about 2.43 mg g^{-1} with an RSD of 5.7% (n = 5) by HPLC–UV method. It can be seen that the icariin content in capsule obtained by HPLC–FLD is in good agreement with that obtained by HPLC–UV.

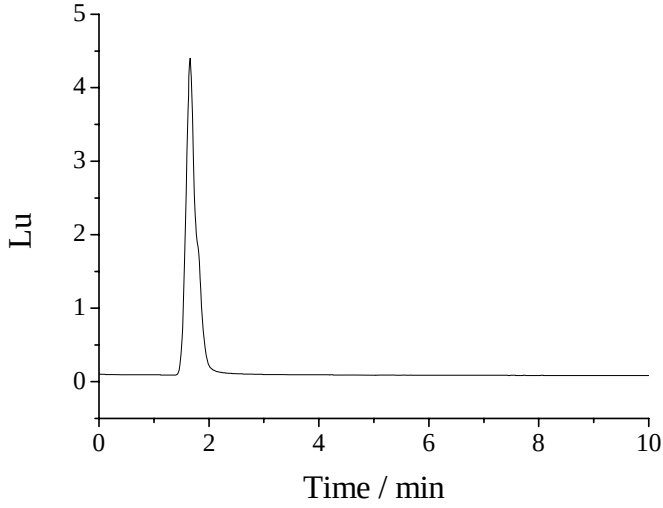


Fig. 7. HPLC–FLD chromatograms of icariin in the bushenqiangshen capsule

In order to further evaluate the validity of the proposed method for the assay of icariin in real samples, a recovery experiment was carried out. The real capsule was spiked with a known amount of icariin, and the recovery was determined by HPLC–FLD. The satisfactory recovery of 95.68–102.96% in the bushenqiangshen capsule indicates that the proposed HPLC–FLD method is reliable for the quantification of the icariin content in the capsule *(Table II)*.

Table II. Recovery data of icariin from bushenqiangshen capsule

Amount added (μg mL^{-1})	Recovery (%)	RSD (%)
0.50	102.31	3.4
5.0	95.68	2.8
25.0	102.96	3.9

Conclusion

In the present study, a novel, sensitive fluorimetric HPLC method for the determination of icariin in bushenqiangshen capsule was developed. The method was validated over a concentration range 0.010–100.0 μg mL^{-1} (r = 0.9991), and offered good accuracy and precision. The results of this study have successfully demonstrated the applicability of this analytical method. The proposed method can simplify the separation conditions to effectively eliminate the interference in matrix and be used for the quality control of traditional Chinese medicinal material such as bushenqiangshen capsules in the future.

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