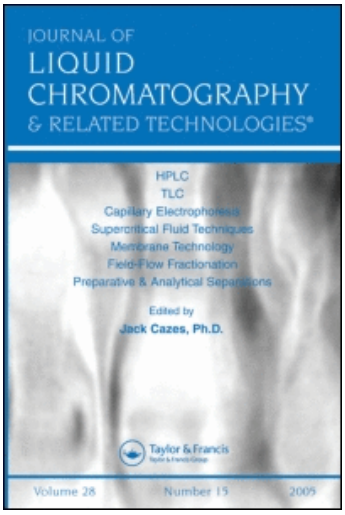

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DETERMINATION OF FIVE BIOACTIVE CONSTITUENTS IN TRADITIONAL CHINESE MEDICINAL PREPARATION SHANDANSHAOYAO TANG BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A reversed phase high performance liquid chromatographic method for the determination of danshensu, protocatechuic acid, protocatechualdehyde, (+)-catechin, and paeoniflorin in traditional Chinese medicinal preparation Shandanshaoyao Tang was established. Danshensu, protocatechuic acid, protocatechualdehyde, and (+)-catechin were separated on a Diamonsil C18 column (250 mm × 4.6 mm I.D.) with a mobile phase consisting of methanol-acetic acid-water (14: 0.2: 86, v/v), and detected by UV at 280 nm.

Paeoniflorin was separated using the same column but with a mobile phase consisting of methanol-acetic acid-water (25: 0.2:

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75, v/v), and detected by UV at 230 nm. The linear calibration ranges were 24.1-241.0 μg/mL, 6.1-61.0 μg/mL, 2.5-25.4 μg/mL, 8.3-83.0 μg/mL and 224-2235 μg/mL for danshensu, protocatechuic acid, protocatechualdehyde, (+)-catechin, and paeoniflorin, respectively.

The relative standard deviations (n=5) and average recoveries (n=5) were 1.45% and 91.4% for danshensu, 1.54% and 100.1% for protocatechuic acid, 0.89% and 95.3% for protocatechualdehyde, 1.78% and 93.6% for (+)-catechin, and 0.68% and 95.3% for paeoniflorin, respectively.

The contents of these five bioactive constituents in Shandanshaoyao Tang were successfully determined by the proposed method.

INTRODUCTION

Traditional Chinese medicinal preparations (TCMP) have been used in eastern Asian countries for centuries and widely adopted for clinical use. It is necessary to establish suitable assay methods to evaluate or control the quality of TCMP. However, due to the complicated components and limited knowledge of the effective compositions of TCMP, the determination of bioactive constituents in TCMP is very difficult. Recently, thin layer chromatography (TLC), high performance liquid chromatography (HPLC),[1-5] and capillary electrophoresis (CE)[6-14] have been widely used to separate and determine the bioactive constituents in TCMP.

Shandanshaoyao Tang is a kind of TCMP used frequently for the treatment of hyperlipidemic symptoms in southeastern China, and is composed of three crude herbs, i.e., Fructus Crataegi, Radix Salviae Miltiorrhizae, and Radix Paeoniae Rubra. In the previous work, we have reported some of the pharmacological actions of Shandanshaoyao Tang.[15] The results show that Shandanshaoyao Tang had significant blood lipid regulation effects on the experimental hyperlipidemic rats. We also studied the chemical constituents of Shandanshaoyao Tang and fifteen compounds were isolated and identified from the water decoction of Shandanshaoyao Tang.[16]

In this study, five major bioactive constituents, i.e., danshensu, protocatechuic acid and protocatechualdehyde in Radix Salviae Miltiorrhizae, (+)-catechin in Fructus Crataegi, and paeoniflorin in Radix Paeoniae Rubra, were selected as the marker constituents of Shandanshaoyao Tang and analyzed by HPLC. The chemical structures of these five compounds are shown in Figure 1.

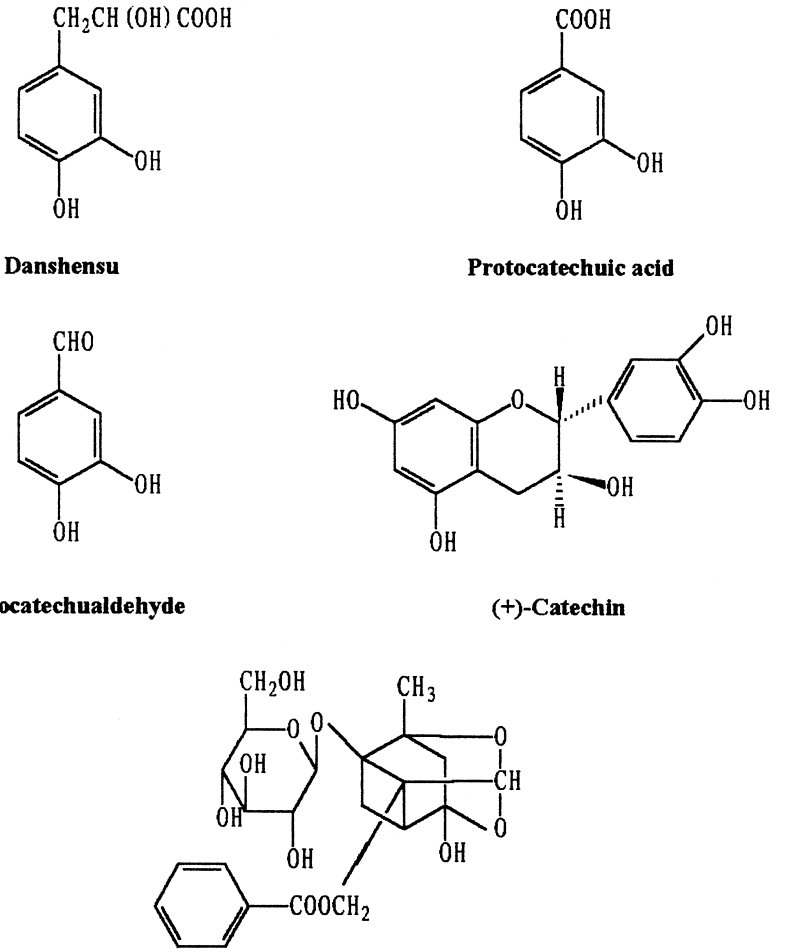

Figure 1. Chemical structures of the five marker constituents.

EXPERIMENTAL

Apparatus

All analyses were performed on a Waters Associates liquid chromatograph (Milford, MA, USA) equipped with a Model 7125 injection valve (Rheodyne,

Cotati, CA, USA) and a Model 510 solvent delivery system connected to a Model SPD-2A spectrophotometric detector (Shimadzu, Kyoto, Japan).

The chromatographic data were recorded and processed with a Model C-R6A chromatographic data system (Shimadzu, Kyoto, Japan). The analytical column was a Diamonsil C18 column (250 mm × 4.6 mm I.D., Dikma, Beijing, China).

Materials and Reagents

Three crude drugs used to prepare Shandanshaoyao Tang were purchased from Tongrentang Pharmaceutical Group Company (Beijing, China) and identified as *Crataegus pinnatifida* Bge.*var.major* N.E.Br. (Shanlihong), *Salvia miltiorrhiza* Bge. (Danshen), and *Paeonia lactiflora* Pall. (Chishao), respectively.

Danshensu was isolated from Radix Salviae Miltiorrhizae and identified by UV, IR, and MS.[16] Protocatechuic acid, protocatechualdehyde, and paeoniflorin were purchased from National Institute for Control of Pharmaceuticals and Biological Products (Beijing, China). (+)-Catechin was obtained from Sigma (St. Louis, MO, USA). Methanol (HPLC grade) was bought from Beijing Chemical Factory (Beijing, China). All other chemicals used were of analytical grade. Deionized and redistilled water was used to prepare all the solutions.

Standard Solutions

Stock solutions of the marker constituents with various concentrations were respectively prepared with 75% ethanol, except danshensu, which was directly prepared using water because it is more stable in water than in an alcoholic solution. Working solutions containing each of the five compounds were prepared by diluting the stock solutions with 75% ethanol.

Sample Preparation

Amounts of individual crude drugs equivalent to a daily dose of Shandanshaoyao Tang (Fructus Crataegi, 10.0 g, Radix Salviae Miltiorrhizae, 7.5 g, and Radix Paeoniae Rubra, 5.0 g) were respectively weighed and cut into thin slices. A ten-fold weight of water was added to the mixture of these three crude drug slices and boiled at 100°C for 2 h. The aqueous solution was filtered while hot. The procedure was repeated three times. The filtrates were combined and then lyophilized into powder.

A 0.500 g sample of the lyophilized powder was accurately weighed and extracted at room temperature with 3 mL of 75% ethanol for 10 min in an ultrasonic bath. The extract was centrifuged at a speed of 4000 rpm (ca. 1600 g) for

10 min. The extraction was repeated three times. The supernatants, after centrifugation, were combined and diluted to 10 mL with 75% ethanol. This solution was passed through a 0.2 μm membrane filter and 10 μL of the filtrate was then injected into the HPLC system for analysis.

RESULTS AND DISCUSSIONS

Chromatographic Conditions

For the determination of danshensu, protocatechuic acid, protocatechualdehyde, and (+)-catechin, the mobile phase was selected to be methanol-acetic acid-water (14: 0.2: 86, v/v). However, in this condition, the retention time of paeoniflorin is very long (longer than 120 min), so a mobile phase containing methanol-acetic acid-water (25: 0.2: 75, v/v) was chosen for the analysis of paeoniflorin.

The detection wavelength was chosen at 280 nm for the measurement of danshensu, protocatechuic acid, protocatechualdehyde, and (+)-catechin, because

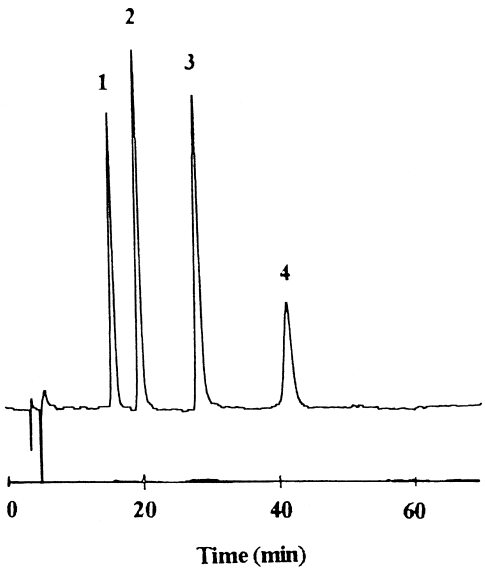

Figure 2. HPLC chromatogram of danshensu, protocatechuic acid, protocatechualdehyde, and (+)-catechin. Separation conditions: column, Diamonsil C18 , 5 μm (250 mm × 4.6 mm I.D.); temperature, 25°C; mobile phase, MeOH-HAc-H_2O (14: 0.2: 86, v/v); flow rate, 0.8 mL/min; detection wavelength, 280 nm. Peaks: **1**, danshensu; **2**, protocatechuic acid; **3**, protocatechualdehyde; **4**, (+)-catechin.

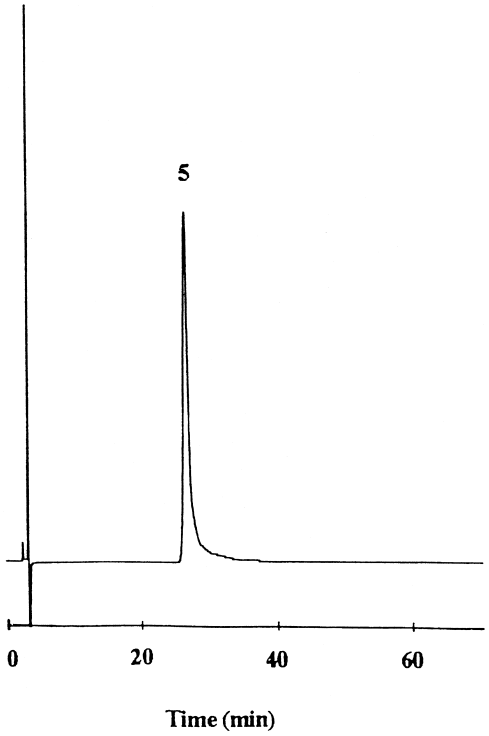

Figure 3. HPLC chromatogram of paeoniflorin. Separation conditions: mobile phase, MeOH-HAc-H_2O (25: 0.2: 75, v/v); flow rate, 1.2 mL/min; detection wavelength, 230 nm; others as in Figure 2. Peak: **5**, paeoniflorin.

the absorbencies of these four compounds are high at this wavelength. For the analysis of paeoniflorin, the detection wavelength was selected at 230 nm. The flow rates were 0.8 mL/min for danshensu, protocatechuic acid, protocatechualdehyde, and (+)-catechin, and 1.2 mL/min for paeoniflorin. The column temperatures for all analyses were kept at 25°C.

The standard HPLC chromatograms are shown in Figure 2 for danshensu, protocatechuic acid, protocatechualdehyde, and (+)-catechin and in Figure 3 for paeoniflorin, respectively.

Calibration Graphs

All calibration graphs were plotted based on linear regression analysis of the integrated peak areas (integration units, Y) versus concentrations (μg/mL, X)

Table 1. HPLC Data for the Calibration Graphs

Compound	Regression Equation	Correlation Coefficient (μg/mL)	Linear Range
Danshensu	Y=1490X-1254	0.9997	24.1-241.0
Protocatechuic acid	Y=3657X-112	0.9996	6.1-61.0
Protocatechualdehyde	Y=10000X-183	0.9992	2.5-25.4
(+)-Catechin	Y=1793X-2787	0.9990	8.3-83.0
Paeoniflorin	Y=2207X-47378	0.9989	224-2235

of the five marker constituents in the standard solution. Each standard solution was analyzed three times. The regression equations, correlation coefficients, and linear ranges for the analysis of the five bioactive constituents are shown in Table 1.

System Suitability Test

The results of reproducibilities (RSD, n=5) of the proposed method, on the basis of integrated peak areas of the standard solutions, were 1.45%, 1.54%, 0.89%, 1.78%, and 0.68% for danshensu, protocatechuic acid, protocatechualdehyde, (+)-catechin, and paeoniflorin, respectively. Different amounts of the five standards were accurately weighed and added into the actual samples of known contents. The mixtures were extracted and analyzed following the proposed procedures. The results of standard addition recovery studies are given in Table 2.

Table 2. Analytical Results of Recoveries (n=5)

Compound	Added (g)	Found (g)	Recovery (%)
Danshensu	0.482	0.441	91.4
Protocatechuic acid	0.244	0.244	100.1
Protocatechualdehyde	0.102	0.097	95.3
(+)-Catechin	0.332	0.311	93.6
Paeoniflorin	3.58	3.41	95.3

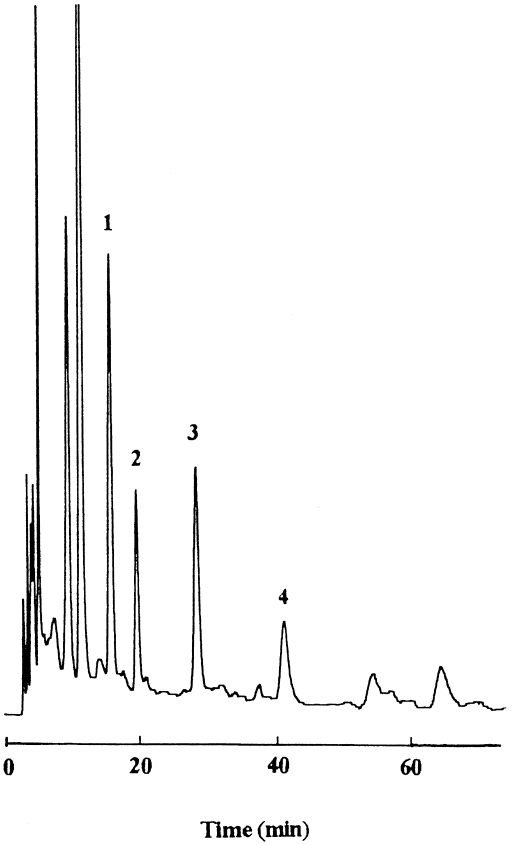

Figure 4. HPLC chromatogram of Shandanshaoyao Tang for the determination of danshensu, protocatechuic acid, protocatechualdehyde, and (+)-catechin. Separation conditions and peaks as in Figure 2.

Sample Analysis

When the sample solutions were analyzed by HPLC under the selected chromatographic conditions, the peaks were identified by comparison of the retention times with those obtained from standard solutions of the five marker constituents. Typical HPLC chromatograms of the actual samples are shown in Figures 4 and 5, respectively. The contents of the five bioactive constituents in Shandanshaoyao Tang are listed in Table 3. The results indicate that the proposed method is suitable for the determination of these five marker constituents in Shandanshaoyao Tang.

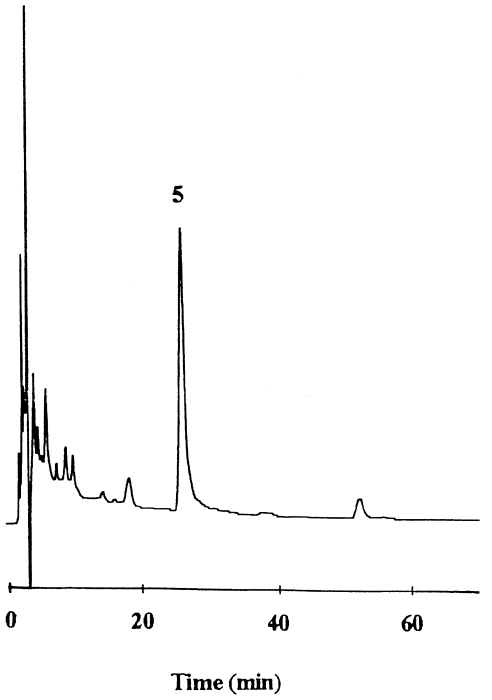

Figure 5. HPLC chromatogram of Shandanshaoyao Tang for the determination of paeoniflorin. Separatin conditions and peak as in Figure 3.

Table 3. Contents of the Five Bioactive Constituents in Shandanshaoyao Tang (n=5)

Compound	Content (mg/g)	RSD (%)
Danshensu	1.423	2.63
Protocatechuic acid	0.099	3.73
Protocatechualdehyde	0.096	4.35
(+)-Catechin	0.330	3.65
Paeoniflorin	10.89	1.96

CONCLUSION

TCMP is usually prepared by water decoction, so the bioactive constituents may be mostly contained in the polar fraction. The five marker constituents ana-

lyzed in this study were all the polar compositions of Shandanshaoyao Tang. With the established procedures, these five bioactive constituents in Shandanshaoyao Tang were successfully separated and determined. The proposed method can be used for the quality assessment and control of Shandanshaoyao Tang.

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