HPLC and Chemometrics for the Quality Consistency Evaluation of Shuanghuanglian Injection

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A high-performance liquid chromatography method with diode array detection was developed for simultaneous analysis of organic acids (chlorogenic acid, caffeic acid, 4,5-dicaffeoylquinic acid) and flavonoids (baicalin, baicalein, wogonin) in Shuang-huang-lian injection, a traditional Chinese medicine being prescribed widely for antibacterial, anti-viral and immunostimulant activity. All calibration curves showed good linear correlation (r² > 0.9997) within the test ranges. Validation proved that the repeatability of the method was good and recovery was satisfactory. The validated method was successfully applied to 24 batches of Shuang-huang-lian injection. The result showed that there was a great variation among different samples. Principal component analysis further proved considerable variations among the samples from eight manufacturers, and suggested that chlorogenic acid and baicalin might have the greatest variations among the 24 batches. In conclusion, these results demonstrated that the method proposed was very useful for the analysis and quality consistency evaluation of Shuang-huang-lian injection.

Introduction

Shuang-huang-lian injection is a traditional Chinese medicine used as intravenous injection, which is extracted from three species of Chinese crude plants such as Lonicera japonica, Scutellaria baicalensis Georgi, and Forsythia suspensa vahl after a series of protocols such as extraction, purification, spraying and sterilized packing (1). It possessed remarkable anti-bacterial, anti-viral and immunostimulant activity. On the basis of previous research on the individual herbs, organic acids in L. japonica (5–7), flavonoids in S. baicalensis Georgi (8) and phenylethanoid glycosides in F. suspensa vahl (9–12) were considered to be the active components of Shuang-huang-lian injection. In China, pharmacopoeia, chlorogenic acid, baicalein and forsythin have been used as the chemical markers for the quality control of Shuang-huang-lian injection (1).

Although the major components of Shuang-huang-lian injection have been identified, no reliable analytical method is available so far. A number of analytical methods have been reported for the quantification of chlorogenic acid, caffeic acid, baicalin and forsythsin (13–16) in raw materials, Chinese traditional medicine preparations and other bio-samples of Shuang-huang-lian injection (17, 18). Among them, HPLC is the most widely used method. However, previous reports focused mainly on single or a few marker constituents, including chlorogenic acid, baicalein and forsythsin. None of these methods was developed with a view to determining multiple active components simultaneously (19, 20). Chlorogenic acid, baicalein and forsythsin are not characteristic components of Shuang-huang-lian injection, as they are also present in many other medicinal materials and their preparations with high contents (21–24). These compounds could not be responsible for the overall pharmacological activities of Shuang-huang-lian injection. Consequently, we believe that determination of more components such as caffeic acid, 4,5-dicaffeoylquinic acid, baicalein, wogonin in Shuang-huang-lian injection would be a better strategy for the comprehensive quality control study of this medicine, because it is widely accepted that therapeutic effect of traditional Chinese medicine (TCM) is usually based on multiple essential components or their combination rather than any single component (25). Considering the above problems, it is absolutely necessary to develop a satisfactory method for simultaneous detection of as many active compounds in Shuang-huang-lian injection as possible to ensure its effectiveness and safety. In this paper, we reported, for the first time, the development of an HPLC method for simultaneous determination of chlorogenic acid, caffeic acid, 4,5-dicaffeoylquinic acid, baicalin, baicalein and wogonin in Shuang-huang-lian injection. The aims of this study were to assess the quality consistency of Shuang-huang-lian injection from eight manufacturers.

Principal component analysis (PCA) is a statistical approach to facilitate an understanding into the causes and effects behind the relationships among multivariate datasets: the score of this method is to generate new principal components (PCs) that are independent of the original variables but shows linear combinations of them, and simultaneously capture most features of the original data. Each PC consists of a set of values called “scores” defining the position of each sample in the new coordinate space, and the other set of values called “loadings” that give the relative contributions of each variable in calculating the scores. Recently, to achieve pattern recognition using PCA, more attention has been paid to data analysis methods in entire chromatogram (26–28), it interprets the chemometric-derived model more directly.

In the present study, a simple and facile HPLC method with diode array detection was developed for simultaneous analysis of six compounds. To assess the quality consistency of Shuang-huang-lian injection from eight manufactures, classification of samples was done based on the contents of active components, using PCA. It is equally applicable to other herbal medicines based on the contents of active components in quality assessment for the purpose of quality control.

Experimental

Reagents and materials

HPLC was performed on an LC-2010A/SPDM series HPLC system (Shimadzu Corporation, Japan), HPLC grade methanol
was purchased from Merck Company (Merck, Darmstadt, Germany), deionized water was prepared using a Milli-Q system (Millipore, MA, USA). Standards of chlorogenic acid, caffeic acid, baicalin, baicalein and wogonin were obtained from the National Institute for Food and Drug Control (Beijing, China). The standard of 4,5-dicaffeoylquinic acid was purchased from Nanchang Beta Biotechnology Co., Ltd. The purities of all compounds were >98%, and their structures are shown in Figure 1. Twenty-four batches of Shuang-huang-lian injection were collected from eight pharmaceutical companies in China, named A, B, C, D, E, F, G and H, respectively. The intermediates of *L. japonica*, *S. baikalensis* Georgi and *F. suspense* vahl were supplied by manufacturer A and used for the preparation of blank samples without *L. japonica*, *S. baikalensis* Georgi, and *F. suspense* vahl. The samples were deposited at the National Institute for Food and Drug Control (Beijing, China). The samples were then the subject of further qualitative and quantitative analysis.

**Preparation of mixed standard solution and sample solution**

Methanol stock solution of mixed standard was prepared, containing chlorogenic acid (1, 0.3680 mg/mL), caffeic acid (2, 0.2512 mg/mL), 4,5-dicaffeoylquinic acid (3, 0.3032 mg/mL), baicalin (4, 0.5452 mg/mL), baicalein (5, 0.3812 mg/mL) and wogonin (6, 0.0838 mg/mL). The flask was sealed by Parafilm (Parafilm, Chicago, IL, USA). The stock solution was further diluted to make working solution. All the solutions were stored in the refrigerator at 4°C before HPLC analysis. Shuang-huang-lian injections were injected directly for HPLC analysis after filtering through a 0.45 μm nylon membrane. Blank samples (test samples without *L. japonica*, *S. baikalensis* Georgi, and *F. suspense* vahl) were prepared, respectively, by mixing other two intermediates according to the formula.

**HPLC conditions**

All analyses were performed on an LC-2010A/SPDM series HPLC system (Shimadzu Corporation, Japan) equipped with a binary solvent manager, sample manager, column compartment, photo diode array detector and LC-Solution software. The separation was performed on an Agilent TC-C18 column (5 μm, 4.6 × 250 mm). The detection wavelength was set at 350 nm. The mobile phase consisted of methanol (A) and 0.25% acetic acid (B). The linear gradient was as follows: 0–15 min, 15–35% A; 15–20 min, 35% A; 20–50 min, 35–100% A, at a flow rate of 1.0 mL/min. The column temperature was maintained at 35°C and the injection volume was 10 μL.

**Results**

**Optimization of method**

To obtain chromatograms with better resolution of adjacent peaks, the chromatographic conditions were optimized. The Agilent TC-C18 column could achieve a better separation than the Illite C18. The detection wavelength was selected as 350 nm, because all the analytes showed a maximum UV absorption around this wavelength. A series of mobile phases, including methanol–water in combination with acetic acid, phosphoric acid or ammonium acetate, were examined. The results showed that methanol–acetic acid is better than other systems, because

![Chemical structures of the six investigated compounds.](https://academic.oup.com/chromsci/article-abstract/52/7/707/523613/708)
these mobile phase additives resulted in an unsteady baseline and the improvement in the resolution was insignificant. Additionally, different linear gradient profiles were applied to improve the separation of Shuang-huang-lian injection by varying the solvent strength during the elution process and the optimum gradient was finally picked through a large number of empirical attempts.

In this experiment, differences in the separations were observed by changing the temperature of the column compartment over 25, 30, 35 and 40°C. The separation of baicalin, baicalein and wogonin was better at lower temperature, whereas the resolution between organic acid and its adjacent peaks was improved at higher temperature. In such cases, it may be preferable to choose 35°C in view of the overall resolution of Shuang-huang-lian injection. Representative chromatograms of reference standard and a typical sample at the conditions described above are shown in Figure 2. The chromatographic peaks were identified by comparing their retention times and UV spectra with those of each reference compound. In addition, spiking samples with reference compounds showed no additional peaks, which further confirmed the identities of the analyte peaks.

Method validation

The linearity, precision, recovery, robustness, limit of detection (LOD) and limit of quantity (LOQ) of the proposed method were evaluated. Calibration curves of chlorogenic acid, caffeic acid, 4,5-dicaffeoylquinic acid, baicalin, baicalein and wogonin were established by plotting the peak area ratios against five different concentrations of the individual analyte. Detector response was linear with $r^2$ between 0.9997 and 0.9999 in ranges of 18.4–441.6 ng/mL for chlorogenic acid, 1.3–30.1 ng/mL for caffeic acid, 1.5–56.4 ng/mL for 4,5-dicaffeoylquinic acid, 10.9–261.6 ng/mL for baicalin, 1.9–45.1 ng/mL for baicalein, 0.2–5.0 ng/mL for wogonin and the residual of the regression line (calculated from the residual standard deviation) ranged from 0.05 to 2.13%, and $t$-test for data were calculated by Microsoft Office Excel (version 2003), the $t$-value varied from 0.94 to 0.98 ($P > 0.05$). The LODs were <0.7 ng/mL, and the LOQs were >1.8 ng/mL (RSDs of $<1.9\%$) (Table I).

Method precision, calculated from the relative standard deviation (RSDs) of peak area ratios, was determined from an injection repeatability ($n = 6$) [with-day ($n = 6$) and between-day ($n = 6$)] assay of the three different concentrations of the working standard solutions. Injection, within-day and between-day precision showed RSDs of $<2.9\%$ for the peak area ratios, respectively (Table II).

Recovery of the method was estimated by spiking different concentrations of chlorogenic acid, caffeic acid, 4,5-dicaffeoylquinic acid, baicalin, baicalein and wogonin into the sample solutions ($n = 3$). The mean recoveries of chlorogenic acid, caffeic acid, 4,5-dicaffeoylquinic acid, baicalin, baicalein and wogonin were 101.2, 100.5, 101.6, 99.7, 100.8 and 100.6%, respectively, with the

![Figure 2. Representative HPLC chromatograms of the standard mixture (A) and Shuang-huang-lian injection (B). 1 = chlorogenic acid; 2 = caffeic acid; 3 = 45-dicaffeoylquinic acid; 4 = baicalin; 5 = baicalein; 6 = wogonin.](https://academic.oup.com/chromsci/article-abstract/52/7/707/523613)
RSDs of <3.0% (Table II). The recovery data indicate that the sample matrices did not affect the quantitation of the investigated analyte in the samples.

Robustness of the proposed method was performed by keeping the chromatographic conditions constant with the following differences: (i) changing the mobile phase composition (0–15 min, 20–40% A; 15–20 min, 40% A; 20–50 min, 40%–100%); (ii) variation in the mobile phase pH (±0.1 pH unit); (iii) changing the flow rate (0.8 and 1.2 mL/min). The robustness data revealed that each peak area was not significantly affected despite the varied mobile phase composition, mobile phase pH and flow rate. The RSDs of these analytical parameters were between 0.50 and 2.42%. Thus, the obtained results indicate that the method is reasonably robust.

The validation data show that the method is appropriate for the analysis of the investigated biologically active organic acids and flavonoids in pharmaceutical formulations.

**Determination of 24 samples**

Twenty-four batches of Shuang-huang-lian injection from eight pharmaceutical manufacturers were tested, as shown in Table III.

**Discussion**

There are eight pharmaceutical manufactures that produce Shuang-huang-lian injection in China. However, this work showed that the chemical variations are large among samples. These variations would be sure to result in the differences in internal quality and pharmaceutical actions. Therefore, the exact comparison and discrimination of these samples were assurance of safety and efficacy of medication. To evaluate and discriminate these samples, PCA was performed based on the contents of six investigated analytes in the 24 tested samples.

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<th>Table II. Precision and Recovery Data</th>
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Without assuming any previous knowledge of sample class, PCA reduces the dimensionality of the original data set by analyzing the correlation among a large number of variables in terms of a small number of underlying factors (PCs) without losing much information. PCA always results in score plots that provide a visual determination of the similarities and dissimilarities among the samples with respect to their experimental variables. From the visualization of the data in a reduced dimensional space by this method, the samples can be separated and discriminated. The score plots of PC1 versus PC2 based on the contents of six components in these 24 samples were examined for the separation or cluster relating to different batches of Shuang-huang-lian injection.

The 24 batches of Shuang-huang-lian injection from eight pharmaceutical manufacturers were tested. As shown in Table III, there were remarkable differences among the compositions of the six bioactive compounds in different samples, the contents of six compounds varied with an RSD of 2.35–8.50%; this variation would be sure to result in the differences of internal quality and pharmaceutical actions. The data were further analyzed by PCA using Unscrambler 9.7 software, singular value decomposition (SVD) was introduced to extract the PCs. As presented in Figure 3, the scores plot (Figure 3A), where each coordinate represented a sample, showed the clear distribution of the 24 samples. From the scatter points, samples were mapped in the space spanned by the first two principle components PC1 and PC2; they could explain 80% of the variability. It was obvious that all samples were classified into three classes, which were just classified according to different factories. The results showed that a great variation existed among the samples from different factories. The differences may arise from the different sources of the crude drugs, the detailed parameters of manufacturing technology and so on. More details will be investigated. Samples produced by B, D, E, G and H were almost in the same
The results indicated that samples produced by these manufacturers resembled each other in chemical characteristic as a group. Furthermore, the loading plot for PCA (Figure 3B) indicated that chlorogenic acid and wogonin might have the greatest influence on the variation among the 24 samples. By controlling the contents of these two compounds, we could better control the internal quality of Shuang-huang-lian injection; moreover, this would be helpful for us to find out the possible reason and carry out corresponding measures.

**Conclusion**

The effective materials exerting efficacy in TCM are complicated, which may be compatible to or may antagonize with each other, and the difference in the effectiveness of the products from different supplier lies in the variations in the chemical constituents and...
their relative contents. Thus, determination of maximum number of compounds was a commonly used strategy for quality control. However, it is difficult to evaluate the overall effectiveness. Our study demonstrated the approach that simultaneous determination of bioactive compounds conjunction with PCA not only offered a powerful way to quality consistency evaluation of Shuang-huang-lian injection but also might optimize a formula in the process of production by quantitative analysis of some important constituents for the purpose of quality control.

Acknowledgment

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