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Simultaneous Separation and Determination of Four Bioactive Constituents in Traditional Chinese Medicinal Tablet Xinkeshu by HPLC-DAD

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A reversed-phase high performance liquid chromatographic method for simultaneous determination of four bioactive constituents was established for the quality control of traditional Chinese medicinal tablet Xinkeshu. Danshensu, protocatechualdehyde, puerarin and daidzein were successfully separated on a Diamonsil C_{18} column (150×4.6 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 ℃. The mobile phase was a mixture of acetonitrile and 0.02 mol/L potassium dihydrogen phosphate employing gradient elution at a flow rate of 0.8 mL/min. Detection was accomplished with a diode-array detector and detection wavelength was set at 280 nm before 22 min, then it was varied to 250 nm. The four constituents were identified by comparing their retention time and UV spectra in the range of 190—400 nm with those of authentic standards. The linear calibration ranges were 15.4—123.2, 3.11—25.22, 21.6—172.8, and 0.26—3.47 μg/mL for danshensu, protocatechualdehyde, puerarin and daidzein, respectively. The detection limits were 0.03, 0.04, 0.10, 0.03 μg/mL for danshensu, protocatechualdehyde, puerarin, and daidzein, respectively. The contents of these four bioactive constituents in Xinkeshu tablet were successfully determined by the proposed method.

Keywords danshensu, protocatechualdehyde, puerarin, daidzein, Xinkeshu, HPLC-DAD

Introduction

In recent years there has been a renaissance of interest in use of traditional Chinese medicinal preparation (TCMP) because of the realization that traditional Chinese medicine can act in a synergistic manner in the human body, and can provide unique therapeutic properties with minimal or no undesirable side-effects.[1] The modernization research on TCMP is in process in China, and the first step is to establish simple and reliable analytical technologies and methodologies for the quality control of TCMP. An effective method for quality control of TCMP is bioactive constituent analysis. But such work is still difficult due to the complicated constituents and limited knowledge of the effective compositions. It was reported that thin layer chromatograph (TLC),[2-4] high performance liquid chromatograph (HPLC),[5-10] high-speed counter-current chromatography (HSCCC),[11] and capillary electrophoresis (CE)[12-14] have been applied to determine the bioactive constituents in traditional Chinese medicine.

Xinkeshu tablet is a kind of TCMP used widely for the treatment of coronary heart disease, which is composed of five crude drugs, *i.e.*, *Fructus Crataegi, Radix Salviae Miltiorrhizae, Radix Puerariae, Radix Notoginseng* and *Radix Aucklandiae*. Pharmacological experiment showed that Xinkeshu tablet possessed the effects of fortifying the body's ability against the absence of oxygen, reducing blood pressure, lightening heart's burden and decreasing oxygen consumption of cardiac muscle.[15,16] Although Xinkeshu tablet has been used for years, no satisfactory quality-control method has been reported up till now.

Danshensu and protocatechualdehyde are main water-soluble bioactive constituents from *Radix Salviae Miltiorrhizae*. Puerarin and daidzein are isoflavones from *Radix Puerariae*. Puerarin is a mark bioactive constituent in *Radix Puerariae* and daidzein is also an important bioactive component. Hence the four bioactive constituents were selected for simultaneous analysis for the quality control of Xinkeshu tablet. It was reported that puerarin and daidzein in plants and medicines have been determined by micellar electrokinetic capillary chromatography,[12,17] CE,[14,18] HPLC[19] and TLC Double-Wave Scanning,[20] and danshensu and protocatechualdehyde have been analyzed by HPLC.[5,21]

In this study, HPLC method was selected because it had better reproducibility than capillary chromatography and better separation ability than TLC Dou-

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ble-Wave scanning. The four bioactive constituents, danshensu, protocatechualdehyde, puerarin and daidzein, in Xinkeshu tablet were simultaneously analyzed by HPLC-DAD. This proposed method, as a simple, sensitive, reliable and efficient one, could be used for the quality control of Xinkeshu tablet and routine analysis. The chemical structures of the four substances are shown in Figure 1.

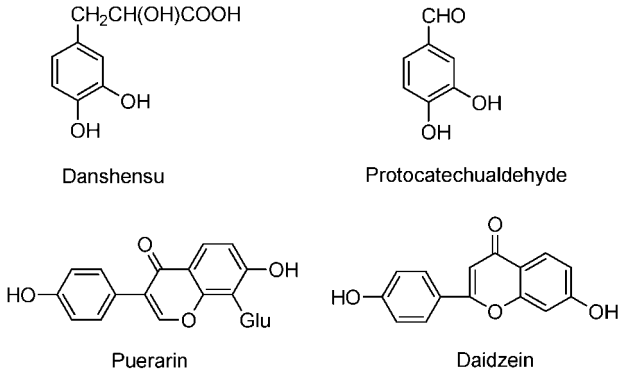

Figure 1 Chemical structures of the four bioactive constituents.

Experimental

Reagents and materials

Danshensu, puerarin and protocatechualdehyde were purchased from China National Institute of the Control of Pharmaceutical and Biological Products (Beijing, China). Daidzein was purchased from Sigma (St. Louis, MO, USA). Acetonitrile and methanol were of HPLC grade. Redistilled water was used to prepare all the solutions. Xinkeshu tablets were obtained from Tongrentang Pharmaceutical Group Company (Beijing, China).

Apparatus and chromatographic conditions

HPLC was performed on an HP G1311A Quat Pump System equipped with an HP G1315 diode array detector and an HP G1328A manual injector. Detector was set at 280 nm before t=22 min and 250 nm after t=22 min. Satisfactory separation of the four constituents was obtained on a Diamonsil C_{18} column (150×4.6 mm i.d., 5 μm, Dikma, Beijing, China) with a guard column (12.5×2.1 mm i.d., 5 μm) packed with the same material at 25 ℃. The mobile phase was set at a flow rate of 0.8 mL/min with a linear solvent gradient of A-B [A, acetonitrile; B, 0.02 mol/L KH_2PO_4] varying as follows: 0 min, 5∶95; 12 min, 12∶88; and 28 min, 30∶70 (*V*/*V*).

Standard solutions

Stock solutions of protocatechualdehyde, puerarin, and daidzein were prepared with methanol, and danshensu was directly prepared using water (pH=3) considering its stability. The various concentrations were within the range of 15.4—123.2, 3.11—25.22, 21.6—172.8, and 0.26—3.47 μg/mL for danshensu, protocatechualdehyde, puerarin and daidzein, respectively. Calibration curves were plotted subsequently for linear regression analysis of the peak area with concentrations.

Selection of extraction solvent

Twenty pieces of Xinkeshu tablets were ground and mixed well after the sugar-coats were divested. Total weight of the Xinkeshu powder was 5.875 g. 100 mg of this sample was extracted twice (8 and 6 mL, successively) with five various solvents, methanol, 1% acetic acid, 30% ethanol, 50% ethanol and 80% ethanol, respectively, in ultrasonic bath, each twenty minutes. All these solvents were adjusted to pH=3 with hydrochloric acid. The extract was centrifuged at a speed of 8000 rpm for 10 min. The supernatants, after centrifugation, were combined and diluted to 15 mL with the same solvent. These solutions were passed through 0.45 μm syringe filters before use.

Sample determination and recovery studies

100 mg of Xinkeshu powder was extracted twice (8 and 6 mL, successively) with 30% ethanol (pH=3) and processed as above. A sample volume of 20 μL was injected for HPLC analysis.

Two different amounts of the four stock solutions were added into the sample powder. Then the powder was dried, extracted and processed as above. The added concentrations of authentic standards were 24.6 and 12.3 μg/mL for danshensu, 7.76 and 4.14 μg/mL for protocatechualdehyde, 43.2 and 21.6 μg/mL for puerarin, and 1.068 and 0.534 μg/mL for daidzein, respectively. All samples were filtered through 0.45 μm syringe filters and injected for HPLC analysis to calculate the concentration of danshensu, protocatechualdehyde, puerarin and daidzein from their calibration graphs.

Results and discussion

Study of separation conditions

Using Diamonsil C_{18} stationary phase, various kinds of mobile phases were tested to achieve optimal separation. Methanol/0.02 mol/L KH_2PO_4 was tested as gradient elution liquid, and the proportion of mixture was adjusted in a broad range. But the four analytes could not be separated simultaneously.

To optimize separation conditions, two-phase system, acetonitrile/0.02 mol/L KH_2PO_4, was applied as gradient elution liquid, which greatly reduced analysis time and allowed baseline separation for the four analytes. Efficient detection was obtained by selecting two stages of detection wavelength according to the retention time and the ultraviolet spectrum of every component. The detection wavelength was set at 280 nm before t=22 min, then it was varied to 250 nm.

The diode array detector facilitated the identification and confirmation of these four constituents. The four constituents were identified by comparing their retention time and UV spectra in the range of 190—400 nm with authentic standards. Figure 2(a) shows HPLC

chromatogram of danshensu, protocatechualdehyde, puerarin and daidzein. Figure 2(b) presents a chromatogram showing the separation of the constituents with the retention times of 6.1 min for danshensu, 18.7 min for protocatechualdehyde, 24.4 min for puerarin, and 35.4 min for daidzein at a flow rate of 0.8 mL/min. When the sample solution was injected and analyzed, the whole analysis was finished within 40 min.

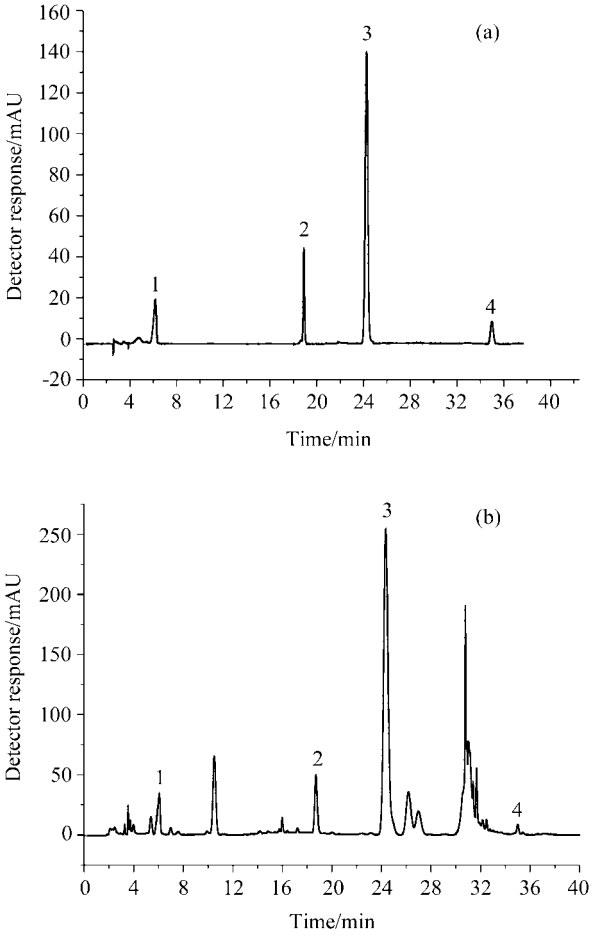

Figure 2 (a) HPLC Chromatogram of danshensu (1), protocatechualdehyde (2), puerarin (3) and daidzein (4). (b) HPLC Chromatogram of 30% ethanol (pH=3) extract of Xinkeshu tablet. HPLC conditions, column: Diamonsil C_{18} column, 150×4.6 mm i.d., 5 μm; mobile phase: A-B [A=acetonitrile, B=0.02 mol/L KH_2PO_4], 0 min, 5∶95; 12 min, 12∶88; and 28 min, 30∶70 (*V*/*V*); flow rate: 0.8 mL/min; detection wavelength: 0 min, 280 nm; and 22 min, 250 nm.

Study of extraction solvent

The selection of solvent influenced the analysis of the four bioactive constituents in Xinkeshu tablet. The extraction effect of the four constituents using different solvents which were adjusted to pH=3 with hydrochloric acid was studied. The contents of danshensu, protocatechualdehyde, puerarin, and daidzein in Xinkeshu were determined to 0.704%, 0.115%, 1.064%, and 0.006% while extracted with 1% acetic acid; 0.703%, 0.121%, 1.147%, and 0.015% while extracted with 30% ethanol; 0.591%, 0.119%, 1.092%, and 0.013% while extracted with 50% ethanol; 0.453%, 0.121%, 0.822%, and 0.014% while extracted with 80% ethanol; 0%, 0.121%, 0.953%, and 0.015% while extracted with methanol. With 30% ethanol, except for daidzein, the other constituents yielded the best extraction rates. Therefore, 30% ethanol (pH=3) was used as extraction solvent throughout this study.

Calibration graph

Calibration graphs were constructed in the range of 15.4—123.2 μg/mL for danshensu, 3.11—25.22 μg/mL for protocatechualdehyde, 21.6—172.8 μg/mL for puerarin, and 0.26—3.47 μg/mL for daidzein. The regression equations of these curves and their correlation coefficients were calculated as follows: danshensu, $y=2.158+12.993x$ ($r=0.99999$); protocatechualdehyde, $y=-65.496+74.865x$ ($r=0.99938$); puerarin, $y=106.682+91.576x$ ($r=0.99998$); daidzein, $y=6.826+137.260x$ ($r=0.99975$). It showed good linear relationships between the peak area and the concentrations. A signal three times higher than the noise peak height was regarded as the detection limit. The detection limits of these four constituents were: 0.03, 0.04, 0.10, 0.03 μg/mL for danshensu, protocatechualdehyde, puerarin, and daidzein, respectively.

Reproducibility test

To assess the precision of these methods, standard solutions of danshensu, protocatechualdehyde, puerarin, and daidzein were injected respectively five times for intra-day analysis and a 5-day period analysis. The coefficient variations of intra-day and inter-day studies were less than 4.5% and 6.5%, respectively. The precision as well as accuracy of this assay was satisfactory (Table 1).

Sample analysis and recovery test

The HPLC chromatogram of the actual sample was shown in Figure 2(b). The contents of the four bioactive constituents in Xinkeshu tablet (No. 020793) are listed in Table 2. The results indicated that the proposed method is suitable for the determination of these four bioactive constituents in Xinkeshu tablet.

Different amounts of the four standards were added into the actual samples of known contents. The mixture were extracted and analyzed following the proposed procedures. The results of standard addition recovery studies are given in Table 3.

Table 1 Intra-day and inter-day assay variations of danshensu, protocatechualdehyde, puerarin and daidzein

Constituent	Concentration/ ($\mu g \cdot mL^{-1}$)	Intra-day RSD*/%	Inter-day RSD*/%
Danshensu	15.4	0.70	3.70
	61.6	0.25	0.93
	123.2	0.20	0.80
Protocatechualdehyde	3.11	2.61	4.18
	13.58	1.23	1.90
	25.22	0.15	0.65
Puerarin	21.6	1.75	2.41
	64.8	0.28	0.71
	172.8	0.30	0.39
Daidzein	0.267	4.48	6.29
	1.334	1.46	2.55
	3.469	0.72	1.46

*$n=5$

Table 2 Contents of the four bioactive constituents in Xinkeshu tablet

Constituent	Content/($mg \cdot g^{-1}$)	RSD*/%
Danshensu	7.028	1.06
Protocatechualdrhyde	1.214	0.54
Puerarin	11.47	0.85
Daidzein	0.146	2.21

*$n=5$

Table 3 Recoveries of danshensu, protocatechualdehyde, puerarin, and daidzein in Xinkeshu tablet

Constituent	Amount added/ ($\mu g \cdot mL^{-1}$)	Amount found/ ($\mu g \cdot mL^{-1}$)	Recovery*/%
Danshensu	24.6	24.1	98.0±0.8
	12.3	11.9	96.7±1.3
Protocatechualdehyde	7.76	7.84	101.0±0.6
	4.14	4.22	101.9±0.9
Puerarin	43.2	42.1	97.5±1.2
	21.6	21.3	98.6±0.7
Daidzein	1.068	1.019	95.4±1.3
	0.534	0.513	96.1±2.1

*$n=5$

Conclusion

A sensitive HPLC-DAD method was developed and validated for the simultaneous analysis of four bioactive constituents, danshensu, protocatechualdehyde, puerarin and daidzein in Xinkeshu tablet. Accurate and precise results were obtained. The good reproducibility and detection limit ensured that the proposed method could be used as an alternative tool for the quality control of Xinkeshu tablet.

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