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A High-Performance Liquid Chromatography Method for the Simultaneous Determination of Five Index Components in Danhong Injection

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Abstract

The purpose of this study was to establish a high-performance liquid chromatography (HPLC) method for the simultaneous determination of sodium danshensu, protocatechuic aldehyde, rosmarinic acid, salvianolic acid B, and 4-coumaric acid in Danhong injection. The chromatographic method employed was as follows: the column was a Welch Ultimate XB-C18 column (250 mm × 4.6 mm, 10 μm), the mobile phase was a gradient elution of 0.4% formic acid aqueous solution (A) and acetonitrile (B), the detection wavelengths were 280 nm for sodium danshensu, protocatechuic aldehyde, and salvianolic acid B and 326 nm for 4-coumaric acid and rosmarinic acid, the sample volume was 10 μL, the flow rate was 1.0 mL/min, and the column temperature was 35˚C. This method can realize the separation and determination of sodium danshensu, protocatechuic aldehyde, rosmarinic acid, salvianolic acid B, and 4-coumaric acid within 50 minutes. The linear relationships of the five peak areas and their concentrations are good (R^2 > 0.9997). The precision RSD values are all less than 1.0%. The reproducibility RSD values are all less than 1.3%. The stability RSD values are all less than 2.2%. The recovery values ranged from 92.4% to 99.4%. This method is simple, accurate, and reproducible. It can be used for the determination of sodium danshensu, protocatechuic aldehyde, rosmarinic acid, salvianolic acid B, and 4-coumaric acid in Danhong injection.

Keywords

Danhong Injection, High Performance Liquid Chromatography, Phenolic

Acid, Flavonoids

1. Introduction

Danhong injection is a traditional Chinese medicine formula prepared from two medicinal herbs of Danshen (*Salviae miltiorrhizae Radix et Rhizoma*) and Honghua (*Carthami flos*) through processes such as water extraction and alcohol precipitation [1] [2]. It has the efficacy of promoting blood circulation and removing blood stasis, as well as regulating channels and collaterals [3] [4]. The main components of Danhong injection include phenolic acids, flavonoids, quinones, nucleosides, sugars, fatty acids, and pigments [5] [6]. Among them, the phenolic acid components of Danshen include danshensu, protocatechuic aldehyde, rosmarinic acid, and salvianolic acid B, which have activities such as anti-myocardial ischemia, expanding coronary arteries, reducing intracellular cholesterol synthesis, and inhibiting lipoprotein oxidation [7] [8] [9]. The flavonoid components of Honghua include 4-coumaric acid and others, which have antioxidant, anti-inflammatory, and immune regulation activities [10]. The phenolic acid components of Danshen and the flavonoid components of Honghua are considered to be the main active ingredients in Danhong injection.

Yang Jing *et al.* reported the application of the UPLC-UV method in the stability study of multiple active components in Danhong injection, including sodium danshensu, protocatechuic aldehyde, salvianolic acid B, rosmarinic acid, adenine, thymine, cytosine and uracil [11]. Dou Jiaojiao *et al.* applied an HPLC-DAD device to determine six components, including danshensu, protocatechuic aldehyde, salvianolic acid B, caffeic acid, and rosmarinic acid [12]. Zhang Xiaodong *et al.* used ultra high performance liquid chromatography to determine the content of danshensu and protocatechuic aldehyde in Danhong injection [13]. You Huilian used HPLC to determine the content of four water-soluble components in Danhong Injection, namely sodium danshensu, protocatechuic aldehyde, salvianolic acid B, and rosmarinic acid [14]. Li Guosong *et al.* developed a UPLC method to determine the content of five components in Danhong Injection, including sodium danshensu, protocatechuic aldehyde, protocatechuic acid, rosmarinic acid, and salvianolic acid B [15]. However, the methods in the literature mainly focused on the determination of phenolic acids and nucleosides, lacking the quantitative research on the flavonoid components of Honghua. Therefore, it is necessary to develop new analytical methods.

In this study, a fast and effective analysis method was established by using two UV detection wavelengths simultaneously to detect the five main active components in Danhong Injection, including sodium Danshensu, protocatechuic aldehyde, 4-coumaric acid, rosmarinic acid and salvianolic acid B. This method is expected to be helpful to improve the quality control level of Danhong Injection.

2. Instruments, Materials, and Methods

2.1. Instruments

High-performance liquid chromatography (1260, Agilent Company, USA) equipped with a degasser, quaternary gradient pump, automatic sampler, column temperature box, and UV detector; water bath pot (XMTE-8112, Shanghai Jinghong Experimental Equipment Co., Ltd.); pure water machine (Direct Q, Hangzhou Jingyuan Technology Co., Ltd.); electronic balance (SE6001, Aarhus Instruments Co., Ltd.); precision electronic balance (XSR105, Mettler Toledo, Switzerland); and ultrasonic cleaning machine (LMDTC, LUMTECH, Germany) were used.

2.2. Materials

Acetonitrile (chromatographically pure, Merck, Germany), methanol (chromatographically pure, Merck, Germany), and formic acid (chromatographically pure, Shanghai Jizhi Biochemical Technology Co., Ltd.) were used. Danhong injection was provided by Shandong Danhong Pharmaceutical Co., Ltd. Salvianolic acid B (HPLC > 98%, Shanghai Ronghe Pharmaceutical Technology Development Co., Ltd., batch number: 200602), sodium danshensu (HPLC ≥ 98%, Shanghai Ronghe Pharmaceutical Technology Development Co., Ltd., batch number: 171027), rosmarinic acid (HPLC ≥ 98%, Shanghai Ronghe Pharmaceutical Technology Development Co., Ltd., batch number: 150901), protocatechuic aldehyde (HPLC > 98%, Shanghai Yuanye Biotechnology Co., Ltd., batch number A28GS147304), and 4-coumaric acid (HPLC > 98%, Shanghai Aladdin Biochemical Technology Co., Ltd., batch number: H2121244501-98-4) were purchased and used.

2.3. Chromatographic Conditions

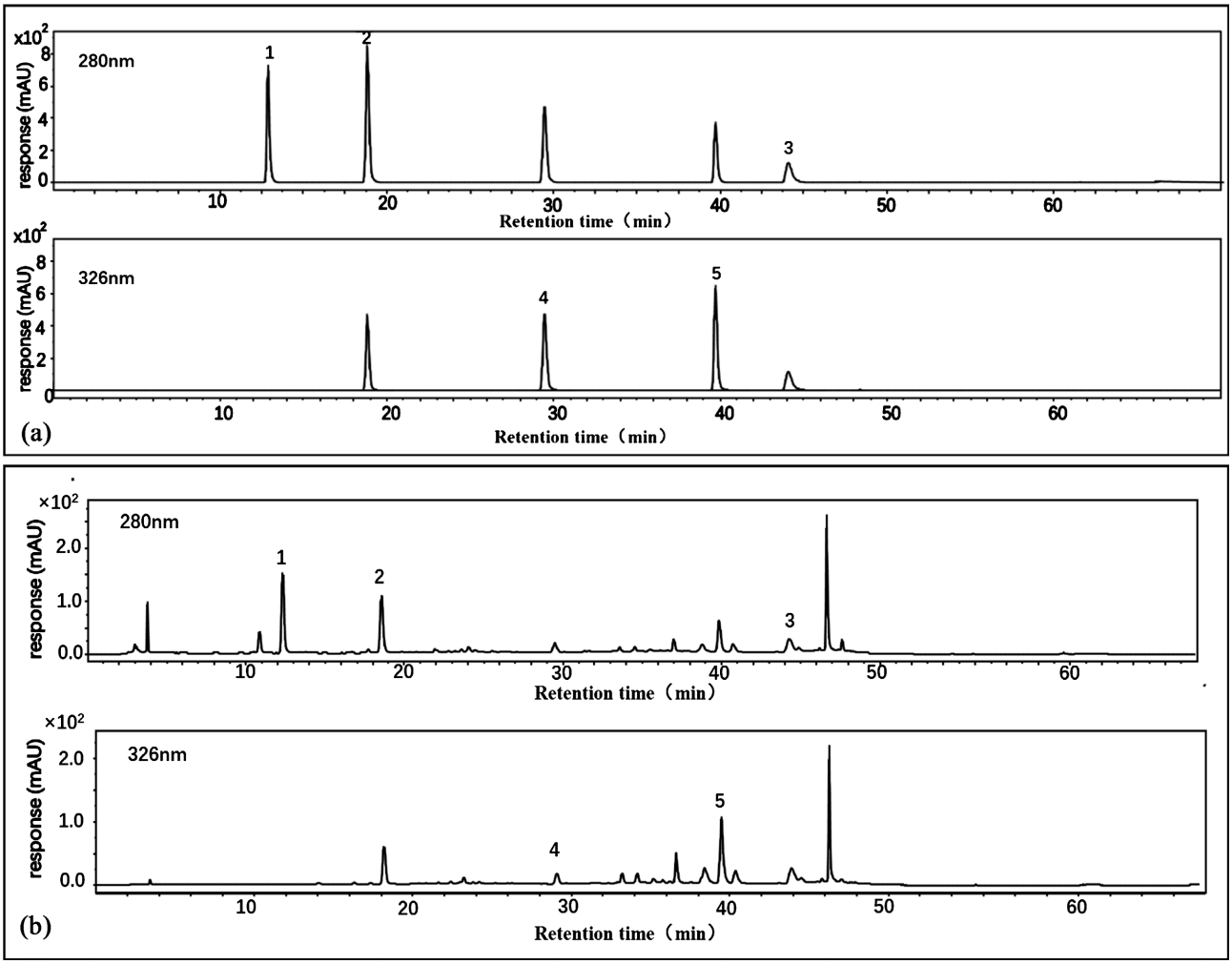
Welch Ultimate XB-C18 chromatographic column (250 mm × 4.6 mm, 5 μm) was used. The mobile phase was composed of 0.4% formic acid water (A) and acetonitrile (B). A gradient elution was used. The elution procedure is shown in Table 1. The detection wavelengths were 280 nm (sodium danshensu, protocatechuic aldehyde, salvianolic acid B) and 326 nm (4-coumaric acid, rosmarinic acid). The flow rate was 1.0 mL/min. The column temperature was 35˚C. The injection volume was 10 μL. The chromatography figures are shown in Figure 1.

2.4. Preparation of Mixed Reference Solution

Appropriate amounts of each reference standard, sodium danshensu, protocatechuic aldehyde, rosmarinic acid, salvianolic acid B, and 4-coumaric acid, were weighed accurately. They were placed in a volumetric flask, and a 20% acetonitrile aqueous solution containing 0.4% formic acid was added to dilute to the mark to prepare mixed reference solutions with concentrations of 1.34, 0.266, 0.356, 0.328, and 0.500 mg/mL, respectively.

Table 1. Gradient elution procedure.

time (min)	A (%)	B (%)
0 - 15	98 - 90	2 - 10
15 - 20	90 - 84	10 - 16
20 - 25	84 - 84	16 - 16
25 - 33	84 - 74	16 - 24
33 - 40	74 - 74	24 - 24
40 - 50	74 - 40	24 - 60
50 - 60	60 - 10	60 - 90
60 - 70	10 - 98	90 - 2



1. Sodium Danshensu 2. Protocatechuic aldehyde 3. Salvianolic acid B 4. 4-Coumaric acid 5. Rosmarinic acid.

Figure 1. High-performance liquid chromatography. (a) Mixed reference substance; (b) Test sample.

2.5. Preparation of Sample Solution

Two milliliters of Danhong injection were accurately measured and placed in a

10 mL volumetric flask. A certain volume of 20% acetonitrile aqueous solution containing 0.4% formic acid was added. Then the volumetric flask was placed in an ultrasonic environment and heated. The mixture was cooled to room temperature, and then the volume was adjusted, shaken well, and filtered to obtain the sample solution.

3. Results

3.1. Investigation of the Linear Relationship

The mixed reference solution prepared in Section 2.4 was diluted gradiently to prepare a series of mixed reference solutions with different concentrations. The samples were analyzed under the above mentioned chromatographic conditions. The standard curve was calibrated using the concentration as the horizontal coordinate (X) and the peak area as the vertical coordinate (Y). The regression equation was calculated. The results are shown in Table 2, indicating that sodium danshensu, protocatechuic aldehyde, rosmarinic acid, salvianolic acid B, and 4-coumaric acid all have good linear relationships within their respective linear ranges.

3.2. Precision Test

The sample solutions were prepared according to the method described in Section 2.5. The same sample solution was injected continuously 6 times, and then the peak areas of each component were determined under the chromatographic conditions described above. The RSD values of the peak areas of sodium danshensu, protocatechuic aldehyde, salvianolic acid B, 4-coumaric acid, and rosmarinic acid were 0.61%, 0.63%, 0.97%, 0.54%, and 0.69%, respectively, all of which are less than 1.0%. This indicates that the instrument precision was good.

3.3. Repeatability Test

The sample solution was prepared according to the method described in Section2.5. Six samples were prepared in parallel and injected into the chromatographic system to determine the contents of each component under the chromatographic conditions described above. The RSD values of the contents of sodium danshensu, protocatechuic aldehyde, salvianolic acid B, 4-coumaric acid, and rosmarinic acid were 0.85%, 0.75%, 1.28%, 0.87%, and 1.08%, respectively,

Table 2. Linear relationship inspection.

Name	Linear equation	R^2	Linear range (mg/mL)
Sodium danshensu	Y = 6749.18X + 45.8	0.9999	0.041 - 1.31
Protocatechuic aldehyde	Y = 44313.2X + 71.3	0.9997	0.008 - 0.261
Salvianolic acid B	Y = 10344.9X + 4.00	0.9999	0.011 - 0.348
4-Coumaric acid	Y = 38777.1X + 5.41	1.000	0.002 - 0.049
Rosmarinic Acid	Y = 27828.3X + 46.3	0.9998	0.010 - 0.321

all of which are less than 1.3%. This indicates that the repeatability of this method was good.

3.4. Stability Test

The sample solution was prepared according to the method described in Section 2.5. The solutions were analyzed under the chromatographic conditions described above after being prepared for 0, 2, 4, 8, 12, and 24 hours. The RSD values of the peak areas of sodium danshensu, protocatechuic aldehyde, salvianolic acid B, 4-coumaric acid, and rosmarinic acid were 2.18%, 0.63%, 0.40%, 1.03%, and 1.09%, respectively, all of which are less than 2.2%. This indicates that the sample solution was stable within 24 hours.

3.5. Sample Recovery Test

With a precision pipette, 2 mL of Danhong injection was taken and placed in a 10 mL volumetric flask. A total of 6 samples were prepared, and each sample was added to a certain concentration of mixed reference solution. Then, a 20% acetonitrile aqueous solution containing 0.4% formic acid was added to adjust the volume to the mark. The solution was mixed well, and used as the test solution. Under the chromatographic conditions described above, the recovery rate was calculated by injecting the test solution into the chromatographic system. The results are shown in Table 3. The recovery rates of sodium danshensu, protocatechuic aldehyde, salvianolic acid B, 4-coumaric acid, and rosmarinic acid were between 98.2% and 101%, 95.0% and 96.6%, 93.6% and 97.7%, 93.5% and 100%, and 91.1% and 94.3%, respectively. The RSD values of all components were less than 3%. The results showed that the recovery values of the five components are good.

3.6. Content Determination

Ten batches of samples were prepared according to the method described in Section2.5. Then their contents were measured. The results are shown in Table 4. The average contents of sodium danshensu, protocatechuic aldehyde, salvianolic acid B, 4-coumaric acid, and rosmarinic acid were 1.36 mg/g, 0.173 mg/g, 0.308 mg/g, 0.0299 mg/g, and 0.248 mg/g, respectively. Among them, the content of sodium danshensu was the highest, ranging from 1.24 to 1.40 mg/g, while the content of 4-coumaric acid was the lowest, ranging from 0.0283 to 0.0316 mg/g.

4. Discussions

4.1. Selection of Detection Wavelength

In this study, the UV spectra of the analytes were measured, as shown in Figure 2. Sodium danshensu, protocatechuic aldehyde, and salvianolic acid B have strong UV absorption at 280 nm. While 4-coumaric acid and rosmarinic acid have strong UV absorption near 320 nm. Therefore dual wavelength detection was selected in this work.

4.2. Acidity of the Mobile Phase

Our research group used the liquid–liquid equilibrium method to determine the dissociation constants of various salvianolic acids [16]. Among them, the pK_a

Table 3. Results of sample addition recovery test.

Name	Added amount (mg)	Measured amount (mg)	Rate of recovery (%)	Average recovery rate (%)	RSD (%)
Sodium danshensu	2.56	5.17	101	99.4	1.34
	2.56	5.13	100		
	2.56	5.08	98.2		
	2.56	5.10	98.9		
	2.56	5.08	98.3		
	2.56	5.11	99.2		
Protocatechuic aldehyde	0.316	0.621	96.6	95.4	0.616
	0.316	0.617	95.3		
	0.316	0.616	95.2		
	0.316	0.617	95.5		
	0.316	0.616	95.0		
	0.316	0.616	95.1		
Salvianolic acid B	0.806	1.54	96.1	95.5	1.49
	0.806	1.53	94.4		
	0.806	1.56	97.7		
	0.806	1.54	95.7		
	0.806	1.54	95.5		
	0.806	1.52	93.6		
4-Coumaric acid	0.0686	0.137	100	98.0	2.39
	0.0686	0.135	98.0		
	0.0686	0.136	99.0		
	0.0686	0.135	98.3		
	0.0686	0.135	98.5		
	0.0686	0.132	93.5		
Rosmarinic Acid	0.535	1.01	94.2	92.4	1.66
	0.535	1.01	94.3		
	0.535	0.997	91.1		
	0.535	0.997	91.2		
	0.535	0.997	91.1		
	0.535	1.00	92.8		

Table 4. Content determination results.

Batch number	Sodium danshensu (mg/g)	Protocatechuic aldehyde (mg/g)	Salvianolic acid B (mg/g)	4-Coumaric acid (mg/g)	Rosmarinic acid (mg/g)
22061020	1.39	0.182	0.392	0.0313	0.273
22061021	1.37	0.179	0.369	0.0311	0.271
22061014	1.34	0.186	0.241	0.0292	0.242
22062015	1.30	0.170	0.297	0.0312	0.231
22082017	1.32	0.172	0.303	0.0286	0.246
22082033	1.53	0.195	0.372	0.0316	0.245
22041025	1.36	0.157	0.270	0.0302	0.240
21102001	1.24	0.150	0.341	0.0289	0.261
22051028	1.34	0.165	0.258	0.0285	0.252
22041023	1.40	0.175	0.232	0.0283	0.218
Average value (mg/g)	1.36	0.173	0.308	0.0299	0.248
Standard deviation (mg/g)	0.0765	0.0134	0.0582	0.00131	0.0172

value of Danshensu ranges from 3.49 to 3.80. The pK_a value of rosmarinic acid ranges from 2.75 to 3.12. The pK_{a1} value of salvianolic acid B ranges from 2.24 to 2.40, and the pK_{a2} value ranges from 3.20 to 3.63 [16]. Mobile phase A was 0.4% formic acid, and its pH value was tested to be 2.37. At this pH value, most of the above mentioned components exist in their molecular form. Due to the use of reversed-phase chromatography for separation, the presence of salvianolic acid components in molecular form is more advantageous for the chromatographic column to retain them, thus enabling better separation of these phenolic acids.

4.3. Chemical Constituents in Danhong Injection

Sodium danshensu is a degradation product of salvianolic acid B. When at a higher pH value or temperature, salvianolic acid B degrades faster and forms danshensu and lithospermic acid [17] [18] [19] [20]. In the production of Danhong injection, water extraction is used, which can accelerate the degradation of salvianolic acid B to produce sodium danshensu [21] [22]. Compared to Salvianolic acid B, sodium danshensu is a smaller molecular weight product with higher water solubility. Danshensu is less likely to form precipitation in an injection. From **Table 4**, it can be seen that the content of sodium danshensu in Danhong injection is relatively high, while the content of salvianolic acid B is relatively low, which is beneficial for the stability of Danhong injection during its shelf life.

In Chinese Pharmacopia (2020 Edition), hydroxysafflor yellow A is the indicator component of Honghua. In previous work, it is observed that the content

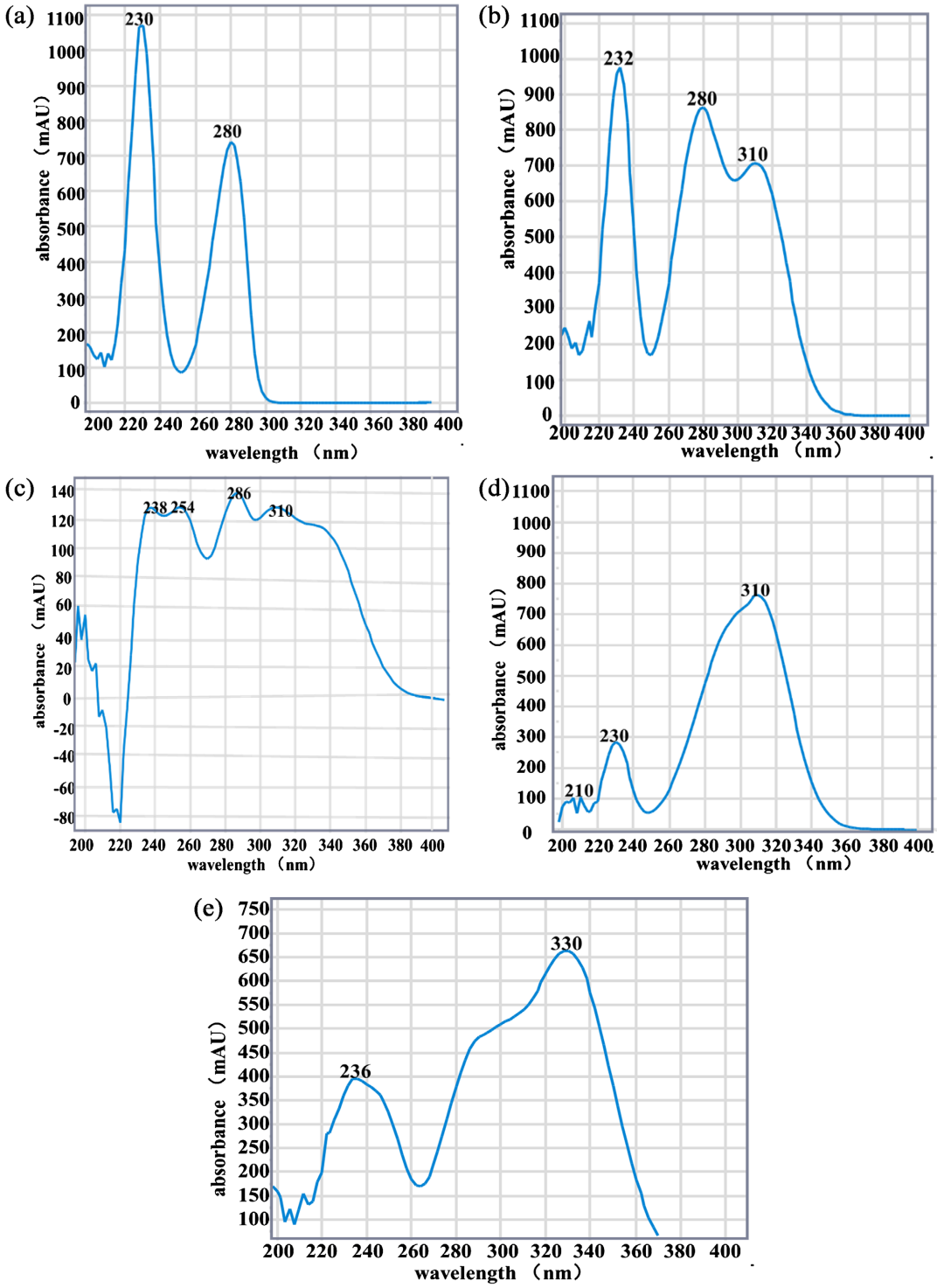


Figure 2. UV Spectra of Various Components. (a) Sodium danshensu; (b) Protocatechuic aldehyde; (c) Salvianolic acid B; (d) 4-Coumaric acid; (e) Rosmarinic acid.

of hydroxysafflor yellow A was higher than that of rosmarinic acid in the water extract of mixed Danshen and Honghua [23]. However, the content of hydroxysafflor yellow A in Danhong injection is very low. Accordingly, hydroxysafflor yellow A is not determined in this work. 4-Coumaric acid is a degradation

product of hydroxysafflor yellow A. Heating and pH adjustment processes in industrial production may be the causes of hydroxysafflor yellow A degradation.

5. Conclusion

In this study, a method for the determination of sodium danshensu, protocatechuic aldehyde, salvianolic acid B, 4-coumaric acid, and rosmarinic acid in Danhong injection using HPLC equipment was established. The method employed a Welch Ultimate XB-C18 column (250 mm × 4.6 mm, 5 μm) as the stationary phase, with a gradient elution system composed of 0.4% formic acid-water (A) and acetonitrile (B) as the mobile phase. Detection wavelengths were set at 280 nm and 326 nm. The method has been validated to be accurate, reliable, and reproducible. This method can be used for the quantification of the five analytes in Danhong Injection. The method can simultaneously determine the contents of four phenolic acids and a flavonoid component, which is beneficial for improving the quality control level of Danhong Injection. In future, the methods of quantitative analysis of multi-components by single marker (QAMS) are expected to be developed for simultaneously determining components in Danhong injection with smaller amount of reference substance.

Data Availability Statement

This published article includes all data generated or analyzed during this study.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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