Clematis huchouensis TAMURA: A Traditional Chinese Herbal Medicine and Its Quality Control Using a High Performance Liquid Chromatography Technique

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A simple, specific and reliable high performance liquid chromatography (HPLC) method has been developed and validated for study of fingerprint chromatograms of extracts from the whole plant of Clematis huchouensis TAMURA (CHT) for quality control of a traditional Chinese medicinal (TCM) herb. An Agilent C18 column was used to separate extracts in this protocol and detection was made by ultraviolet absorbance at 340 nm. The column temperature was maintained at 25 °C. A mobile phase consisting of (a) water (with 30 mM KH2PO4) and (b) CH3CN, (c) CH3OH was found to be suitable for this separation at a flow rate of 0.8 µl min-1 with gradient elution. Under the described conditions, a fingerprint profile of 8 compounds was collected within 35 min, which made the HPLC method unique and interesting. The fingerprint chromatograms had good stability, precision and reproducibility. Moreover, eco-climatic (habitat) effects were studied by comparison of fingerprint chromatograms obtained from extracts of CHT collected from two habitats, with rutin as a reference marker peak. The protocol developed is quite suitable for differentiation of extracts of CHT and can be used as a quality control method for this herb and a model for other herbal drugs.

Key words  traditional Chinese medicine; Clematis huchouensis; rutin; HPLC fingerprint; flavonoid

Medicinal plants have been used for thousands of years in virtually all cultures as a source of medicine. Traditional Chinese medicines (TCMs) are used by a huge majority of people in China and other countries for curing various diseases. These TCMs have served as a source of alternative medicines, new pharmaceuticals, and healthcare products.

Gorman1) drew attention to the power of Chinese folk medicinal potions in treating maladies from eczema and malaria to respiratory disorders. TCMs are highly complex, being composed of many herbal contents. The multiherbal compositions of TCMs however, create analytical problems in terms of quality control. Previously, the quality control of herbal medicines was controlled solely on the basis of one or a few constituents. However, basic information on many herbal medicines is lacking. Due to this acceptance or competition of TCMs in western medical markets seems to be difficult and problematic. Maintaining quality standards is a basic requirement for a drug to be competitive and trusted in the marketplace. Therefore, there is a need to develop a method to monitor the total quality of TCMs in an efficient way.

Fingerprint analysis is a protocol that provides chemical information, including chromatograms, spectrograms and other graphic representation of analytical data. This technique is more effective for comparing and evaluating quality control of drugs.2,3) Such fingerprints have been used in different drug studies and hence proved to be a valuable informative tool for quality control.4—13) The TCM herb Clematis huchouensis TAMURA (CHT) belongs to the family Ranunculaceae which is distributed in Zhejiang, Hunan, Jiangsu and Jiangxi provinces of China. TCM prepared from this herb has been used for rheumatoid arthritis.14) In folklore traditions, herbal preparations from this plant are considered to have anti-tumor/anti-cancer, anti-inflammatory, anti-allergy, anti-thrombotic, anti-mutagenic and other bioactivities in conjunction with other herbs. Due to its effective role as a TCM, it is being extensively researched. A key problem is how to display and control the total quality of a herbal medicine originating in different habitats and comprising many constituents with variable quantities and properties. It has flavonoids as its major compounds, which may be the reason for its effectiveness in the above-mentioned diseases,15,16) but flavonoids may be precursors of some toxic substances in photosynthesis and cellular energy transfer processes,17,18) Therefore, due to these variable and contrasting properties, flavonoids were selected in our study as quality control markers (QCM). As far as we know, no simple and reliable method has yet been developed for fingerprint analysis of extracts of CHT. The qualitative and quantitative eco-environmental effects on chemical constituents of plants were studied for herbal specimens originating from two habitats (Hangzhou and Jiangsu) using the parameters relative retention time (RRT) and relative peak area (RPA).

MATERIALS AND METHODS

Chemicals and Materials  Acetonitrile and methanol were of analytical grade (Merck, U.S.A.). HPLC grade water was prepared using a Milli-pore water purification system (Millipore, MA, U.S.A.). TCM herbs were obtained from Hangzhou and Jiangsu provinces. The specimens were authenticated and their herbaria were prepared and deposited at the College of Chinese Medicine Science and Engineering, Zhejiang University, Hangzhou, China.

Apparatus  The LC system used consisted of a low pressure gradient pump (Model 616, Waters, Milford, MA, U.S.A.), a helium degasser system and an auto sampler (Model 717, Waters) with a 200 µl loop and a diode array detector (model 996) with a 10 mm path length flow cell. Separation was made at ambient temperature by using an Agilent column C18 (250 mm, 4.6 mm, 5 µm). Data were stored on a
personal computer based data system (Millennium version 32 Waters).

**Preparation of Reference Standards and Working Solutions** Rutin, a reference standard, was purchased from Guo Yao Ji Tian Hua Xue Shi Ji You Xian Company; Shanghai, China. Rutin stock solution was prepared by dissolving (10 mg) in MeOH (5 ml) and diluting to 10 ml and stored at 4 °C. A working solution was made by diluting stock solution with mobile phase.

**Mobile Phase** A Binary mobile phase consisting of acetonitrile: methanol: and water, at a ratio of 80 : 10 : 10 (v/v) was used. The mobile phase was degassed by sonication in an ultrasonic bath before use. The mobile phase flux rate was 0.8 ml min⁻¹.

**Extraction Procedure** Dry herbal material ca. 50 g was extracted in 350 ml of 70% ethanol for 2 h (twice) by refluxing. The extract collected was mixed, filtered and concentrated in rotavapour. The concentrated extract was then adsorbed on polyresin D-101 in the column and eluted with water, 20% ethanol, and 70% ethanol to collect the sugar, saponin and flavonoid fractions, respectively. The 70% fraction was further partitioned using Sephadex column eluting with methanol. TLC was used to monitor the presence of flavonoids in different fractions. The fractions containing flavonoids were mixed, concentrated, and stored at −20 °C until further use. The sample was dissolved in methanol and centrifuged (10000 rpm for 10 min) before injection into the HPLC system.

**Results and Discussion**

**UV Spectra and Chromatographic Separation** The chromatograms obtained from extracts of CHT were UV detected from 280—360 nm. The results showed that the peaks were moderate and adequately separated, while the baseline was steady and stable at 340 nm. To examine the effects of solvents on the chromatographic peaks, various binary mobile phases in different gradients were used; however, many produced poor baselines and insufficient separation of the peaks. The above mentioned mobile phase (acetonitrile: methanol: water) was found to be the most appropriate one in this chromatographic analysis. Since it produced steady and stable baselines and better peak separation, therefore, it was used during this analytical process. The effects of temperature on detection were investigated. Temperature had no ambient impact on the retention time or peak area of the chromatograms. However, constant temperature conditions (25 °C) were good in this analysis process. Therefore, the temperature of the column was maintained constant at 25 °C throughout the analysis.

**Selection of Reference Peak and Common Peak Fractions** Rutin, one of the major active constituents in CHT extract, was selected as the reference standard for equalizing and evaluating the quality of the herb. In our experimental chromatographic conditions, the reference marker peak for rutin has an area approximately 24.81% of the total peak area. Moreover, the retention time and peak area of rutin were stable and reproducible. Rutin was therefore selected as the reference peak and reference marker for RRT and RPA in this study.

In this fingerprint analysis, chromatographic peaks with the same RRT were selected as the common peak fractions in different samples. Five samples of the CHT collected from Hangzhou and Jiangsu were analyzed. There were almost 34 peaks in the HPLC chromatogram and 15 peaks were common peaks in five analyzed batches. Among the common peaks, only those 8 peaks that had a retention time of more than 7.2 min, a single peak area equal or greater to 2.54% of total peak area, and the most importantly that contained no co-eluting substances were selected as representative peaks for this fingerprint chromatogram profile. Hence, the peak profile of the 8 components made up the fingerprint of extracts from whole plant of CHT. Among them, the rutin peak was used as a reference peak and reference marker. The area of all 8 peaks accounted for more than 60.61% of the total peak area. The relative retention times and relative peak areas are shown in Table 1.

**Stability, Precision and Reproducibility of Experiment** The stability of the fingerprint analysis was assessed by analyzing the same sample at 0, 2, 4, 8, 10 and 12 h. The results showed that the relative standard deviation (RSD) of the 8 peak fraction assays was less than 0.75% of the relative retention time with rutin as the reference peak less than 2.4% for relative peak area, respectively.

The precision of the fingerprint analysis was examined by 5 repeated extractions of CHT. The relative standard deviation ranged from 0.125 to 0.40% for relative retention time and from 0.52 to 1.55% for relative peak area. The reproducibility of the fingerprint analysis was evaluated by assaying of 5 samples according to the described protocol. The results showed that relative standard deviations of the 8 common peak fraction assays were less than 0.75% for relative retention time and less than 2.4% for relative peak area.

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>Relative retention time</th>
<th>Relative peak area</th>
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<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>1</td>
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<td>0.3791</td>
</tr>
<tr>
<td>2</td>
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<td>1.2514</td>
</tr>
<tr>
<td>7</td>
<td>1.3106</td>
<td>1.3121</td>
</tr>
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Rutin was the standard and reference marker peak with RRT and RPA values =1.
Fingerprint Comparison of Extracts of CHT from Hangzhou and Jiangsu

The plant specimens obtained from Hangzhou and Jiangsu were analysed to visualize the eco-environmental effects on the bioconstituents. After fingerprint analysis, the qualitative results of the peak groups were very similar in the two group samples. However, they differed considerably on a quantitative basis. This study revealed that on a qualitative basis, the major components of two specimens of the herb collected from Hangzhou and Jiangsu were approximately the same. However, the quantitative ratio of some components was significantly different in two samples originating from diverse habitats. In particular, quantitative differences between peaks 1, 2, 3, 4, 5 and 6, and the reference peak were prominent. In extracts of CHT collected from Hangzhou, the concentration of the peaks was lower. The reference fraction rutin also varied (ratio of peak area = 1.3264, Fig. 1). In contrast to this situation, the concentrations of almost all of the common peak fractions were significantly higher in the extracts of CHT collected from Jiangsu (relative peak area = 0.5777, Fig. 2). Therefore, further study is needed to determine the qualitative and quantitative differences in the chemical constituents of CHT herb due to variations in the eco-climatic conditions in 2 habitats. This quantitative variation of constituents may be due to altitude, temperature, and/or edaphological differences, as Jiangsu plants are located at different topography and altitude than plants species in Hangzhou. This suggests that the type of habitat affects the composition of the herbal bioconstituents. Further studies are required to investigate and confirm how altitude and topography affect the chemical constituents in plants and how the quality of herbal medicines may vary from one habitat to another.

CONCLUSION

Traditional medicinal herbs are integral components of research and developing in the pharmaceutical industry. Such research can be useful for the isolation and direct use of active medicinal constituents, in the development of semi-synthetic drugs, or in the active screening of natural products to yield synthetic pharmacologically active compounds from CHT and other medicinal herbs with quality good assurance. This high performance liquid chromatographic fingerprint analysis protocol which is simple, convenient and stable, can be used to monitor the quality of CHT medicinal herbs by the identification of flavonoids, which are major bioconstituents. At the same time, such fingerprint chromatogram analysis also contributes to greater scientific and medical understanding of herbal medicines and promotes a new way of studying TCMs. Furthermore, it has revealed that eco-climatic variation affects the chemical constituents of plants. It is therefore very promising for further pharmacological and biochemical experiments that focus on evaluating the mechanism by which environmental variation affects the chemical constituents in plants viz a viz quality of a medicinal herb.

REFERENCES