DETERMINATION OF CHLOROGENIC ACID, BAICALIN AND FORSYTHIN IN SHUANGHUANGLIAN PREPARATIONS BY HPLC-DAD

LI YANG^{1*}, QINGHUA YAN², HAIZHEN MO³, XIAOMIN LI⁴, QING WANG¹

¹ Department of Experimental Center, Henan Institute of Science and Technology, Xinxiang Henan 453003, China

² Department of Life Science and Technology, Xinxiang Medical University, Xinxiang Henan 453003, China

³ School of Food Science, Henan Institute of Science and Technology, Xinxiang 453003, China

⁴ School of Technique and Electricity, Henan Institute of Science and Technology, Xinxiang 453003, China

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ABSTRACT

High performance liquid chromatography coupled with diode array detection (HPLC-DAD) was developed for the simultaneous determination of three bioactive components - chlorogenic acid, baicalin and forsythine - in traditional Chinese medicinal Shuanghuanglian preparations. The analytes were extracted successfully using 80% aqueous methanol for 15 min in an ultrasonic bath. The optical chromatographic separation conditions were performed on a Finnigan Surveyor HPLC system with an Agilent Zorbax ODS C18 (4.6×150 mm, particle size 5 µm) at 30 °C using a gradient elution program with a mixture of acetonitrile and 0.2% phosphoric acid (v/v) as mobile phase within 25 min. Method validation results were obtained through the generation of calibration curves with the mixed standard solutions and a standard addition method, regression equations revealed linear relationships (correlation coefficients 0.9965-0.9985) between the peak area and concentration of the three analytes. The average recoveries were 90-106% (n= 6) with standard deviation below 3.37%. The developed HPLC assay was specific and could be readily utilized for comprehensive evaluation of Shuanghuanglian preparations.

Keywords: HPLC-DAD; Shuanghuanglian preparation; Chlorogenic acid; Baicalin; Forsythine.

1 INTRODUCTION

As already known, Traditional Chinese Medicines (TCMs) and their preparations have been used for centuries in the practice of treating human diseases, medicated diet and dietetic therapy, especially in some Asian and African countries. Due to the effective therapeutic performance and low toxicity, herbal medicines have attracted considerable attention in many fields^{1,2}. Shuanghuanglian preparations (SHLs), including oral liquid, injection powder, capsule, and tablet, belong to a family of refined traditional Chinese preparation. It comprises several herbs including *Flos Lonicerae*, *Radix Scutellariae*, and *Fructus Forsythiae*³⁻⁵. Pharmacological action has demonstrated that it is a bacteriocide, diminishes inflammation, antiviral and is useful for treating fever, cough, pneumonia etc⁶. Chlorogenic acid, baicalin and forsythine are the most important active compounds in Shuanghuanglian preparation (Chemical structures are shown in Fig.1). However, knowledge regarding the composition of the bioactive ingredients in Chinese medicinal preparations is limited. Therefore, it is necessary to establish an appropriate analytical method for determination of the content of the three compounds.

Figure 1. Chemical structures of the main components detected in Shuanghuanglian preparations

HPLC-DAD has been proved to be a powerful approach for rapid characterization of constituents in natural products. DAD as sensitive detectors can provide abundant structural information, which facilitates the structural identification of unknown compounds⁷⁻¹⁰. In this paper, we applied HPLC-DAD to identify, separate and determine the three bioactive compounds in Shuanghuanglian preparation. The experimental results obtained from the characteristic peaks of HPLC-DAD chromatograms and all total scan spectra from 200 to 600 nm were analysed for qualitative and quantitative assay of the three analytes.

2 EXPERIMENTAL

2.1 Materials and reagents

Chlorogenic acid, baicalin and forsythine were purchased from the Chengdu Scholar Bio-Tech. Co., Ltd., China. Acetonitrile and methanol (HPLC grade) used in the study were purchased from Fisher Scientific (Pittsburgh, PA, USA). Phosphoric acid (analytical grade) was purchased from Beijing Chemicals Reagents Plant, China. Shuanghuanglian tablet and oral liquid were purchased from a traditional Chinese medicine store in Xinxiang, China and identified by Yang li-juan at School of Pharmacy of Xinxiang Medical University. An ultra-pure water system (SG Ultra Clear System, Wasseraufbereitung und Regenerierstation GmbH, Germany) was used to produce ultra pure water with specific conductivity down to 0.055 µS/cm for the analysis of HPLC. All solutions and samples were filtered before analysis and degassed.

2.2 Apparatus

A Finnigan Surveyor HPLC (Thermo Finnigan, MA, USA), consisting of a quaternary solvent delivery system, an on-line degasser, an autosampler, a column temperature controller and photodiode array detector were used for acquiring chromatograms and UV spectra. HPLC separation was performed on a Zorbax ODS C18 (Agilent, 4.6×150 mm, particle size 5 µm), preceded by a Zorbax C18 guard column (Agilent, 4.6×12.5 mm, particle size 5 µm).

2.3 Chromatographic conditions

The mobile phase was composed of acetonitrile (A) and 0.2% phosphoric acid (v/v) (B) with a gradient elution program of 78-60% (A) in 0-15 min, 60-78% (A) in 15-20 min and 78-78% (A) in 20-25 min. The total analysis time was 25 min. The sample injection volume was 25 µL. The flow rate was 0.8 mL/min. The column temperature was maintained at 30 °C. The on-line UV spectra recorded from 200 to 600 nm were used for peak characterization, and the detection wavelength was set at 331 nm were used for chlorogenic acid analysis, and at 277 nm were used for baicalin and forsythine similarity analysis.

2.4 Extraction of Shuanghuanglian preparations

An appropriate amount of each of the five Shuanghuanglian preparations (tablet) was extracted with 80% aqueous methanol for 15 min in an ultrasonic bath, and then centrifuged at 2500 rpm/min for 5 min. The extraction was

repeated three times. The extracts were combined and filtered through filter paper, and then diluted to 25 mL in a volumetric flask with 80% aqueous methanol. Blank solutions were prepared in the same way as the samples. All solvents were filtered through a 0.45 μm micro-filter prior to injection. The samples were analyzed immediately after the pre-treatment. Six injections were performed for each sample. Shuanghuanglian oral liquid was analyzed directly through a 0.45 μm micro-filter prior to injection.

3 RESULTS AND DISCUSSION

3.1 Optimization of extraction procedure and chromatographic conditions

In order to guarantee “specificity”, “stability” and “reproducibility” of the HPLC-DAD chromatograms, variables involved in the procedure such as extraction method and chromatographic conditions were optimized. According to the optimization of extraction procedure of samples by Yan Qinghua, 80% aqueous ethanol was chosen for the extraction processing of Shuanghuanglian tablets¹¹.

The extraction procedure was further optimized by investigating the influence of following factors as solvent volume, extraction time and extraction repetitions. Based on the results tested above, ultrasonic extraction with 80% ethanol for 15 min chosen for the three analytes could not only be efficiently extracted but also well resolved from background peaks.

The chromatographic conditions were optimized to obtain an ideal HPLC-DAD chromatogram with good resolution and reasonable analysis time. Total scan spectras from 200 to 600 nm was performed to choose the characteristic absorption maximum for the three analytes to achieve more characteristic peaks as well as a smooth baseline. Fig. 2 showed the characteristic absorption maximums at 331 nm for chlorogenic acid, at 277 nm for baicalin, at 230 and 278 nm for forsythine. In order to improve the sensitivity for the quantitative analysis of forsythine, 278 nm as the characteristic absorption maximum was detected.

Figure 2. Total scan spectras of chlorogenic acid, baicalin and forsythine from 200 to 600 nm.

Different mobile phase compositions including $\text{MeOH-H}_2\text{O}$ (HCOOH , CH_3COOH), $\text{CH}_3\text{CN-H}_2\text{O}$ (HCOOH , CH_3COOH), $\text{MeOH-CH}_3\text{CN-H}_2\text{O}$ (HCOOH , CH_3COOH) were investigated, the chromatograms showed the resolution of the three analytes was poor or the peak tailing of the three analytes was serious. Finally, a mobile phase consisting of acetonitrile and 0.2% phosphoric acid was chosen for the determination of chlorogenic acid, baicalin and forsythine in Shuanghuanglian preparations. Typical HPLC-DAD chromatograms of the mixed standard solutions of chlorogenic acid, baicalin and forsythine are presented in Fig. 3.

Figure 3. The chromatograms of the mixed standard solution of chlorogenic acid (2.45 min), baicalin (7.62 min) and forsythine (8.01 min).

3.2 Quantitative analysis and application

Linear relationships between the concentrations of the three analytes and the corresponding peak areas were found in the concentration range 0.25–420 $\mu\text{g/mL}$ for chlorogenic acid and for 0.20–370 $\mu\text{g/mL}$ and 0.5–170 $\mu\text{g/mL}$ for forsythine, respectively. chlorogenic acid, baicalin and forsythine standard materials were dissolved in the mobile phase yielding the same concentration of 500 $\mu\text{g/mL}$. Then, 0.5, 1.00, 5.00, 10.00, 15.00 and 20.00 $\mu\text{g/mL}$ of the mixed standard solutions were prepared to yield a series of working solutions for the calibration curves. The regression equations of these curves and their correlation coefficients (r) were calculated as follows: chlorogenic acid, $Y = 65152X + 332202$ ($r = 0.9981$); baicalin, $Y = 605142X + 78771.3$ ($r = 0.9965$); and forsythine, $Y = 106362X + 27826.2$ ($r = 0.9985$); where Y and X are the peak area and the concentration ($\mu\text{g/mL}$) of the analytes, respectively. The method was validated for reproducibility of the retention time and the peak area of the analytes. The relative standard deviation (RSD) values of the retention time and the peak area of each peak for six replicate injections were 0.12–2.13%, 1.34–3.37% and 0.84–2.98%, respectively. Recovery was determined by the standard addition method for chlorogenic acid, baicalin and forsythine to be 91–103%, 95–106% and 90–102%, respectively.

3.3 Determination of components in Shuanghuanglian preparations

The method described above has been applied successfully to the separation and determination of chlorogenic acid, baicalin and forsythine in Shuanghuanglian tablet and oral liquid. Fig. 4 presents the chromatograms of chlorogenic acid, baicalin and forsythine in Shuanghuanglian tablet sample. The analytes were clearly identified by comparing each peak retention time, and spiked standards in actual samples were used to further confirm the identities the three analytes. The contents of chlorogenic acid, baicalin and forsythine determined in the extracts of Shuanghuanglian preparations are listed in Table 1. From this table, it can be seen that the amounts of each analyte were markedly different in the test samples, which is probably due to different sources of the herbs, different preparative processes or different composition formulas. Therefore, quality control is critical for these traditional Chinese medicinal preparations.

Table 1. Results of chlorogenic acid, baicalin and forsythine in Shuanghuanglian preparations ($n=6$).

Compound	Chlorogenic acid	Baicalin	Forsythine
Shuanghuanglian tablet (mg/g)	2.75	0.62	0.33
Shuanghuanglian oral liquid ($\mu\text{g/mL}$)	0.44	3.82	0.95

Figure 4. The chromatograms of the Shuanghuanglian tablets sample. chlorogenic acid (2.45 min), baicalin (7.62 min) and forsythoside (8.01 min).

4 CONCLUSION

The results demonstrated that the HPLC-DAD method is a simple, reliable and sensitive for determination chlorogenic acid, baicalin and forsythoside in Shuanghuanglian preparations. This procedure, suitable for routine analyses, consists of direct injection, a simple sample pre-treatment, followed by an HPLC quantification step. The method was characterized by good precision, linearity and accuracy. The optimized procedure was a good alternative for simultaneous analysis of bioactive components in traditional Chinese medicinal preparations. Furthermore, the method also promised to be applicable to the quality control of Chinese medicine.

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