Development of an HPLC Fingerprint for Quality Control and Species Differentiation of Uncaria rhynchophylla (Miq.) ex Havil

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Summary. The stems of Uncaria rhynchophylla (Miq.) ex Havil have a long history of use in traditional Chinese medicine to treat diseases and improve health. There is evident evidence that alkaloids constituents are mainly responsible for the beneficial effects of this plant medicine. The amounts of the major bioactive alkaloids in this plant vary widely with species, habitat, and as such, and establishment of a high-performance liquid chromatography (HPLC) fingerprint for quality control of this herbal medicine is of particular importance. The most alkaloids are used for medicine treatment and research. On the basis of the chromatographic data, a consistent HPLC fingerprint pattern containing 15 common peaks was obtained. Among these common peaks, four were identified as rhynchophylline, isorhynchophylline, corynoxeine, and isocorynoxeine. On the basis of this HPLC fingerprint and principal-components analysis, the quality of fifteen samples from different producing areas of China was objectively assessed. To summarize, the data described in this study offer valuable information for quality control and proper use of U. rhynchophylla (Miq.) ex Havil.

Key Words: Uncaria rhynchophylla (Miq.) ex Havil, HPLC fingerprinting, Similarity Evaluation System

Introduction

Herbal medicines have been increasingly appreciated as effective remedies in many countries [1]. For better development of herbal medicines for effective therapy, it is imperative to control the quality of the herbal medicines. Fingerprint technology has recently been introduced and accepted by the WHO and the State Food and Drug Administration (SFDA) of China as a strategy for evaluation of the quality of herbal medicines and their products [2]. Among these fingerprinting techniques, chromatographic fingerprinting is a very useful and popular analytical approach because it emphasizes the systemic characterization of sample composition [3]. Chromatographic methods currently available for fingerprinting include high-performance liquid chromatography (HPLC), and thin-layer chromatography (TLC) [4–6].

Rhynchophylla is the dried rattan of Uncaria rhynchophylla. For its unique effectiveness, this plant has been widely used in China and Japan to treat...
diseases and to improve health. Rhynchophylla alkaloids are the major constituents of U. rhynchophylla [7]. More than 15 have been isolated, and among which the most abundant includes rhynchophylline, isorhynchophylline, corynoxeine, and isocorynoxeine. Rhynchophylla alkaloids are largely responsible for the beneficial effect of Rhynchophylla extracts [8]; there is evidence that alkaloids have antihypertension, anticonvulsant, and sedative, etc. effects [9–12].

Many approaches have recently been developed for the qualitative and quantitative analysis of the major alkaloids in U. rhynchophylla; among these, HPLC and TLC are most frequently reviewed elsewhere [13–15]. Compared with TLC, HPLC has the advantages of high analytical efficiency and precision. To optimize the conditions used to obtain the HPLC fingerprint, the effect of solvent, drug particle size, extraction method, analysis time, and elution conditions were scrutinized. Nonetheless, the problem of whether the established HPLC fingerprint could be used for effective evaluation of the quality of U. rhynchophylla from different producing areas, or whether this method was superior to others for assessment of the species differences, remained largely unsolved. In this study, we combined chemometric methods, for example, similarity evaluation and principal-component analysis, with HPLC–photodiode-array (DAD) detection to develop a specific and valid chromatographic fingerprinting approaching for quality assessment and species differentiation of U. rhynchophylla.

**Experimental**

**Chemical and Reagents**

The major alkaloids in U. rhynchophylla, rhynchophylline, isorhynchophylline, corynoxeine, and isocorynoxeine (Fig. 1) were isolated from U. rhynchophylla as described elsewhere. Reference substance was purchased from Chengdu Must Bio-Technology Co., Ltd. (Chengdu, China).

The chemical structures of these compounds were characterized by HPLC and mass spectrometry (MS). The purity of the compounds, as determined by HPLC, was >98%.

HPLC grade methanol was purchased from Fisher Scientific (Pittsburgh, PA, USA). High-purity deionized water (18.24 MΩ cm) was obtained from a Labconco system. AR-grade glacial acetic acid from Tianjin Jingbei Reagent and triethanolamine from Tianjian Hongyan Reagent were used for preparation of the mobile phase. Ethanol used for plant extraction was of AR-grade and purchased from Shanghai Chemical Corporation of China.
Fig. 1. Chemical structures of the reference compounds in Uncaria rhynchophylla

Plant Materials

U. rhynchophylla (samples 1–10) was collected in Hunan, Hubei, Hebei, Shanxi, Guangxi, Fujian, Jiangxi, Guangdong, Shaanxi, and Anhui province, China. U. rhynchophylla (Miq.) ex Havil, belonging to family Rubiaceae Uncaria, a downward curved hook or single hook in the stems, was identified as Chinese herbal medicine Uncaria by Xiaofeng Niu Associate Professor by the Department of Pharmacy, Xi’an Jiaotong University.
Table I. Details of the herbal materials collected

<table>
<thead>
<tr>
<th>No.</th>
<th>Producing area</th>
<th>Collection year</th>
<th>No.</th>
<th>Producing area</th>
<th>Collection year</th>
</tr>
</thead>
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<tr>
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<td>2010</td>
<td>S6</td>
<td>Hebei</td>
<td>2010</td>
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<tr>
<td>S2</td>
<td>Guangxi</td>
<td>2010</td>
<td>S7</td>
<td>Shanxi</td>
<td>2010</td>
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<tr>
<td>S3</td>
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<td>2010</td>
<td>S8</td>
<td>Guangdong</td>
<td>2010</td>
</tr>
<tr>
<td>S4</td>
<td>Fujian</td>
<td>2010</td>
<td>S9</td>
<td>Jiangxi</td>
<td>2010</td>
</tr>
<tr>
<td>S5</td>
<td>Hubei</td>
<td>2010</td>
<td>S10</td>
<td>Shaanxi</td>
<td>2010</td>
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</tbody>
</table>

HPLC Equipment and Conditions

The high-performance liquid chromatography was from Shimadzu Corporation (Kyoto, Japan). This included a LC-20AD pump, a DGU-20A3 degasser, a SIL-20A autosampler, a CTO-20A column oven, and an SPD-M20A diode array detector. Samples were separated on a Monolithic Speed ROD RP-18e column (4.6 mm i.d. × 50 mm). The mobile phase was a gradient prepared from H₂O (component A) and CH₃OH (component B), and the conditions used for gradient elution were the following: 0–3 min, 30% CH₃OH; 3–15 min, 40% CH₃OH; 10–15 min, 50% CH₃OH; 15–25 min, 55% CH₃OH; 25–30 min, 75% CH₃OH; 30–35 min, 75% CH₃OH; 35–40 min, 30% CH₃OH; 40–50 min, 30%. The flow rate was 1.0 mL min⁻¹. After the 75% methanol, the mobile phase was switched to 30% methanol for 10 min to equilibrate the column, and then the next sample was injected. The injection volume was 20 μL. The column temperature was 25 °C to obtain chromatograms with a good separation. The detection wavelengths are 254 nm.

Sample Preparation

_H. Rhynchophylla_ was pulverized to be a coarse powder. Samples (100 g) were extracted with 70% ethanol (500 mL) for 2 h by extraction under reflux (85 °C). Extracts were filtered and evaporated to semi-dryness, and the residues (0.05 g) which were obtained from the semi-dryness of the extracts were dissolved in 2 mL chromatographic pure methanol. Solutions were clarified by centrifugation (8000×g, 10 min) before analysis.
Data Analysis

Similarity analysis was performed by use of the professional software “Similarity Evaluation System (SES) for Chromatographic Fingerprint of Traditional Chinese Medicine” recommended by the SFDA of China. This software is currently not commercially available, but the SFDA of China allows free use of this software, solely for quality control of Chinese herbal medicine. The relative standard deviation (RSD) of the software is evaluation of the precision of the chromatogram value.

Results and Discussion

The Extraction Conditions

To achieve maximum recovery of the components of Rhynchophylla, the extraction conditions including 70% ethanol solvent and extraction under reflux method were optimized.

Validation of the Method

Data analysis of the chromatographic fingerprint was performed by use of SES software, and similarities between chromatograms were used to evaluate fingerprint quality. The reproducibility of the method was valuated by analysis of six replicates of the same sample. The same sample solution was also analyzed at different times (0, 2, 4, 8, 24, and 48 h) after preparation to test its stability. The result from the reproducibility test revealed that the RSD of average similarity of the chromatograms obtained from six replicate analyses was less than 2%. In the stability test, the RSD of average similarity of the different chromatograms was less than 0.7%. Therefore, the analytical method used in this study is reproducible, and samples are stable during the test period.

Establishment of the Common Pattern of the HPLC Fingerprint of U. rhynchophylla

Under the HPLC conditions, a mixture of the alkaloid series reference standards rhynchophylline, isorhynchophylline, corynoxeine, and isocorynoxeine was separated in one chromatogram (Fig. 2). Ten batches of Rhynchophylla samples collected from Hanzhong in Shaanxi province were analyzed by HPLC with the purpose of establishing a common HPLC fingerprint pattern for Rhynchophylla. It is worth mentioning that, on the basis of the
principle of fingerprinting, when the relative standard deviation (RSD) of peaks’ relative retention times for all batches of samples is less than 1%, these peaks belong to the same substance and can be assigned as a “common peak.” In this study, more than 20 peaks were separated in all batches of samples, but only 15 peaks could be regarded as common peaks.

![Chromatograms](image)

*Fig. 2.* Chromatograms obtained from the standard compounds (A) and common HPLC fingerprint pattern for *Uncaria rhynchophylla* (B2). The compounds corresponding to peaks a to d in A are corynoxeine, isorhynchophylline, isocorynoxeine, and rhynchophylline, respectively, and also corresponding to peaks 10, 11, 12, and 13 in B2 are corynoxeine,isorhynchophylline, isocorynoxeine, and rhynchophylline, respectively.

*Table II.* Similarity values of the ten batches of samples

<table>
<thead>
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<th>No.</th>
<th>Similarity value</th>
<th>No.</th>
<th>Similarity value</th>
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<tr>
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<td>S6</td>
<td>0.998</td>
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</tr>
<tr>
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</tr>
<tr>
<td>S5</td>
<td>0.998</td>
<td>S10</td>
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</tr>
</tbody>
</table>
Development of an HPLC Fingerprint

On the basis of the HPLC conditions established for fingerprinting, 10 samples (S1–S10) collected from different producing areas were analyzed. The overlaid chromatographs shown in Fig. 3 provide a general profile of the compounds in these samples. By comparing the similarity of these chromatograms with the standard HPLC fingerprint (Table III), it is not difficult to find that the samples (S1, S6, and S10) can assemble into one group, the samples (S2, S3, S5, and S9) can assemble into one group, and the samples (S4, S7, and S8) can assemble into one group.

<table>
<thead>
<tr>
<th>No.</th>
<th>Similarity value</th>
<th>No.</th>
<th>Similarity value</th>
</tr>
</thead>
<tbody>
<tr>
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<td>S6</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>0.807</td>
<td>S9</td>
<td>0.592</td>
</tr>
<tr>
<td>S5</td>
<td>0.590</td>
<td>S10</td>
<td>0.047</td>
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</tbody>
</table>

Finally, by taking advantage of the optimized HPLC conditions, we compared the chromatographic fingerprint of *U. rhynchophylla* with the standard HPLC fingerprint. As shown in Fig. 4, the chromatographic fingerprint of *U. rhynchophylla* was substantially different from that of the four corynoxeine, isorhynchophylline, isocorynoxeine, and rhynchophylline from Shaanxi, which are higher than the shoot from Shanxi province, and also are missing the components a, b, and c in sample 7. Although the two samples have some similar chemical constituents, and usually regarded as alternative drugs in the clinic, they have some differences in composition and have different content. We still need to further study the specific structure of the different components in the *Uncaria* from different areas. From the results, we still need further efforts to improve the conditions of the *U. rhynchophylla* (Miq.) ex Havil in Pharmacopoeia of China (2010).
Fig. 3. Overlaid chromatograms obtained from 10 samples from different locations. Samples 1–10 were from Hunan, Guangxi, Anhui, Fujian, Hubei, Hebei, Shanxi, Guangdong, Jiangxi, and Shaanxi.

Fig. 4. Differences between the standard HPLC fingerprint and the chromatograms obtained from sample 7. Three peaks a, b, and c in sample 7 are missing from common pattern. The peak d rhynchophylline is lower than in common pattern.
Conclusion

In this work, an HPLC fingerprinting method was developed for evaluation of the quality of *U. rhynchophylla*. Twenty common peaks were identified in the chromatographic fingerprint of *U. rhynchophylla*. By using this common pattern as the HPLC fingerprint, the quality of fifteen batches of *U. rhynchophylla* was evaluated. This approach was also successfully used to identify species and collection time differences between herbal plants. This proposed method is simple and reliable, with high precision, stability, and repeatability, and can thus be adopted for quality control of herbal products.

Acknowledgments

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References


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