Simultaneous Determination of Protocatechuic Acid, Syringin, Chlorogenic Acid, Caffeic Acid, Liriodendrin and Isofraxidin in _Acanthopanax senticosus_ HARMS by HPLC-DAD

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A high performance liquid chromatography (HPLC) method was developed for the first time to quantify simultaneously the six major active ingredients in _Acanthopanax senticosus_ (Rupr. et Maxim.) HARMS, namely protocatechuic acid, syringin, chlorogenic acid, caffeic acid, liriodendrin and isofraxidin. The analysis was performed by a reverse phase gradient elution with an aqueous mobile phase (containing 0.05% phosphoric acid) modified by acetonitrile and diode-array multiple-wavelength UV detector (DAD). Six regression equations showed good linear relationships between the peak area of each marker and concentration. The recoveries of the markers listed above were 92.3%, 93.9%, 90.3%, 93.1%, 94.3% and 90.7%, respectively. The relative standard deviation of intra-day and inter-day were less than 2.7% and 3.1%, respectively. This method was validated for specificity, accuracy, precision and limits of quantification. Medicinal materials of ten commercial brands were analyzed and found to contain different amounts of the six bioactive markers. The method developed can be used for the quality control of _Acanthopanax senticosus_ (Rupr. et Maxim.) HARMS.

Key words HPLC-DAD; syringin; chlorogenic acid; caffeic acid; liriodendrin; isofraxidin

Traditional Chinese medicine (TCM) which uses natural therapeutic agents under the guidance of the theory of traditional Chinese medical science has been applied by TCM practitioners for several thousands years and has been increasingly popular. _Acanthopanax senticosus_ (Rupr. et Maxim.) HARMS (ASH) is a well-known TCM in China, which has been officially listed in Chinese Pharmacopoeia for a long time. It possesses various pharmacological effects, such as antibacterial, antifatigue, anti-oxidant and anti-tumor activities.1—7

ASH has been shown to contain many effective constituents, including protocatechuic acid, syringin, chlorogenic acid, caffeic acid, liriodendrin and isofraxidin (ingredient 1—6) and etc.8—13 Although many HPLC methods have been developed for the determination of one or two constituents,14—17 up to date, there have been few reports on the simultaneous determination of multiple constituents in ASH. Moreover one or two constituents could not be responsible for overall pharmacological activities of ASH. In the present study, a rapid and simple HPLC method was established for the quality control of ASH and successfully applied for the assessment of ten commercial samples.

**MATERIALS AND METHODS**

**Reagents and Materials** ASHs were purchased from TCM shops of different places of China. The standards of protocatechuic acid, syringin, chlorogenic acid, caffeic acid and isofraxidin were all ordered from the Chinese National Institute of Control of Pharmaceutical and Biological Products (Beijing, China). The standard of liriodendrin was isolated by the author from ASH and its structure was fully characterized by chemical and spectroscopic methods (UV, IR, NMR, MS). Purity analysis proved its purity above 98%. Phosphoric acid, methanol and acetonitrile were HPLC grade.

**Chromatographic System** The HPLC system consisted of a Shimadzu LC-10ATVP chromatograph, a SPD-M10AVP detector and column oven. The LC separation was performed on an Agilent SB-C18 150 × 4.6 mm i.d.; 5 μm) protected by a guard C18 column (5 μm). The mobile phase was a stepwise gradient of water (0.05% v/v phosphoric acid)–acetonitrile (0.01 min, 94 : 6; 50 min, 65 : 35). The column temperature was maintained at 35 °C. The analysis were carried out at a flow-rate of 0.9 ml·min⁻¹ with DAD detection from 200—370 nm.

**Preparation of Standard Solutions** To prepare a standard containing ingredient 1—6, accurately weighed amounts of each compound were dissolved in methanol to give serial concentrations of 0.35—3.52, 5.76—57.65, 11.19—167.8, 1.61—16.12, 4.76—47.62 and 0.38—3.75 μg·ml⁻¹, respectively. Calibration graphs were plotted after linear regression of the peak area with concentrations. Chromatogram are presented in Fig. 1A.

**Preparation of Sample Solutions** ASHs were powdered to a homogeneous size in a mill, sieved through a No. 40 mesh, and dried at 40 °C in the oven for 6 h. The powder sample (1.0 g) was extracted with 40 ml ethanol for 30 min in an ultrasonic bath, and then filtered. The resulting solution was evaporated to dryness in vacuum. The residue was dissolved in 10 ml of methanol using a volumetric flask. All samples were filtered through a 0.45 μm Millipore filter before injecting 20 μl for HPLC analysis.

**RESULTS AND DISCUSSION**

Calibration graphs for ingredient 1—6 were obtained over the ranges 0.35—3.52, 5.76—57.65, 11.19—167.8, 1.61—16.12, 4.76—47.62 and 0.38—3.75 μg·ml⁻¹, respectively. The regression equations are 

\[ y = 1.2E + 0.5x + 88582, \quad y = 1.6E + 0.5x + 32026, \quad y = 7.2E + 0.5x - 93468, \quad y = 1.4E + 0.5x - 45806, \quad y = 8.6E + 0.4x - 10881, \quad y = 4.4E + 0.4x - 2526, \] 

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son, 25%, 50%, 75% and 100% ethanol solution were also tried for extraction. The contents of protocatechuic acid, syringin, caffeic acid and liriodendrin seem to stable when the ethanol increases to 75%. The highest extraction efficiency of chlorogenic acid in ASH was achieved with 75% ethanol and the best solvent for isofraxidin, which was used as marker compound of identification of ASH in Chinese Pharmacopoeia, was 100% ethanol. If ASH sample was extracted with methanol contained water, more interfering peaks disturbed analysis of the marker peaks. The chromatographic results indicated that the best extraction was obtained with 100% ethanol solution, which was in consistent with Chinese Pharmacopoeia. Extraction time and solvent volume of processing procedure, which may affect medicine quality, were also optimized by the developed method. The results indicated that the contents of the six constituents tend to stable with the time prolongation and volume increasing. From experiments it could be concluded that the best extraction process was using 40 times ethanol for 30 min.

In this study, the six marker components of ASH could not be separated effectively by using the isocratic mobile solvents. In order to find an easy way to analyze the components, we employed a gradient solvent system (acetonitrile and phosphoric acid solution), which can effectively separate six makers simultaneously. Different combinations of the two-solvent gradient were investigated. An increase in aqueous increased the separation between syringin and chlorogenic acid.

Full UV spectra (200—370 nm) of ASH were obtained from the DAD detector. Though the UV absorption maximum of six marker components were different, all could be resolved with baseline separation at 220 nm and the better resolution of chlorogenic acid, caffeic acid and isofraxidin could be obtained at 340 nm. Therefore, a monitoring wavelength at 220 nm was used for the quantitative determination of protocatechuic acid, syringin and liriodendrin, while monitoring wavelength at 340 nm was used for the quantitative determination of chlorogenic acid, caffeic acid and isofraxidin. Chromatograms are shown in Figs. 1B and C.

Ten commercial samples were obtained from various provinces and cities in China and were analyzed using this method. The data presented in Table 1 shows that all six compounds were found in ten samples and that the contents of ingredient 1—6 in individual sample can vary considerably, which might be derived from the plant origins, environmental factors and some other factors, such as collecting season, drying process and storage conditions. In Chinese Pharmacopoeia, only the content of syringin was used as marker compound to evaluate the quality of ASH and the limit was no less than 0.05%. However, the assessment of herb quality by using one or two constituents was not suitable. In the present study, a more overall assessment method was established to explore the quality control method of TCM.

CONCLUSION

The described method was successfully applied to the simultaneous determination of protocatechuic acid, syringin, chlorogenic acid, caffeic acid, liriodendrin and isofraxidin in ASH for the first time. The assay was found to be rapid, linear, accurate and reproducible. Results indicate that the developed HPLC-DAD method can be readily utilized as a
quality control method (for both qualitative and quantitative analysis) of ASH.

REFERENCES