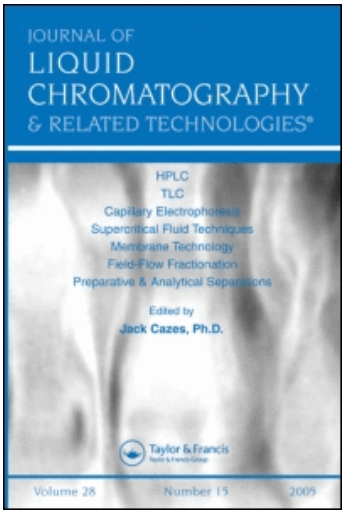

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Abstract: A high performance liquid chromatographic method was developed and applied to the simultaneous determination of puerarin and danshensu in a traditional Chinese medicinal preparation containing *Pueraria lobata* and *Salvia miltiorrhiza* as its major herbal constituents. Elution was performed on a C_{18} column at 27°C with acetonitrile–water (0.5% acetic acid) at different proportions according to a time scheduled programme, and pumped at a flow rate of 1.0 mL/min. Indomethacin was used as an internal standard. High resolution was obtained between the analytes and the internal standard. The intra-day and inter-day precisions were better than 1.9%

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and 2.2%, respectively. The coefficient of determination for each analyte was above 0.997. It is a simple and sensitive analytical method with good accuracy and reproducibility.

Keywords: Puerarin, Danshensu, *Pueraria lobata*, *Salvia miltiorrhiza*, HPLC

INTRODUCTION

Traditional Chinese medicinal preparations are prepared with several natural Chinese herbs based on the philosophy of traditional Chinese medicine, which is a complete system of healing that has been used in China for about 3000 years.[1] Since China was exposed to western influence in the early 1900s, treatment of diseases in China has been shifted more to orthodox medicine.[2] Some of the diseases diagnoses and treatments were replaced by orthodox medicine. Traditional Chinese preparations remains widely use amongst Chinese communities, for the good therapeutic efficacy and the absence of severe side effects. In fact, they are becoming more and more popular throughout the world.[3]

Pueraria lobata (Gegen) and *Salvia miltiorrhiza* (Danshen) are two traditional Chinese medicinal herbs that are widely used in China to promote health and cure cardiovascular diseases. Their therapeutic value, including reduction of blood pressure, lipid lowering effects, anti-oxidant, and improvement of the microvascular circulation, is proven in ancient Chinese literature and current pharmacological studies.[4–6] Therefore, many newly developed traditional Chinese medicinal preparations and health products use Gegen and Danshen as their major herbal components.

Studies on the chemical compositions of both Gegen[7,8] and Danshen[9–11] have been reported extensively in literature, revealing a number of active constituents for each herb. Amongst those constituents, puerarin in Gegen and danshensu in Danshan have been demonstrated to have significant cardiovascular protective effects (see Figure 1 for the chemical structures of puerarin

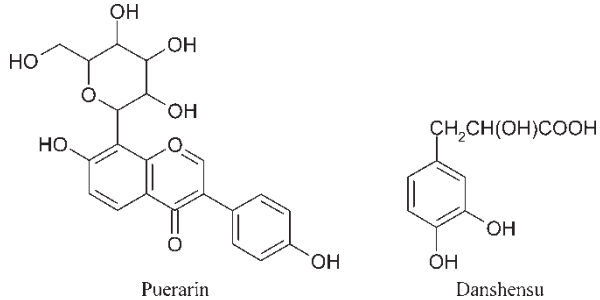

Figure 1. Chemical structures of puerarin and danshensu.

and danshensu). Puerarin has been reported to significantly dilate coronary arteries, protect ischemic brain tissue, alleviate myocardial injury, induce angiogenesis, reduce infarct area in the heart with myocardial infarction, as well as to improve microcirculation in both animals and patients.[12–18] Danshensu has also been shown to have multifold effects on cardiovascular diseases, including blood circulation promotion, coronary arteries dilation, platelet aggregation inhibition, endothelial cell protection, preventing myocardial hypertrophy, and protecting the myocardium form reperfusion injury.[10,19–21] Puerarin and danshensu not only have therapeutic effects, but also are the characteristic constituents of Gegen and Danshen, respectively. Therefore, quantitative analysis of these constituents is significant for the quality control of their related commercial traditional Chinese medicinal preparations. In spite of the wide application of both Gegen and Danshen in commercial traditional Chinese preparations and health products, only a few high performance liquid chromatographic methods have been reported for the simultaneous quantification of both marker constituents in related products. However, each had drawbacks limiting ease of use for their complicated sample preparation procedures and long analytical period.[22,23]

The present paper describes the development of a simple and sensitive high performance liquid chromatography (HPLC) method for the simultaneous determination of puerarin and danshensu. The simultaneous detection and determination of both analytes was facilitated and improved by the use of internal standard, namely indomethacin, and the use of a simple one step extraction. The developed method was validated for its application to analyze a commercial traditional Chinese preparation, Jingtongping tablet, in which the major herbal ingredients are Gegen and Danshen. In China, it has been used in a clinical setting for about a decade.

EXPERIMENTAL

Reagents and Chemicals

Puerarin and danshensu were obtained from Beijing Union Pharmaceutical Factory (Beijing, China) and National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), respectively. Indomethacin, an internal standard, was purchased from Sigma (MO, USA). Jingtongping tablets were provided by Peking University Third Hospital (Beijing, China). A specimen of the tablet was kept at the State Key Laboratory of Chinese Medicine and Molecular Pharmacology, Shenzhen, China. All organic solvents for chromatography (HPLC grade) were purchased from Merck, Germany. All other reagents are analytically pure. Distilled, deionized water was obtained in a PURELAB Option-R system (ELGA, UK).

Instrumentation

The chromatographic analysis was performed on an Agilent 1100 Series system (Agilent Technologies, Waldbronn, Germany) in conjunction with a quaternary pump, an autosampler, an autoelectronic degasser, a thermostated column compartment, a diode array multiple wavelength detector, and a computer with a ChemStation software program for analysis of the HPLC data. The separation was carried out on a Baseline C_{18} column (4.6 mm $\times$ 250 mm i.d., 5.0 μm particle size).

Chromatographic Conditions

A gradient mobile phase consisting of A (acetonitrile) and B (0.5% acetic acid) was used to run the separation. The elution program was set as follows: 7% A and 93% B for 0–9 min, 13% A and 87% B for 9–18 min, 73% A and 17% B for 18–30 min. The mobile phase was degassed automatically using the electronic degasser system. The flow rate was set at 1 mL/min, the column temperature at 27°C and the wavelength of a UV detector at 280 nm. An auto sampler was utilized for sample injection with injection volume of 20 μL.

Calibration Curves

The stock solutions of puerarin (1 mg/mL) and indomethacin (1 mg/mL) were prepared in methanol, while danshensu (0.5 mg/mL) was dissolved in distilled, deionized water. They were stored at -20°C for less than a month. Working solutions were prepared daily in their corresponding solvents by appropriate dilutions of the stock solutions. Standard samples contained 12.5, 25, 50, 75, 100 μg/mL puerarin and danshensu. Indomethacin, an internal standard, was added to the individual standard sample to give the final concentration of indomethacin, which is equal to 10 μg/mL. Each sample was analyzed in triplicate. Standard curves were established by determining the peak area ratios (puerarin to indomethacin, danshensu to indomethacin) of the HPLC chromatograms.

Repeatability

The precision of the chromatographic determination for the developed method was determined following the method described above.[24,25] It was expressed as a relative standard deviation (RSD) and was calculated by triplicate injections of both puerarin and danshensu (25, 50, 75 μg/mL in methanol) (intra-day and inter-day). The standard sample was prepared and analyzed within

24 hrs for intra-assay precision. The inter-assay precision was determined using three independent experiments in different days. For each calibration curve, the calibration concentrations were back calculated from their related peak area ratio (analyte to internal standard). The deviation from the nominal concentration was defined as accuracy.

Sample Preparation

The powder of Jingtongping tablet (0.1 g) was extracted with 40 mL methanol for 30 min at room temperature in an ultrasonic bath, and to each sample was added indomethacin in the ultimate concentration of 10 μg/mL as the internal standard. The contents in the flask were filtered through Whatman #1 filter paper (Whatman Co., UK). One milliliter of the resulting solution was filtered through 0.45 μm PVDF syringe filter (Gelman Co., USA) and was used as the sample for HPLC analysis.

Recovery

The recovery was evaluated by adding known amounts (200 μg) of individual standards into an accurately weighed powder of Jingtongping tablet (0.1 g). The mixture was extracted and analyzed using the method stated above. Extractions of the tablet powder with and without prior addition of each standard compound were both conducted in triplicate. The extraction recoveries of each compound were calculated as:

$$\text{Recovery } (\%) = 100 \times (\text{amount found} - \text{original amount})/\text{amount spiked}$$

RESULTS AND DISCUSSION

Calibration Curves

Based on the concentrations of puerarin and danshensu present in the pharmaceutical product, the calibration range was constructed through consideration of the practical range. The linearity of the current analytical method for determination of puerarin and danshensu was evaluated by analyzing a series of different concentrations of each compound. In this study, five concentrations were chosen, ranging from 12.5 μg/mL to 100 μg/mL for both compounds. Each concentration was repeated three times. This approach provides information on the variation in peak area between samples of the same concentration. The linearity of the calibration graphs was validated by the high value of the coefficient of determination (r^2) and the intercept value that was not statistically ($P < 0.05$) different from zero (Figure 2). The standard

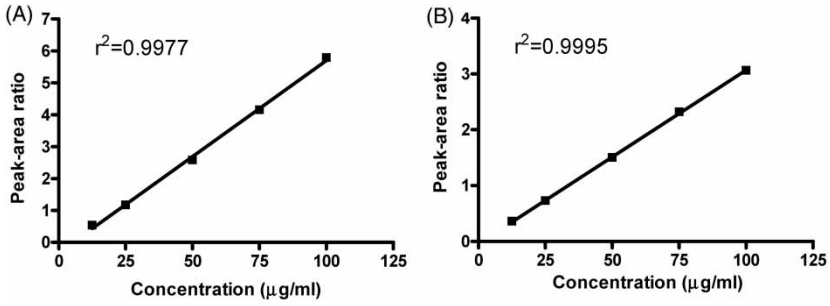

Figure 2. Calibration curves of puerarin (A) and danshensu (B).

curve of puerarin is $y = 0.0602x - 0.3158$, $r^2 = 0.9977$; that of danshensu is $y = 0.0311x - 0.0388$, $r^2 = 0.9995$; where y is the peak area ratio, x is the concentration in μg/mL. The results showed that good linearity was achieved over the ranges for the analysis.

Table 1. Intra- and inter-day precision and accuracy of danshensu and puerarin

Concentration added (μg/mL)	Concentration measured (μg/mL) mean ± S.D.[a]	RSD (%)[b]	Accuracy (%)[c]
Intra-day precision			
Danshensu			
25	24.67 ± 0.24	0.99	98.68
50	49.51 ± 0.15	0.30	99.02
75	75.76 ± 0.24	0.31	101.01
Puerarin			
25	24.70 ± 0.11	0.43	98.80
50	48.01 ± 0.90	1.88	96.02
75	74.23 ± 0.19	0.25	98.97
Inter-day precision			
Danshensu			
25	24.74 ± 0.35	1.42	98.96
50	48.45 ± 1.05	2.18	96.90
75	75.87 ± 0.37	0.48	101.16
Puerarin			
25	24.86 ± 0.32	1.27	99.44
50	48.31 ± 0.78	1.62	96.62
75	74.63 ± 0.63	0.84	99.51

[a]Mean average of three determination, S.D. means standard deviation.

[b]RSD (%) (relative standard deviation) = (S.D./mean) × 100.

[c]Accuracy (%) = [1 − (mean concentration measured − concentration spiked)/concentration spiked] × 100.

Precision and Accuracy

The reproducibility of the method was examined by analyzing three standard solutions in three consecutive days and calculating the RSD and accuracy. The intra-day precision was lower than 1.88%, and the accuracy ranged from 96.02% to 101.01%. The inter-day precision ranged from 0.48% to 2.18%, and the accuracy from 96.90% to 101.16% (Table 1). In addition, the data for each concentration level were evaluated by one way analysis of variance. There is no statistically significant difference between the mean results obtained from one level of day to another at the 95% confidence level. It indicated that the method is reliable and accurate.

Recovery

The extraction recoveries of the two analytes (n = 3) from spiked tablet powder were satisfactory. The recovery of puerarin was 98.82% with RSD of 1.75% (n = 3); while that of danshensu was 99.86% with RSD of 1.63%

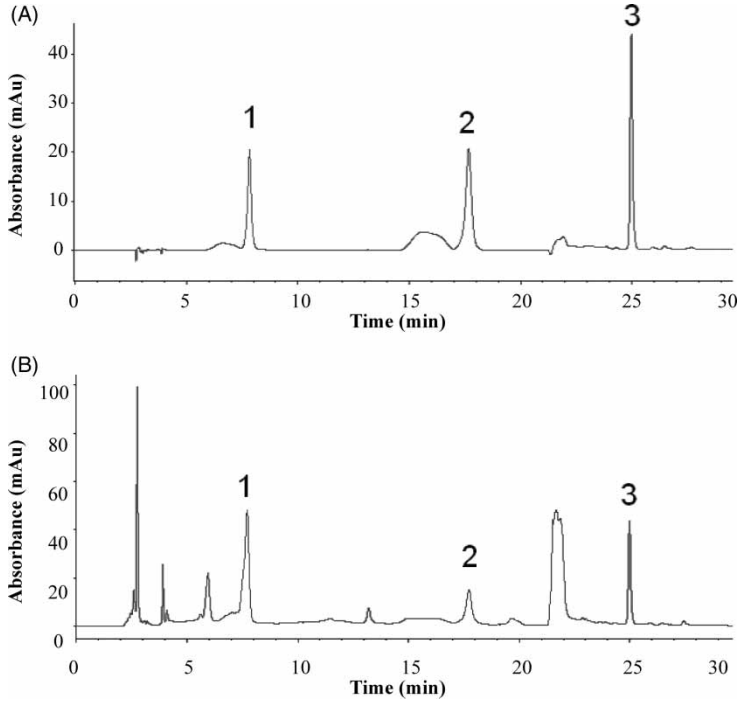

Figure 3. Typical HPLC chromatograms of standard mixture (A) and Jintongping tablet extract (B); danshensu (1), puerarin (2), and indomethacin (3).

(n = 3). The high recoveries for both analytes and the low RSD suggested that the developed method was highly reliable and consistent.

Application of the Method

The validated HPLC-UV method has been successfully performed for the simultaneous detection of puerarin and danshensu in Jingtongping tablet. The detector wavelength was set at 280 nm, and there was no interference for the two components and the internal standard, indomethacin. The retention time of danshensu, puerarin, and indomethacin were 7.89, 17.70, and 24.98 min, respectively. The representative chromatograms are shown in Figure 3. The content of danshensu and puerarin in one gram of Jingtongping tablet was 32.08 mg (RSD = 2.09%) and 6.61 mg (RSD = 1.36%), respectively.

CONCLUSIONS

This developed method is a simple and selective analytical method with good accuracy and reproducibility for the simultaneous determination of two active components (puerarin and danshensu) in Jingtongping tablet. This HPLC assay can be readily utilized as a suitable quality control method for the determination of puerarin and danshensu in Jingtongping tablet and other drugs, with puerarin and danshensu as their major active ingredients. For the prominent cardiovascular protective effects of health product preparations with both Gegen and Danshen,[5] more and more related health products would emerge in the worldwide market, especially in China. Therefore, having a simple and sensitive quality control method based on major marker ingredients is indispensable. The current developed method is, undoubtedly, a good starting point for the development of quality control methods on other commercial traditional Chinese medicinal preparations and health products with Gegen and Danshen as their major herbal components.

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