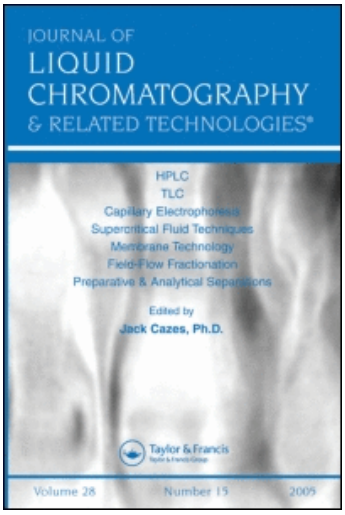

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Simultaneous Separation and Determination of Five Bioactive Components in Traditional Chinese Medicinal Formula, Guanxin II, by HPLC

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ABSTRACT

A reversed phase high performance liquid chromatographic method was established for the simultaneous determination of the bioactive constituents in traditional Chinese medicinal (TCM) formula Guanxin II. Danshensu, protocatechuic acid, protocatechualdehyde, paeoniflorin, and ferulic acid were successfully separated on an Agilent Zorbax extend-C_{18}narrow-bore column (150 × 2.1 mm i.d., 5 μm) with a guard column (12.5 × 2.1 mm i.d., 5 μm) packed with the same material at 25°C. The mobile phase was a mixture of methanol and 0.5% acetic acid

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employing gradient elution at a flow rate of 0.15 mL/min. Detection was accomplished with a diode-array detector and chromatograms were recorded at 230 nm, 262 nm, 280 nm, and 322 nm. The compounds were identified by comparing their retention times and UV spectra in the 200–400 nm range with authentic standards. Regression equations revealed good linear relationship (correlation coefficients: 0.99938–0.99996) between the peak areas of the constituents and their concentrations. The relative standard deviations ($n = 5$) of retention time and peak area were less than 0.73% and 1.13%, respectively. The average recoveries ($n = 3$) were between 97.57% and 100.68%. The proposed method has been successfully applied to the simultaneous determination of the above bioactive constituents in Guanxin II decoction.

INTRODUCTION

Traditional Chinese Medicine (TCM) has been widely adopted for clinical use in eastern Asian countries for over 2000 years. In the long history of its development, TCM has demonstrated its great vitality because of its firm clinical foundation, significant therapeutic effects, and specific system of theory based on clinical practice. Nowadays, more and more countries pay attention to TCM,[1,2] especially for dubious and complicated cases, such as cancer and cardiovascular diseases.[3]

Among many TCMs for the treatment of cardiovascular diseases, Guanxin II is a famous formula.[4] Due to its better performance and fewer side effects, as confirmed in long-time clinical use, Guanxin II is widely adopted for the treatment of coronary heart diseases and angina pectoris. Pharmacological studies reveal that Guanxin II has the clinical effects of promoting blood circulation to remove blood stasis, clearing away heat, relieving vexation, nourishing blood, tranquilizing the mind, and cooling the blood to relieve carbuncles, dilating coronary artery and increasing coronary flow.

Composed of five crude herbs, i.e., Radix Salviae Miltiorrhizae (Chinese name: Danshen), Radix Paeoniae Rubra (Chishao), Rhizoma Chuanxiong (Chuanxiong), Flos Carthami (Honghua), and Ligni Dalbergiae Odoriferae (Jiangxiang), Guanxin II is usually administered by water decoction, and the water-soluble constituents are the major part of the decoction. It is reported[5] that the water-soluble bioactive components in Salviae Miltiorrhizae are danshensu, protocatechuic acid, and protocatechualdehyde, while paeoniflorin and ferulic acid are the bioactive components for Radix Paeoniae Rubra and Rhizoma Chuanxiong, respectively. Accordingly, in this paper, danshensu, protocatechuic acid, protocatechualdehyde, paeoniflorin, and ferulic acid (their chemical structures are shown in Fig. 1) were selected as the marker constituents of Guanxin II. Although a number of assays have been reported

for the analysis of some of the above compounds in crude drugs and other traditional Chinese medicinal preparations,[6–10] they are not practical for the simultaneous determination of the five components in Guanxin II. So, for the therapeutic monitoring of Guanxin II and for the quality control of the manufacturing process of Guanxin II in the future, it is needed to develop an efficient method for the simultaneous determination of the five bioactive components.

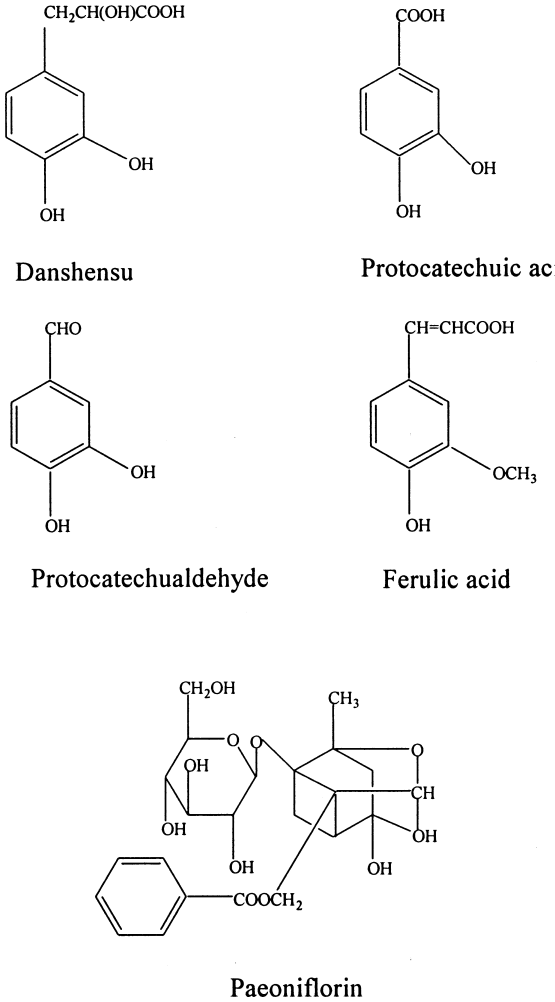

Figure 1. Chemical structures of the five bioactive components.

This paper describes, for the first time, the simultaneous determination of danshensu, protocatechuic acid, protocatechualdehyde, paeoniflorin, and ferulic acid in the water decoction of Guanxin II by HPLC. Additionally, comparisons were made among the HPLC chromatograms of Guanxin II decoction and single drug decoctions prepared by each of the above mentioned crude herbs.

EXPERIMENTAL

Materials and Reagents

Five crude drugs, i.e., Radix Salviae Miltiorrhizae, Radix Paeoniae Rubra, Rhizoma Chuanxiong, Flos Carthami, and Ligni Dalbergiae Odoriferae used to prepare Guanxin II decoction were purchased from Jiashitang pharmaceutical store (Beijing, China).

Authentic standards of danshensu, protocatechuic acid, protocatechualdehyde, paeoniflorin, and ferulic acid were purchased from National Institute for Control of Pharmaceuticals and Biological Products (Beijing, China). All other reagents were of analytical grade. Double-distilled water was used to prepare all the solutions.

Apparatus and Chromatographic Conditions

All analyses were performed on an HP1100 liquid chromatograph (Hewlett Packard, USA), which consisted of a quaternary pump, an on-line degasser, a column thermostat, a model 0497 injection valve (sample loop 20 μL) and a photodiode-array detector. The chromatographic data were recorded and processed with an HP chemstation software. The analytical column was an Agilent Zorbax extend-C_{18} (150 × 2.1 mm i.d., 5 μm) narrow-bore column, and before it, a guard column (12.5 × 2.1 mm i.d., 5 μm) with the same packing materials was used. The column temperature was controlled at 25°C.

The mobile phase was a mixture of methanol and 0.5% (v/v) Hac, employing gradient elution, as shown in Table 1. The flow rate was 0.15 mL/min. The column effluents were monitored simultaneously at 230 nm, 262 nm, 280 nm, and 322 nm. Injection volume was 5 μL.

Preparation of Standard Solutions

Standard stock solutions of danshensu, protocatechuic acid, and protocatechualdehyde were prepared in 1% (v/v) HAc, for these compounds are

Table 1. Gradient elution programme using mobile phase A and B.

Time (min)	Flow rate (mL/min)	Mobile phase A[a] (%)	Mobile phase B[b] (%)
0	0.15	8	92
10	0.15	8	92
20	0.15	25	75
50	0.15	25	75

[a]Methanol.
[b]0.5% Acetic acid–water solution.

more stable[11] in acidic solution and can be stored for at least one month at 4°C. Paeoniflorin was prepared in methanol and ferulic acid was prepared in methanol–1% HAc (1 : 1, v/v). Working standard solutions containing each of the five compounds were prepared by diluting the stock solutions with methanol–1% HAc (25 : 75, v/v).

Preparation of Sample Solutions

Guanxin II Decoction

Amounts of individual crude drugs (cut into thin slices) equivalent to a daily dose of Guanxin II (Radix Salviae Miltiorrhizae 30 g, Radix paeoniae Rubra 15 g, Rhizoma Chuanxiong 15 g, Flos Carthami 15 g, and Ligni Dalbergiae Odoriferae 15 g) were respectively weighed and all put into a 2500 mL beaker; a 10-fold weight of boiling water was added to the beaker and the above five crude drug slices were macerated in water for 30 min. Then the solution was heated and kept boiling for 30 min. The aqueous solution was filtered through five layers of gauze while hot. A 6-fold weight of water was added to the dregs and the above procedure was repeated. Then the filtrates were combined and allowed to cool to room temperature and adjusted to 500 mL by adding water. A 1.0 mL aliquot of the solution was removed and adjusted to 2 mL by adding water, the solution was then blended and centrifuged at a speed of 15,000 rpm/min for 5 min. The supernatant was passed through a 0.45 μm syringe filter, and 5 μL of the filtrate was injected into the HPLC system for analysis.

Single Crude Drug Decoction

Single-crude drug decoction of each crude drug equivalent to a daily dose was separately prepared according to the above procedure.

RESULTS AND DISCUSSION

Selection of the Mobile Phase

Employing an Agilent Zorbax extend-C_{18} narrow-bore column (150×2.1 mm i.d., 5 μm) with a same packing material guard column, various kinds of mobile phases were tested to achieve optimal separation of the five bioactive components in the complex matrix. It was found that pH and organic modifier concentration of the mobile phase substantially affected the retention behaviour of the five bioactive components. As danshensu, protocatechuic acid, and ferulic acid are ionizable weak acids, asymmetrical peaks were observed due to their disassociation equilibrium on the column. To solve this problem, both the ion-suppression chromatography and the ion-pair chromatography could be adopted. Considering the convenience of operation, an ion-suppression technique was used by adding HAc into the mobile phase. Employing methanol as mobile phase A and HAc–water solution as B, different concentrations of Hac (0.25%, 0.5%, 1.0%, 1.5%, and 2.0%) were tested for the elution of the compounds. As a result of the observation, we selected 0.5% HAc as optimal.

The chromatographic conditions were adjusted in order to provide an HPLC procedure capable of separating the five bioactive components of Guanxin II in one run. Accordingly, we examined the effect of methanol concentration on the retention of the bioactive components. As shown in Fig. 2, there is a wide range of capacity factors for the five compounds, and isocratic elution does not produce an appropriate separation of them. In order to elute the relatively strong retained peaks paeoniflorin and ferulic acid at proper retention time, and permit retention and resolution of the very early eluting peaks, danshensu and protocatechuic acid, a gradient elution programme has to be employed. After several series of preliminary experiments, a simple linear gradient-eluting programme for the simultaneous determination of the five bioactive components over a moderate timeframe was then generated (Table 1). In order to get reproducible retention time, prior to the next injection, the column was solvent conditioned by passing the initial solvent through the column until the baseline stabilized.

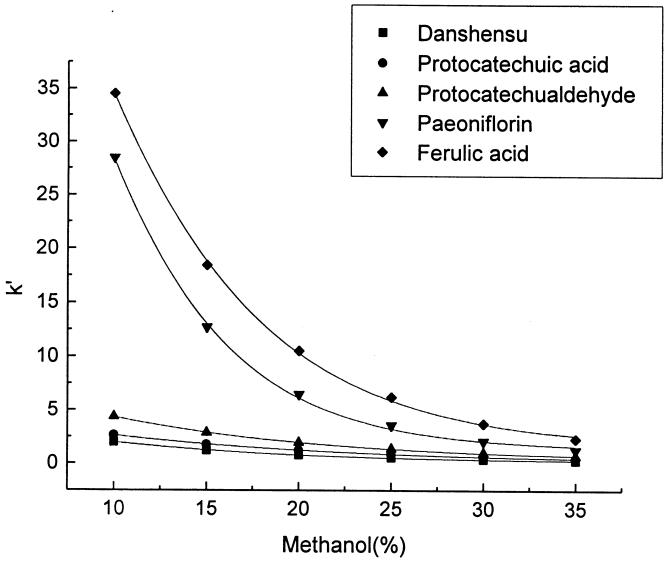

Figure 2. Effect of methanol content in the mobile phase on the retention of the compounds.

Selection of the Detection Wavelength and Identification of the Compounds

The on-line UV spectra of the five bioactive components are shown in Fig. 3. As maximally efficient detection can be obtained by selecting the wavelength where the component has the maximum absorption, in this study, four different detection wavelengths were set according to the maximum absorption of the components. Protocatechuic acid, paeoniflorin, and ferulic acid were detected at 262 nm, 230 nm, and 322 nm, respectively. As for danshensu and protocatechualdehyde, although they have larger absorbance at 230 nm, the peak areas could not be measured accurately due to the unstable baseline, therefore, 280 nm was selected for the determination of danshensu and protocatechualdehyde.

Photodiode-array detection was used in the experiment so that UV spectra of the bioactive constituents could be compared with those of the authentic standards. Identification of the five compounds was performed by characterizing the sample peak in terms of retention time and UV spectrum. The excellent agreement between standard and sample spectra found in all analyzed samples of Guanxin II indicates that, under the proposed analytical conditions,

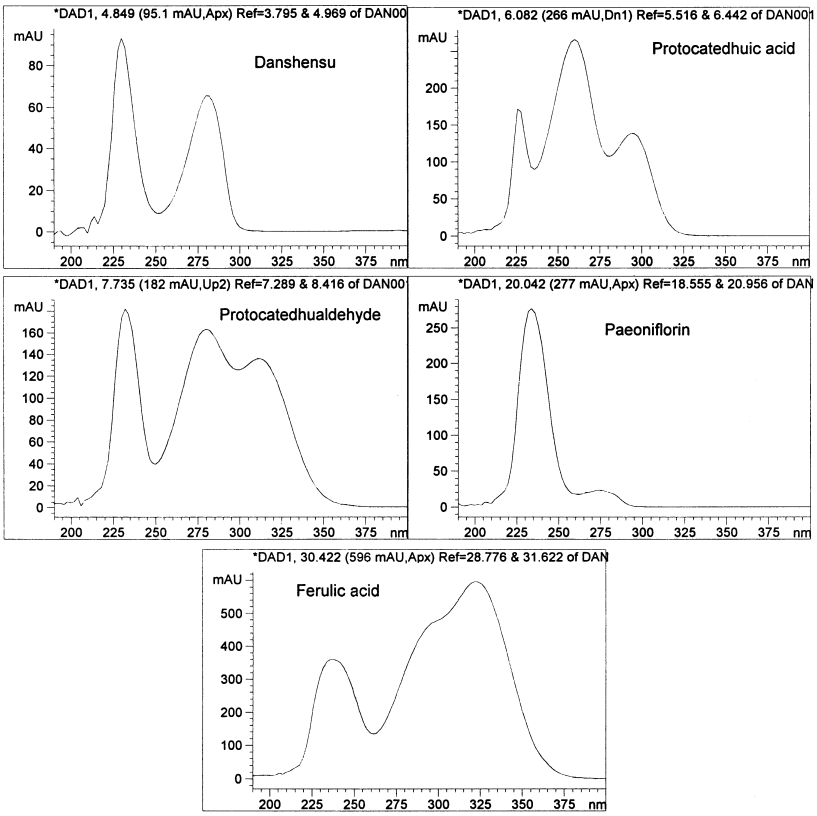

Figure 3. On-line UV spectra of the five bioactive components.

separation of the five bioactive components is not subjected to interference by other components in the matrix. Typical chromatograms of the standards and Guanxin II decoction recorded at different wavelengths are depicted in Fig. 4.

To ensure the specificity and selectivity of the method, and to compare the contents of bioactive components in single-drug decoction and Guanxin II decoction, we prepared single-drug decoction of each individual crude herb. The chromatograms recorded at 280 nm are shown in Fig. 5. It can be seen that no peak was detected at the specific retention time of the bioactive components in blank decoction prepared only by Flos Carthami or Ligni Dalbergiae Odoriferae. Therefore, the above conditions can be used for quantification of the bioactive components in Guanxin II.

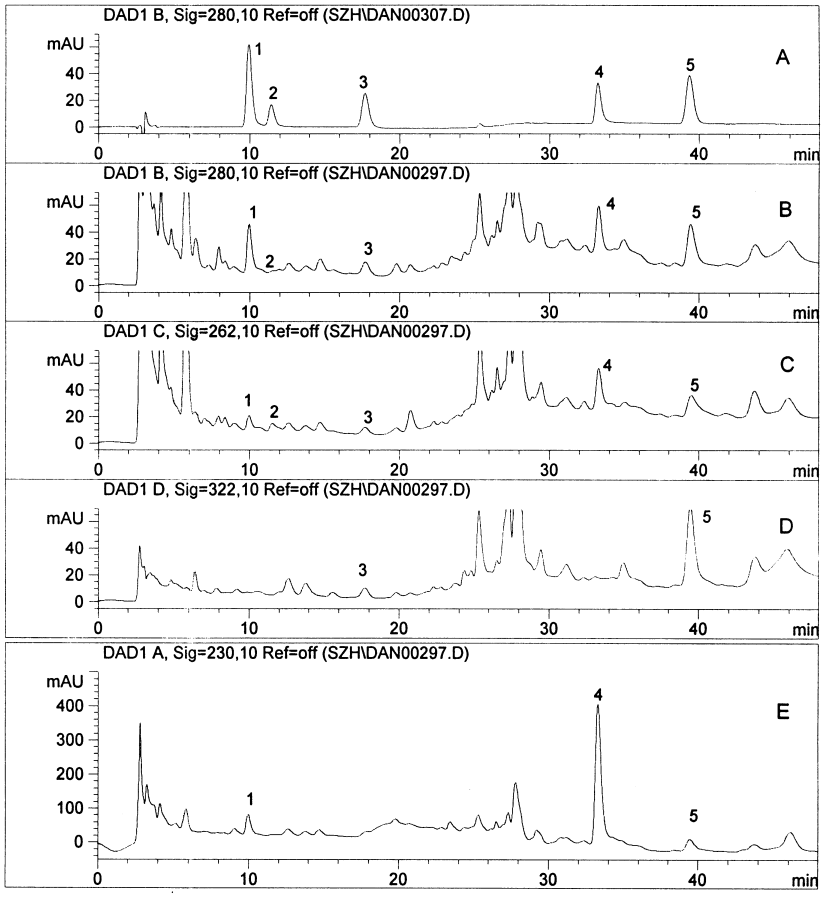

Figure 4. HPLC chromatograms of the authentic standards and Guanxin II decoction. (A) Authentic standards detected at 280 nm; (B)–(E) Guanxin II decoction detected at 280 nm, 262 nm, 322 nm, and 230 nm, respectively. 1. Danshensu, 2. Protocatechuic acid, 3. Protocatechualdehyde, 4. Paeoniflorin, 5. Ferulic acid. HPLC conditions: column: Agilent Zorbax extend-C_{18} narrow-bore column (150 × 2.1 mm i.d., 5 μm) connected with a same packing material guard column (12.5 × 2.1 mm i.d., 5 μm) at 25°C. Mobile phase: a mixture of methanol and 0.5% (v/v) acetic acid, employing gradient elution at a flow rate of 0.15 mL/min (see Table 1). Detection was accomplished with a diode-array detector and chromatograms were recorded at 230 nm, 262 nm, 280 nm, and 322 nm.

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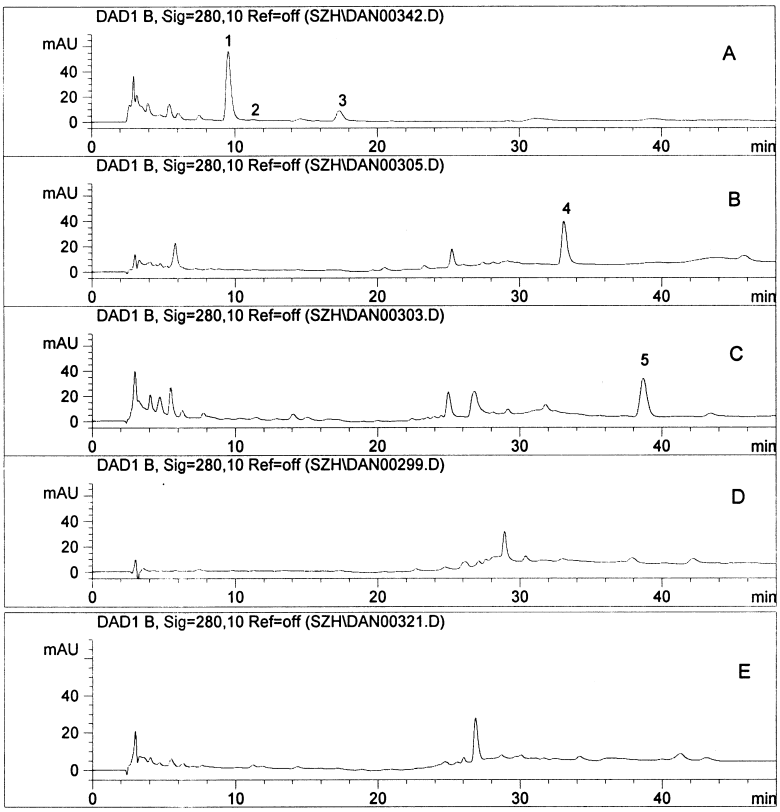

Figure 5. Chromatograms of single-crude drug decoctions. (A) Radix Salviae Miltiorrhizae decoction, (B) Radix Paeoniae Rubra decoction, (C) Rhizoma Chuanxiong decoction, (D) Ligni Dalbergiae Odoriferae decoction, (E) Flos Carthami decoction. 1. Danshensu, 2. Protocatechuic acid, 3. Protocatechualdehyde, 4. Paeoniflorin, 5. Ferulic acid. Chromatographic conditions as in Fig. 4.

Calibration Graphs and the Limit of Detection

All calibration graphs were plotted based on linear regression analysis of the integrated peak areas (Y) vs. concentrations (μg/mL, X) of the five bioactive components in the standard solution at six different concentrations. The regression equations, correlation coefficients, and linear ranges for the analysis of the five bioactive compounds are shown in Table 2.

The limit of detection (LOD) value was calculated as the amount of the injected sample, which gave a signal-to-noise ratio of 3. The LOD values of the method for the five bioactive components are also listed in Table 2.

Repeatability Test

The results of repeatability of the proposed method, on the basis of retention time and integrated peak area of the standard solutions, are listed in Table 3. The results indicated that there is little variability in the instrumental response and, thus, showed very good repeatability.

Recovery Test

Recovery experiments were carried out by adding authentic standards at three different concentrations to aliquots of the blank decoction prepared only by Flos Carthami and Ligni Dalbergiae Odoriferae. The results are reported in Table 4. It can be seen that the average recoveries lay between 97.57% and 100.68%, indicating that the proposed method has an adequate degree of accuracy for the determination of the five bioactive components in the samples.

Sample Analysis

The newly established method has been applied to the determination of the five bioactive components in Guanxin II decoction and single crude drug decoctions. The contents ($n = 3$) of the five bioactive components in a daily dose of the decoctions are depicted in Fig. 6. It can be seen that danshensu

Table 2. HPLC data for the calibration graphs.

Compound	Linear regression	Linear range (μg/mL)	r	LOD (g/mL)
Danshensu	$Y = 24.94 + 23.75X$	15.64–156.40	0.99985	0.86
Protocatechuic acid	$Y = 21.88 + 113.73X$	0.55–5.52	0.99953	0.12
Protocatechualdehyde	$Y = 73.39 + 159.14X$	1.16–11.55	0.99978	0.16
Paeoniflorin	$Y = 711.30 + 41.85X$	80.72–807.20	0.99938	2.34
Ferulic acid	$Y = 122.44 + 146.45X$	2.58–25.80	0.99996	0.12

Table 3. Repeatabilities of retention time and peak area of the bioactive constituents ($n = 5$).

	Retention time		Peak area	
Compound	SD (min)	RSD %	SD	RSD %
Danshensu	0.072	0.73	21.74	0.97
Protocatechuic acid	0.078	0.68	4.56	1.13
Protocatechualdehyde	0.12	0.69	6.48	0.55
Paeoniflorin	0.16	0.49	141.86	0.71
Ferulic acid	0.28	0.72	20.20	0.72

and paeoniflorin in single-drug decoction are apparently more than that in Guanxin II decoction; the contents of ferulic acid in Guanxin II and Chuanxiong decoction are almost the same, while the contents of protocatechuic acid and protocatechualdehyde in Guanxin II decoction are much more than in Danshen decoction. A possible reason is that the co-existing components in Guanxin II accelerate the dissociation of danshensu and paeoniflorin,

Table 4. Analytical results of recoveries ($n = 3$).

Compound	Added (μg/mL)	Found (μg/mL)	Recovery (%)	Mean ± SD (%)	RSD (%)
Danshensu	15.64	15.62	99.87	98.87	1.06
	93.84	92.87	98.97		
	125.12	122.33	97.77		
Protocatechuic acid	0.55	0.56	101.82	100.68	1.40
	3.32	3.29	99.10		
	4.42	4.47	101.13		
Protocatechualdehyde	1.28	1.26	98.44	97.57	2.05
	6.93	6.86	98.99		
	10.20	9.72	95.29		
Paeoniflorin	80.72	78.26	96.95	97.93	1.13
	484.32	480.11	99.13		
	667.20	651.84	97.70		
Ferulic acid	2.62	2.66	101.53	99.32	2.09
	10.48	10.21	97.42		
	20.96	20.75	99.00		

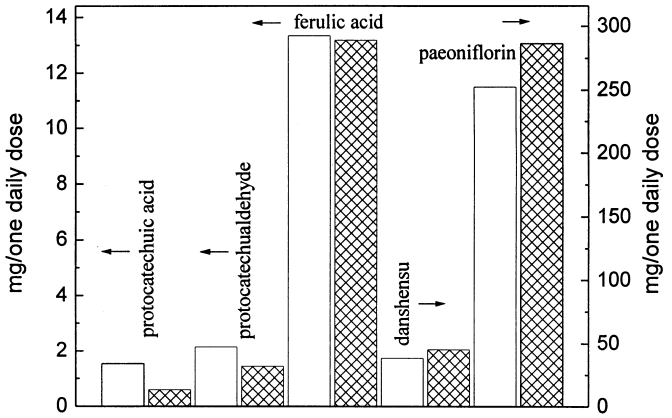

Figure 6. Contents ($n = 3$) of the five bioactive components in Guanxin II (blank columns) and single crude drug decoctions (gridding columns).

whereas the dissolution of protocatechuic acid and protocatechualdehyde in water is promoted in multi-component decoction.

CONCLUSION

A highly selective RP-HPLC method for the simultaneous determination of five bioactive components in traditional Chinese medicinal preparation Guanxin II was developed. The sample preparation method was relatively simple and straightforward, and no extensive clean-up procedure was needed. The established method was precise and accurate, allowing its quality control of the manufacturing process of Guanxin II in the future.

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