Differentiation of Belamcandae Rhizoma and Iridis Tectori Rhizoma by Thin-Layer Chromatography and High-Performance Liquid Chromatography

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**Abstract**

**Objective:** Belamcandae Rhizoma and Iridis Tectori Rhizoma are easily confused with each other. The main objective of this study is to distinguish them using chemical analysis. **Materials and Methods:** Thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) fingerprint methods were established to compare the chemical profile, while HPLC quantitation was used to determine the contents of three isoflavones in thirty batches of Belamcandae Rhizoma and Iridis Tectori Rhizoma samples. **Results:** The two herbs could be distinguished by TLC using acetic acid-n hexane-ethyl acetate (1:90:80 v/v/v) as the mobile phase, according to the fluorescent band under 366 nm at Rf 0.2. In total, 12 compounds were identified in the 24-min HPLC fingerprint. The similarity coefficient between the two herbs was 0.54 ± 0.01. Mangiferin (1), tectoridin (2), iridin (3), irigenin (5), irisflorentin (6), and iristectorin A (9) were the main peaks in Belamcandae Rhizoma, while tectoridin (2) and tectorigenin (4) were the major peaks in Iridis Tectori Rhizoma. The contents of 2 in Iridis Tectori Rhizoma (2.50 ± 0.20 %) were 8.93 times higher than that of Belamcandae Rhizoma (0.28 ± 0.08 %), while the ones of 5 and 6 were slightly lower in Iridis Tectori Rhizoma. **Conclusions:** The study established fast and effective methods to distinguish Belamcandae Rhizoma from Iridis Tectori Rhizoma.

**Keywords:** Belamcandae Rhizoma, high-performance liquid chromatography fingerprint, high-performance liquid chromatography quantitative analysis, Iridis Tectori Rhizoma, thin-layer chromatography

**INTRODUCTION**

Belamcandae Rhizoma (She Gan) and Iridis Tectori Rhizoma (Chuan She Gan) are derived from the rhizome of **Belamcanda chinensis** (L.) DC. and **Iris tectorum** Maxim., respectively.[1,2] They are both used in traditional Chinese medicine (TCM) to treat inflammation, cough, tonsillitis, and pharyngitis.[3] For example, Belamcandae Rhizoma is the main component herb of Shegan-Mahuang Decoction, a widely used TCM formula to treat flu and expectoration,[4] while Iridis Tectori Rhizoma was used to treat cough with Ephedrae Herba or other herbs.[5] Due to their similar Chinese names and functions, these two herbs are easily confused with each other in drug market or in clinical use.[3,6]

The major bioactive components of Belamcandae Rhizoma and Iridis Tectori Rhizoma are isoflavones, which exhibit anticancer, antioxidative, antimitogenic, anti-inflammatory, antiangiogenic, and antibacterial activities.[7-10] Chemical analysis of isoflavones had been reported for these two herbs.[11-18] For example, in total, 35 flavonoids including 30 isoflavones were identified from Belamcandae Rhizoma.

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by HPLC-DAD-ESI-MS<sup>n</sup>. High-performance liquid chromatography-ultraviolet (HPLC/UV) methods were also established to determine 12 and 7 isoflavones in Belamcandae Rhizoma and Iridis Tectori Rhizoma, respectively. The current studies also concentrated on the chemical profiles of different tissues<sup>3</sup> or contents of certain compounds in these two herbs.<sup>30</sup> However, few reports are available to compare the chemical profile of Belamcandae Rhizoma and Iridis Tectori Rhizoma. In addition, the reported methods usually need a long analysis time (50–160 min) or phosphate buffer which were not adaptable for mass spectrometry.<sup>11-18</sup> Thus, fast and effective analytical methods are required to distinguish Belamcandae Rhizoma and Iridis Tectori Rhizoma.

In this study, we established thin-layer chromatography (TLC), HPLC fingerprint, and HPLC quantitative methods to differentiate the two herbs and to evaluate the quality of Belamcandae Rhizoma. These methods were fully validated and then applied to 27 batches of Belamcandae Rhizoma and 3 batches of Iridis Tectori Rhizoma samples.

**Materials and Methods**

**Plant materials**

In total, 27 batches of Belamcandae Rhizoma and 3 batches of Iridis Tectori Rhizoma (SG-1 to SG-30) were purchased from crude drug markets or drug stores in China Table S1. All samples were identified by Dr. Xue Qiao. The voucher specimens were deposited at the School of Pharmaceutical Sciences, Peking University Health Science Center (Beijing, China).

**Chemicals and reagents**

The reference standards mangiferin (1), tectoridin (2), tectorigenin (4), irigenin (5), and irisflorentin (6) were accurately weighed and dissolved in methanol, respectively. The final concentrations were 1020.00 µg/mL, 1040.00 µg/mL, 985.00 µg/mL, 1010.00 µg/mL, 945.00 µg/mL, and 980.00 µg/mL for 1–6, respectively.

The mixed reference stock solution was diluted by 10 folds with methanol and then filtered through a 0.22 µm nylon filter before analysis.

The stock solutions of 6 (160 µL of each) and 2 and 5 (320 µL of each) were mixed and then diluted with methanol to 2 mL to obtain the mixed reference stock solution. The final concentrations of 1–6 were 81.60 µg/mL, 166.40 µg/mL, 157.60 µg/mL, 80.80 µg/mL, 75.60 µg/mL, and 78.40 µg/mL, respectively. The mixed reference stock solution was serially diluted with methanol to obtain the calibration solutions (dilution factor = 2, 4, 8, 16, 32, 64, and 128). The limit of detection (LOD) was defined as the signal-to-noise (S/N) ratio of 3, whereas the limit of quantification (LOQ) was required to the S/N ratio of 10.

**Preparation of mixed reference standards for fingerprint**

The stock solutions of 1, 4, 5, and 6 (160 µL of each) and 2 and 3 (320 µL of each) were mixed and then diluted with methanol to 2 mL to obtain the mixed reference stock solution. The final concentrations of 1–6 were 81.60 µg/mL, 166.40 µg/mL, 157.60 µg/mL, 80.80 µg/mL, 75.60 µg/mL, and 78.40 µg/mL, respectively. The mixed reference stock solution was serially diluted with methanol to obtain the calibration solutions (dilution factor = 2, 4, 8, 16, 32, 64, and 128). The limit of detection (LOD) was defined as the signal-to-noise (S/N) ratio of 3, whereas the limit of quantification (LOQ) was required to the S/N ratio of 10.

**Preparation of samples**

An aliquot of 100 mg of Belamcandae Rhizoma or Iridis Tectori Rhizoma powder (through a 60-mesh sieve) was extracted with 25 mL methanol in an ultrasonic water bath (50 kHz, 500 W) at room temperature for 120 min. After cooled to room temperature, the samples were weighed and adjusted to the original mass with methanol. The extract was passed through a filter with a 0.22-µm membrane before analysis.

**Preparation of method validation samples**

Samples used for precision, repeatability, and stability tests were prepared following “Preparation of sample solutions.” Accuracy was evaluated by standard addition tests, where Belamcandae Rhizoma samples (SG-2) were mixed with reference standards of tectoridin, irigenin, and irisflorentin.
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at three concentrations (around 50%, 100%, and 150% of the concentration in sample SG-2, n = 3). Recoveries were calculated by the formula: recovery (%) = (found amount-original amount)/spiked amount × 100%.

High-performance liquid chromatography conditions
A Waters Alliance e2695 HPLC System (Waters, Milford, MA, USA) was used, consisting of a quaternary solvent delivery system, an online degasser, an autosampler, a column temperature controller, and photodiode array coupled with an analytical workstation (Empower™ 3, build 3471, Waters). The samples were separated on an Agilent Zorbax SB-C$_{18}$ column (4.6 mm × 250 mm, 5 μm) equipped with an Agilent Zorbax SB-C$_{18}$ guard column (4.6 mm × 12.5 mm, 5 μm). The mobile phase was composed of acetonitrile (A) and 0.1% formic acid in water (B). The gradient elution was as follows: 0–16 min, 20%–45% A; 16–21 min, 45% A; 21–22 min, 45%–95% A; and 22–24 min, 95% A. The injection volume was 10 μL. The flow rate was 1.0 mL/min, and column temperature maintained at 40°C. The wavelength was set at 280 nm and 266 nm for fingerprint and quantitative analysis, respectively.

Liquid chromatography–mass spectrometry analysis
A Vanquish UHPLC™ system (Thermo Fisher Scientific Inc., USA) was used for LC analysis. Samples were separated on an Acquity UPLC HSS T3 column (100 mm × 2.1 mm I.D., 1.8 μm) equipped with an Acquity UPLC HSS T3 VanGuard precolumn (5 mm × 2.1 mm I.D., 1.8 μm) (Waters, USA). The column temperature was 40°C. The mobile phase consisted of acetonitrile (A) and water containing 0.1% formic acid (v/v; B). The gradient program was as follows: 0–16 min, 5%–55% A; 16–21 min, 55% A; 21–22 min, 55%–100% A; and 22–24 min, 100% A. The flow rate was 0.3 mL/min. Mass spectrometry analysis was performed on a Q-Exactive Focus hybrid quadrupole-orbitrap mass spectrometer (Thermo Scientific, San Jose, USA) equipped with a heated electrospray ionization source. The mass spectrometer was operated in the negative and positive ion mode. The MS parameters were set as follows: spray voltage: 3.5 (+) or 3.0 (−) kV; sheath gas flow rate: 45 arb; auxiliary gas: 10 arb; capillary temperature: 350°C; S-lens RF level: 60 V; scan mode: full MS (resolution 70,000), and MS/MS (resolution 17,500); scan range: m/z 100–1200. The stepped collision energies were 15, 30, and 45 eV. The three most abundant ions were selected as the precursor ions to obtain MS/MS spectra in each cycle.

Statistical analysis
The retardation factor ($R_f$) was the ratio of the distance from the point of application to the center of the band and the distance travelled by the solvent front from the point of application. HPLC fingerprint similarity indices were calculated by CASE software (Similarity Evaluation System for Chromatographic Fingerprint of TCM, Version 2004A), which was recommended by Chinese Pharmacopoeia Commission. Data were presented as the mean ± standard deviation for quantitative analysis. Statistical significance values were determined by the t-test with Microsoft Excel (Microsoft Corporation, Washington, USA). A value of $P < 0.05$ was considered statistically significant.

LC/MS data were processed using Xcalibur™ 4.1 software (Thermo Fisher, USA).

RESULTS AND DISCUSSION

Morphological characteristics
The prepared slices of Belamcanda Rhizoma are usually irregular or strip-shaped pieces, with grayish yellow brown, brown or black brown surface, wrinkled texture, branched, numerous annular striations, stem scars, and root fragments. The fracture is light yellow or bright yellow, and the central parenchyma has a pitted appearance due to the numerous primary vascular bundles, some of these can be seen as rings.[1,19] The prepared slices of Iridis Tectori Rhizoma are usually irregular pieces, with grayish yellow brown, or brown surface, and sometimes there are ring lines, sunken, or dot-like raised fibrous root marks.[2] These above characteristics are consistent with the description of the Chinese Pharmacopoeia and European Pharmacopoeia,[11,12,19] indicating that they could be distinguished by their appearance. Nonetheless, when used as powders or extracts, these two herbs are still difficult to be distinguished. Thus, we used chemical analyses to differentiate them.

Thin-layer chromatography analysis
Optimization the thin-layer chromatography conditions
Different stationary phases (silica gel F$_{254}$ TLC plate and polyamide plate) were tested, and silica gel F$_{254}$ TLC was chosen for its better resolution. Several mobile phases had been reported for the TLC of Belamcanda Rhizoma. Chloroform, butanone, and methanol (3:1:1, v/v/v) were used in Chinese pharmacopoeia[1] where chloroform and butanone are toxic. Acetic acid, cyclohexane, and ethyl acetate (1:20:80, v/v/v) were used in European pharmacopoeia,[19] which provided a good resolution. However, the $R_f$ value of iridin reached 0.86, which was too high for TLC. Benzene-ethyl acetate (8.5, v/v/20) and petroleum ether-ethyl acetate (1:1, v/v)[21] were also reported, where irisflorentin and iridin were not well separated. Therefore, the mobile phase was optimized based on European pharmacopoeia, where cyclohexane was replaced by n-hexane and the ratio of the solvents was adjusted to 1:90:80. The $R_f$ value of irisflorentin and iridin was 0.30 and 0.56, respectively. Finally, different examination methods were tested, and the plates were examined under ultraviolet light at 254 nm and 366 nm or examined under ultraviolet light at 366 nm after sprayed with 10% of H$_2$SO$_4$ in 96% ethanol.

Optimization of the extraction method
The sample extraction procedure was optimized including extract methods (ultrasound, soaking, and heating in water bath), solvents (methanol, 50% methanol v/v, ethanol, and ethyl acetate), and extraction time (5, 10, 20, 30, and 40 min). As a result, 1.0 g sample powder was extracted with 10 mL methanol in an ultrasonic bath at room temperature for 20 min.
Method validation

Specificity

The chromatograms of the reference solutions exhibited two quenching bands representing irisflorentin and iridin with \( R_f = 0.30 \) and 0.56, respectively, while the test solutions exhibited the same quenching bands at the same position and color.

Stability

The stability was evaluated by comparing the chromatograms of the test solution and the reference solution at 0 and 24 h at room temperature, respectively. The bands were observed in the same position and color after 24 h-storage, indicating that the test and reference solutions were stable.

Precision

Intraday precision was investigated by developing three plates using the same sample (SG-2), successively. Interday precision was investigated by analyzing the same sample on 3 consecutive days. The intermediate precision was investigated by comparing chromatograms of the same sample prepared independently by two operators. Two bands in the chromatogram obtained with the test solution correspond in position and color to the bands obtained with the reference solution. Moreover, the relative standard deviation (RSD) of the precision tests was 2.40%–4.72%, which was calculated based on \( R_f \) of irisflorentin and iridin. The result showed that the intraday precision, interday precision, and intermediate precision met the requirement of Chinese Pharmacopoeia, which were not higher than 10%.\(^{[22]}\)

Ruggedness

TLC plates from different manufacturers (Haiyang, Aladdin, and Liang Chen Gui Yuan) or different batches (Haiyang, Lot number 20191010, 20190806, 20190709) were tested. Different batches of n-hexane (Huihai, Lot number 20190527, 20180309, 20181005) were also tested for ruggedness. The results showed that TLC plates from different manufacturers or different batches, as well as solvent from different batches, provided similar chromatograms. The RSD of the \( R_f \) value for irisflorentin and iridin ranged from 2.60% to 5.77%. The above results indicated the robustness of the TLC method.

Thin-layer chromatography analysis of Belamcandae Rhizoma and Iridis Tectori Rhizoma

The optimized TLC method was then used to analyze 12 Batches Belamcandae Rhizoma and three batches Iridis Tectori Rhizoma [Figure 1]. For all samples, two bands in the chromatogram obtained with the test solution correspond in position and color to the bands obtained with the reference solution. For Belamcandae Rhizoma, two fluorescent bands at \( R_f = 0.2 \) and 0.4 were observed under 366 nm; a light yellow and a dark red band at \( R_f = 0.4 \) and 0.7 were observed after treated with 10% of \( \text{H}_2\text{SO}_4 \), respectively. Compared with Belamcandae Rhizoma, there was no fluorescent band observed under 366 nm at \( R_f = 0.2 \), and no bands at \( R_f = 0.4 \) and 0.7 after treated with 10% of \( \text{H}_2\text{SO}_4 \) in Iridis Tectori Rhizoma samples. These bands could distinguish Belamcandae Rhizoma from Iridis Tectori Rhizoma.

High-performance liquid chromatography fingerprint analysis

Optimization of high-performance liquid chromatography chromatographic conditions

Although the HPLC analysis had been reported for Belamcandae Rhizoma,\(^{[11-18]}\) they usually use a long elution program (50–160 min), or phosphate buffer which was not adaptable for mass spectrometry. In this study, the chromatographic conditions were optimized including different columns (Waters Xierra®MS C\(_{18}\), Waters altantis C\(_{18}\), Agilent Zorbax SB-C\(_{18}\)), elution systems (acetonitrile, methanol), column temperatures (35°C, 40°C and 45°C), and UV wavelength (200–450 nm). Finally, the samples were separated on a Zorbax SB-C\(_{18}\) column using acetonitrile and 0.1% formic acid in water within 24 min. The UV detector was monitored at 280 nm because most of the isoflavones and phenolic acids could be observed under this wavelength.

Optimization of the extraction method

The sample extraction procedure was optimized including the extract methods (ultrasound and reflux), solvents (methanol, 50% methanol, and ethanol), solvent volumes (10, 25, and 40 mL), extraction times (once, twice, and three times), and extraction time (60, 90, 120, and 150 min). As a result, 100 mg...
sample powder was extracted with 25 mL methanol in an ultrasonic bath at room temperature for 120 min for one time.

**Method validation**

**Precision, repeatability, and stability**

The intra- and inter-day precision were evaluated by testing a representative sample of Belamcandae Rhizoma (SG-2) for six times in the same day and in 3 consecutive days, respectively. The RSD values of retention time ranged from 0.08% to 0.73%. The repeatability test was carried out by preparing six sample solutions in parallel with the same batch of Belamcandae Rhizoma sample. The RSD ranged from 0.08% to 0.19%. The stability was evaluated by analyzing the sample (stored at room temperature in the autosampler) every 3 h between 0 and 36 h. The RSD was 0.11%–0.23%. The above results indicated robustness of the HPLC method.

High-performance liquid chromatography fingerprint analysis of Belamcandae Rhizoma and Iridis Tectori Rhizoma

The optimized method was applied to thirty batches of samples. Eleven common peaks, representing twelve compounds, were observed in all samples. Compounds 1–6 were characterized by reference standards, and 7–12 were tentatively identified by LC/MS and previous reports [Figures 2 and 3].[11] For the 27 Belamcandae Rhizoma samples, the correlation coefficient of similarity ranged 0.97–1.00. The similarity coefficient of the three Iridis Tectori Rhizoma samples was only 0.54 ± 0.01 compared with Belamcandae Rhizoma samples. These results indicated that Belamcandae Rhizoma were highly similar in chemical composition, while Iridis Tectori Rhizoma have a different chemical profile. Mangiferin (1), tectoridin (2),...
iridin (3), irigenin (5), irisflorentin (6), and iristectorin A (9) were the main peaks in Belamcandae Rhizoma with similar peak heights. Meanwhile, the major peaks were tectoridin (2) and tectorigenin (4) in Iridis Tectori Rhizoma, and 2 was significantly more abundant than 4 [Figures 2 and 3]. The similarity of twenty batches of Belamcandae Rhizoma from Hebei ranged 0.91–1.00, which was slightly lower than the ones from all 27 batches (0.97–1.00). The results indicated that the chemical profile of Belamcandae Rhizoma was not closely related with their production area.

**High-performance liquid chromatography quantitative analysis**

Isoflavones are major active components in Belamcandae Rhizoma and Iridis Tectori Rhizoma, and they exhibit antioxidative, phytoestrogenic, anti-inflammatory, and antiangiogenic activities.[7] To further explain the difference of Belamcandae Rhizoma and Iridis Tectori Rhizoma, the contents of tectoridin (2), irigenin (5), and irisflorentin (6) were determined.

**Optimization of extraction and high-performance liquid chromatography chromatographic conditions**

The HPLC conditions were optimized as described in “HPLC fingerprint analysis,” except for the detection wavelength. The wavelength was set at 266 nm according to the maximum absorption of tectoridin (2), irigenin (5), and irisflorentin (6). Extraction method and other chromatographic parameters were consistent with HPLC fingerprint analysis.

**Method validation**

Linearity, limit of detection and limit of quantification

Linear regression analysis for the three isoflavones was
performed by external standard method [Table 1]. Tectoridin, irigenin, and irisflorentin showed good linearity ($r^2 = 0.9997$) at a concentration range of 1.30–83.20 μg/mL, 1.18–75.60 μg/mL, and 0.61–39.20 μg/mL, respectively [Table 1]. The LOD of tectoridin, irigenin, and irisflorentin was 16.25 ng/mL, 14.77 ng/mL, and 7.66 ng/mL, respectively. The LOQ values were 81.65 ng/mL, 78.32 ng/mL, and 38.28 ng/mL, respectively.

**Precision, repeatability, and stability**

The intra- and inter-day precision were evaluated by analyzing a representative sample (SG-2) of Belamcandae Rhizoma for six times in the same day and in 3 consecutive days, respectively. The RSD values were between 0.38% and 2.22%, respectively. The repeatability test was evaluated by preparing six sample solutions in parallel with the same batch of Belamcandae Rhizoma sample, and the RSD ranged from 0.79% to 3.79%. The stability was evaluated by analyzing the sample (stored at room temperature in the autosampler) every 3 h between 0 and 36 h. The RSD was 2.96%–3.05%. The above results met the requirements for method validation in Chinese Pharmacopoeia. [23]

**Recovery**

The recovery was validated by adding known amounts of standards at three concentration levels (approximately 50%, 100%, and 150% of the original concentration, $n = 3$) into a selected sample (SG-2). The recovery of the three analytes ranged from 98.08% to 104.82%, which met the requirements for method validation in Chinese Pharmacopoeia. [23] Data for the method validation were summarized in Table 1.

**High-performance liquid chromatography quantitation of Belamcandae Rhizoma and Iridis Tectori Rhizoma**

The thirty batches of samples were tested using the validated method. Contents of tectoridin (2), irigenin (5), and irisflorentin (6) in 27 batches of Belamcandae Rhizoma were 0.28 ± 0.08 %, 0.35 ± 0.03 %, and 0.12 ± 0.02 %, respectively. Contents of these compounds in three batches of Iridis Tectori Rhizoma were 2.50 ± 0.20 %, 0.19 ± 0.04 %, and 0.07 ± 0.01 %. The content of tectoridin was 8.93 times higher in Iridis Tectori Rhizoma than that in Belamcandae Rhizoma ($P < 0.005$). The contents of irigenin and irisflorentin were slightly lower in Iridis Tectori Rhizoma. Therefore, the content ratio of tectoridin could be considered as an important indicator to distinguish Belamcandae Rhizoma and Iridis Tectori Rhizoma [Figure 4].

The content of three isoflavones were 0.75 ± 0.04 %, 0.76 ± 0.11 %, and 0.76 ± 0.07 % in Hubei (2 batches), Henan (3 batches), and Hebei (20 batches), respectively, indicating that there was no obvious difference between production areas. We further analyzed the twenty Belamcandae Rhizoma samples from Hebei province. The content variation for tectoridin and irisflorentin was relatively remarkable with the RSD of 31.42% and 12.69%, respectively. The content of tectoridin in Belamcandae Rhizoma collected in 2017–2019 was 0.42 ± 0.02 %, 0.34 ± 0.08 %, and 0.23 ± 0.05 %, respectively, indicating that the content of tectoridin may be relevant to the storage time.

**Conclusions**

In this study, we elucidated the chemical difference of Belamcandae Rhizoma and Iridis Tectori Rhizoma using TLC and HPLC. In TLC analysis, Belamcandae Rhizoma could be distinguished by the fluorescent band under 366 nm at $R_f = 0.2$. In HPLC fingerprints analysis, 12 common peaks of Belamcandae Rhizoma and Iridis Tectori Rhizoma were identified, and the two herbs showed low similarity (correlation coefficient 0.54 ± 0.01). The main peaks in Belamcandae Rhizoma were mangiferin (1), tectoridin (2), iridin (3), irigenin (5), irisflorentin (6), and iristectorin A (9) with similar peak heights. In contrast, the major peak in Iridis Tectori Rhizoma was tectoridin (2) and tectorigenin (4). In HPLC quantitation, the content of tectoridin in Iridis Tectori Rhizoma (2.50 ± 0.20 %) was 8.93 times higher than that in Belamcandae Rhizoma (0.28 ± 0.08 %), whereas irigenin (0.19 ± 0.04 vs. 0.35 ± 0.03 %) and irisflorentin (0.07 ± 0.01 vs. 0.12 ± 0.02 %) were slightly lower than Belamcandae Rhizoma. The study provided three quick and effective methods to distinguish Belamcandae Rhizoma and Iridis Tectori Rhizoma.

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Conflicts of interest
There are no conflicts of interest.

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