A sensitive high performance liquid chromatography-ultraviolet (HPLC-UV) method for the quantitation of α-obscurine in rat plasma and its application to pharmacokinetic studies

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INTRODUCTION

Lycopodium japonicum Thunb. has definite treating rheumatism bi-complex pain therapeutic effects (Zheng et al., 2006), and has been widely used in clinical. Recent researches indicates that different doses of Lycopodium ethanol extracts can exert an inhibitory effect on secondary reaction of Freund’s adjuvant arthritis of rats and a obviously decreased level of serum cytokines (interlukin [IL], tumor necrosis factor-alpha [TNF-α] and IL-6) (Yin et al., 2008; Wang et al., 2004; Wu and Gu, 2006). It was believed that ethanol extracts of Lycopodium were the effective pharmacological composition. α-Obscurine (Figure 1) was the main component in ethanol extracts, it had the obvious analgesic action in our studies. It is one of Lycopodium alkaloids. The analysis about α-obscurine content determination methods determination methods has not been reported.

A sensitive and simple analysis method for determining α-obscurine in rat plasma was established and its pharmacokinetic in rats was studied, because the pharmacokinetic studies on the active ingredients in traditional Chinese medicinal preparations (TCMPs) will contribute to the elucidation of their mechanisms of action.

EXPERIMENTAL

Materials, reagents, and solutions

L. japonicum Thunb. was obtained from Huaping National Nature Reserve (Guangxi, China). α-Obscurine was prepared from the
herb of *L. japonicum* Thunb. (purity 98.5%, determined by high performance liquid chromatography [HPLC]). Schizandrin, used as IS was purchased from the National Institute for Control of Biological and Pharmaceutical Products (Beijing, China).

HPLC-grade methanol was supplied by Yuwang Reagent Co. Ltd. (Shandong, China). Other reagents were of analytical grade. Water was obtained by double distillation. The content of α-obscurine in the ethanol extract of *Lycopus japonicus* was 78 mg/g.

By dissolving different accurately weighed amounts of standards in methanol solution, the stock solutions were gained as follows: α-obscurine 141.6 μg/ml and the IS 5 μg/ml. Different volumes of each stock solution were transferred into volumetric flasks and then diluted to scale to make working standard solutions with methanol. All these solutions earlier were stored at 4°C.

**Sample preparation**

All plasma samples, quality control samples (QC) samples, and spiked plasma calibration samples were treated in the same manner. To 200 μl plasma, 50 μl IS solution and 1000 μl ether was added. Each tube was mixed thoroughly by vortex mixing for 3 min and then centrifuged at 4000 rpm (800×g) for 10 min. The organic phase was transferred into a test tube and evaporated to dryness at 45°C under a gentle stream of nitrogen. Subsequently, the residual was redissolved in 50 μl of methanol and vortexed for 1 min, then centrifuged at 10000 rpm (5500×g) for 5 min. Finally, 20 μl of the supernatant was directly injected into the HPLC system.

**Instrumentation and chromatographic conditions**

The chromatographic system consisted of an LC-10Atvp liquid chromatograph, and an SPD-10Avp ultraviolet (UV) detector set at 250 nm, all from Shimadzu (Japan). Separation was performed using a prepacked stainless-steel column (200 × 4.6 mm i.d) with Century Sil C18 EPS 5 μm silica. The mobile phase consisted of 390 ml water, 610 ml methanol, 0.5 ml triethylamine and the flow-rate was 1.0 ml/min.

**Validation of the method**

The specificity, linearity, lower limit of quantitation (LLOQ), precision, recoveries and stability of the method were all validated. The specificity of the method was shown through comparing the chromatograms of blank plasma, blank plasma spiked with α-obscurine and IS, and rat plasma sample after oral administration of the extracts of *L. japonicum* Thunb.

 Calibration samples were prepared as follows: to 200 μl of blank rat plasma, 50 μl of the internal standard solution and 20 μl of the standard working solutions were added to yield final concentration ranges at: 0.354 to 14.16 μg/ml α-obscurine in plasma. QC samples (0.4425, 2.832, 11.33 μg/ml) were independently prepared in the same manner for evaluation of accuracy, precision, and extraction recovery of the method.

The recoveries were calculated with the QC samples by comparing the peak areas of α-obscurine in the spiked plasma samples with plasma-free samples containing the same amount of α-obscurine.

Stability of α-obscurine were tested in processed samples after storage at temperature for 24 h or frozen at -20°C for 2 weeks.

**Application to pharmacokinetic study in rats**

Male Wistar rats (200±20 g) were purchased from the Experimental Animals Center of China Medical University. They were kept in an environmentally controlled breeding room for 7 days before the start of the experiments, fed with a standard laboratory water and food. After the rats were given 5 g/kg extracts of *L. japonicum* Thunb. intragastrically, venous blood samples (0.5 ml each) were withdrawn to the heparinized tubes by eye puncture at 0, 0.25, 0.5, 0.75, 1, 2, 3, 6, 12 and 24 h centrifuged at 4000 rpm (800×g) for 10 min. The plasma samples were stored at -20°C until analysis.

**Data analysis**

The plasma concentrations of the analyte at different times were expressed as mean±standard deviation (SD) and the mean concentration-time curves were plotted. All data were probed by use of dug and statistics (DAS) software (version 2.0) for windows.

**RESULTS AND DISCUSSION**

**LC method optimization**

The wavelength was set at 250 nm. The optimal mobile phase consisted of a mixture of methanol:water:triethylamine (61:39:0.05,v/v/v), α-obscurine, IS and endogenous interference in plasma sample solution were well separated. There are no interfering peaks caused by endogenous plasma components. Due to the fact that α-obscurine is such a molecule with double bonds, it should have ultraviolet absorption. Full UV spectra (200 to 400 nm) of sample were obtained by spectrophotometer. The UV absorption maximum of α-obscurine was at 250 nm, which also gave a good absorption of the IS. Consequently, the wavelength was set at 250 nm.

Small amount of triethylamine were added into the mobile phase, because α-obscurine is a natural weak alkaloid.

**Validation of the method**

**Specificity**

Typical chromatograms of blank plasma, blank plasma spiked with α-obscurine and IS, and rat plasma sample
Figure 2. Typical chromatograms obtained from determination of α-obscurine in plasma samples: (a) blank sample; (b) blank plasma spiked with α-obscurine and IS; (c) a rat plasma sample 2.5 h after oral administration of Peaks: 1= IS and 2=α-obscurine.

After oral administration of the extracts of *L. japonicum* Thunb. are as shown in Figure 2. IS and α-obscurine were eluted at 8.628 and 15.253 min, respectively.

**Calibration plot and LLOQ**

The calibration curve for the determination of α-obscurine
Table 1. Precision, accuracy and stability of HPLC analysis of α-obscurine in rat plasma samples (n=5).

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>Precision RSD (%)</th>
<th>Accuracy (%)</th>
<th>Stability RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intra-day</td>
<td>Inter-day</td>
<td>Intra-day</td>
</tr>
<tr>
<td>0.4425</td>
<td>1.8</td>
<td>3.7</td>
<td>101.6±4.3</td>
</tr>
<tr>
<td>2.832</td>
<td>2.6</td>
<td>2.4</td>
<td>96.03±2.6</td>
</tr>
<tr>
<td>11.33</td>
<td>2.3</td>
<td>2.1</td>
<td>98.48±3.8</td>
</tr>
</tbody>
</table>

in rat plasma was linear over the range 0.354 to 14.16 μg/ml. Least squares linear regression analysis was used to determine the slope, intercept and correlation coefficient. The limit of quantification (LOQ) was 0.3 μg/ml. The mean values of regression equation of the analytes in rats plasma were:

Y=2.299X+0.123(γ=0.9995).

**Precision and accuracy**

The precision of the method was studied by estimation of intra- and inter-day relative standard deviations of values by determining LQC, MQC and HQC plasma samples. At least five QC samples were processed and injected on a single day (intra-day) and at different days (inter-day). The precision was evaluated by the relative standard deviation (RSD) of intra- and inter-day assay. The RSD of intra- and inter-day were ≤2.6 and ≤3.7%, respectively (Table 1).

Furthermore, as the sample process involved in extraction by ether, the average extraction and method recoveries of assay were 70.81 to 75.27% and 96.03 to 101.6%, respectively.

**Stability**

Plasma samples left at room temperature for 24 h or frozen at −20°C for 2 weeks were checked. The deviation of the mean test responses were within ±5%, no effect on quantitative detection was observed. The results suggested that rat plasma samples containing α-obscurine can be controlled under normal laboratory conditions. The results are shown in Table 1.

**Pharmacokinetic study of α-obscurine in rats**

Due to the lack of an appropriate analytical method, there was no information about α-obscurine before this study. By using this method for determining α-obscurine in rats plasma, satisfactory results were obtained. It was successfully used for the pharmacokinetic study on extract of L. japonicum Thunb. The mean plasma concentration versus time profiles of α-obscurine is as shown in Figure 3.

The peak of α-obscurine in rat plasma occurred rapidly, which was approximately 0.75 h (Tmax) and it was eliminated from plasma with a MRT of 4.5 h. The values of T1/2 and Cmax were 2.68 h and 6.306 μg/ml, respectively.

Figure 3. Pharmacokinetic profiles of α-obscurine after a single oral doses of 5 g/kg in rats.
The pharmacokinetic results are similar to Huperzine A with swift absorption (T1/2 1.4 h) (Yue et al., 2007). This was probably related to their similar structure, which both contain two N atoms and similar structure to pyridine ring.

**Conclusions**

Generally, a simple and sensitive HPLC method had been established and validated to quantify α-obscurine in rat plasma. To our knowledge, the method has not been reported earlier. The pharmacokinetic results are very useful for evaluating the clinical efficacy of *L. japonicum* Thunb.

α-obscurine medicine/curve appears in the form of double apices phenomena, respectively in oral administration methods after 0.25 and 3 h. According to the current research material, double apices phenomena may be by liver/intestinal material circulation, double part absorbed or stomach/bowel circulation, etc. Various causes may also be by several reasons of joint action of weak alkali medicine (morphine, diazepam) often in the gastrointestinal cycle. α-Obscurine is an alkalescent drug in which the petronas appear, and whether this is caused by gastrointestinal circulation remains to be further studied experimentally.

This paper only reports the pharmacokinetic studies after oral administration of ethanol extract of *L. japonicum* Thunb, the studies on the pharmacokinetic comparison of α-obscurine and the ethanol extract are being tested in our laboratory.

**ACKNOWLEDGEMENT**

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**REFERENCES**


