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Simultaneous detection of seven phenolic acids in Danshen injection using HPLC with ultraviolet detector*

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Abstract: A high-performance liquid chromatographic (HPLC) method with ultraviolet (UV) detector had been developed for simultaneous quantification of danshensu, protocatechuic aldehyde, caffeic acid, salvianolic acid D, rosmarinic acid, salvianolic acid B and salvianolic acid A in Danshen injection. According to the UV spectra of these components, three detection wavelengths have been selected as follows: 280 nm for danshensu and protocatechuic aldehyde, 326 nm for caffeic acid, salvianolic acid D and rosmarinic acid, 286 nm for salvianolic acid B and salvianolic acid A. The limit of detection (LOD) was improved to be in the range of 0.008~0.160 μg/ml. Moreover, excellent linear behavior over the investigated concentration range was observed, with R>0.999 for all the analytes.

Key words: High-performance liquid chromatography (HPLC), Danshen injection (DSI), Phenolic acids, Multi-wavelengths, Quantification

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INTRODUCTION

Danshen (DS), the dried root of *Salvia miltiorrhiza*, is a famous traditional Chinese medicine and widely used to treat coronary heart disease, cerebrovascular disease, bone loss, hepatitis, hepatocirrhosis and chronic renal failure (Wasser *et al*., 1998; Liu *et al*., 2000; Ji *et al*., 2003; Chae *et al*., 2004; Ling *et al*., 2005). There are a number of traditional Chinese medicinal preparations containing DS, such as Danshen tablets, Danshen dripping pills, Danshen injection (DSI) and Xiangdan injection. DSI is made from the aqueous extract of DS and widely used in clinics as a treatment for coronary heart disease, heart-stroke and cerebrovascular disease.

The chemical constituents of DS include both lipophilic and hydrophilic components. The major hydrophilic components are phenolic acids including danshensu (DSS), protocatechuic aldehyde (PA), rosmarinic acid (RA) and salvianolic acids, which are also major pharmacologically active constituents in DSI. Taking the phenolic acids as target compounds, many high-performance liquid chromatographic (HPLC) methods have been established for the quality control of DS and its related traditional Chinese medicinal preparations. For instance, HPLC coupled with ultraviolet (UV) detection (Zhang *et al*., 2005) or diode-array detection (DAD) (Hu *et al*., 2005; Liu *et al*., 2007) together with liquid chromatography-tandem mass spectrometry (LC-MS^n) method and HPLC with a coulometric electrode array system (HPLC-CEAD) (Ma *et al*., 2007) have been established for chemical fingerprint analysis of DS. HPLC-UV methods have been used in the quantification of both four phenolic acids and three lipophilic components in DS (Yuan *et al*., 2005) and seven active components including salvianolic acids, tanshinones and ginsenosides in the Fufang Danshen tablet (Wei *et al*., 2007). An HPLC method with photodiode-array UV detection has been developed to

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quantify three major lipophilic components (cryptotanshinone, tanshinone I and tanshinone IIA) and three major hydrophilic components (DSS, PA and salvianolic acid B (SaB)) (Fig.1) of DS (Zhou *et al*., 2006). An HPLC method with DAD has been established for simultaneous quantification of six phenolic acids (DSS, PA, RA, lithospermic acid, SaB and salvianolic acid A (SaA)) in DS and its related traditional Chinese medicinal preparations (Liu *et al*., 2006). All of the abovementioned papers have indicated that HPLC is a feasible method for quality control of DS and its related preparations including DSI. On the other hand, we realized that the contents of some phenolic acids, such as caffeic acid (CA) and salvianolic acid D (SaD) (Fig.1), are so low that they are difficult to be quantified.

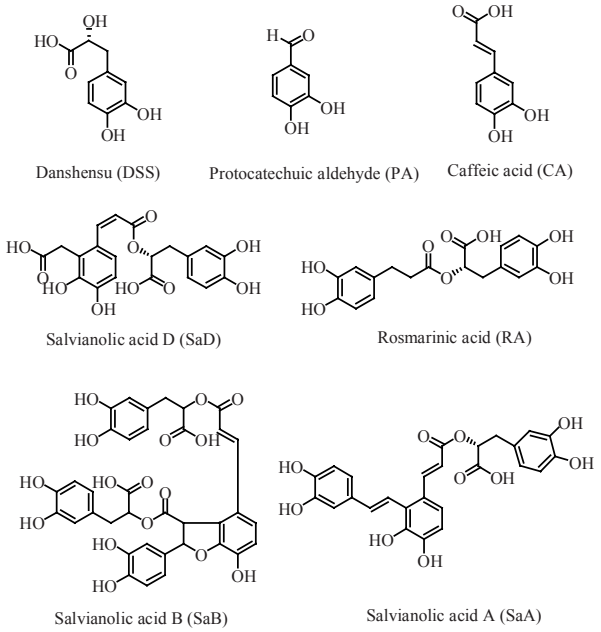

Fig.1 Chemical structures of target compounds in Danshen injection

The present study aimed at developing a more sensitive HPLC method with UV detector for simultaneous quantification of those components which are in low content in the DSI. In the analytical process three wavelengths of 280 nm, 326 nm and 286 nm have been selected to detect DSS and PA, CA, SaD and RA, SaB and SaA, respectively, and the limit of detection (LOD) was improved to be in the range of 0.008~0.160 μg/ml. This method has been applied in determining the amount of these seven compounds, from different batches and producers, in the DSI.

MATERIALS AND METHODS

Chemicals and reagents

DSIs were supplied by a Chinese pharmaceutical manufacturer (Qingchunbao, Zhejiang, China). Standard substances including DSS, PA, CA, RA and SaB were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). SaD and SaA were isolated from aqueous extract of DS. These isolated salvianolic acids were identified on the basis of mass spectrum (MS) data, purities of which were all over 97% by HPLC analysis. The HPLC-grade acetonitrile and formic acid were purchased from Merck (Darmstadt, Germany) and the Tedia Company (Fairfield, USA), respectively. Deionized water used throughout the experiments was produced using a Mill-Q academic water purification system (Milford, MA, USA).

Instrumentation and analytical conditions

The HPLC system HP 1100 series (Agilent Technologies, Waldbronn, Germany) was equipped with Chemstation Software (Agilent Technologies) and was comprised of a quaternary pump, an online vacuum degasser, an autosampler and a thermostated column compartment. A UV detector was used for the chromatographic analysis and a DAD detector was used to obtain UV spectra of seven standard substances. All separations were carried out on a Tigerkin C_{18} column (200 mm×4.6 mm i.d., 5.0 μm particle size) from Dalian Sipore Co., Ltd. (Dalian, China). A linear gradient elution of Eluents A (0.5% (v/v) aqueous formic acid) and B (0.5% (v/v) formic acid in acetonitrile) was used to run the separation. The elution programme was well optimized and conducted as follows: the first linear gradient was 5%~20% Eluent B in the range of 0~10 min, the second one was 20%~25% Eluent B in the range of 10~17 min, and the last one was 25%~55% Eluent B in the range of 17~35 min. Then the system was restored to initial conditions after 5 min. The solvent flow rate was 1.0 ml/min, the injection volume was 20 μl, and the column temperature was maintained at 30 °C. The chromatograms were recorded at 280 nm in the range of 0~13 min, 326 nm in the range of 13~23.5 min, and 286 nm in the range of 23.5~35 min.

Solution preparation

1. Standard solution

The standard stock solutions of DSS (4.05 mg/ml), PA (5.10 mg/ml), CA (1.0 mg/ml), SaD (1.15 mg/ml), RA (2.70 mg/ml), SaB (1.00 mg/ml) and SaA (1.10 mg/ml) were prepared in phosphoric acid-methanol-water (0.5/80/19.5, v/v/v) and stored away from light at 4 °C. Working solutions of the lower concentration were prepared by an appropriate dilution of the stock solution.

2. Sample solution

DSI of 200 μl was diluted to 1 ml with deionized water and the dilution was injected into the HPLC system.

RESULTS AND DISCUSSION

Chromatographic separation

An aqueous acetonitrile solvent system was often used in the HPLC analysis of phenolic acids in DS (Wasser *et al*., 1998; Liu *et al*., 2000; Zhang *et al*., 2005; Zhou *et al*., 2006; Ma *et al*., 2007) and the modifiers were formic acid, acetic acid, or phosphoric acid. Water (0.5% (v/v) formic acid) and acetonitrile (0.5% (v/v) formic acid) were selected as the mobile phases in the experiment. From the UV spectra of these seven phenolic acids (Fig.2), 280 nm for DSS and PA, 326 nm for CA, RA and SaD, and 286 nm for SaB and SaA, were selected as detective wavelengths. Five column temperatures (15, 20, 25, 30 and 35 °C) were investigated and the separation of CA, SaD and SaB was found to be improved when the temperature stepped up. However, base-line separation of DSS was obtained at 30 °C (Fig.3). As a result, the column temperature was determined as 30 °C. The most suitable flow rate was found to be 1.0 ml/min. Finally the chromatograms, after optimization of the mixture of standard solution and the real sample solution, were described as in Fig.4.

Calibration curves and limits of detection

Integrated chromatographic peak areas (Y) were plotted against the corresponding concentrations (X, μg/ml) of the seven constituents in the standard solutions to obtain calibration curves based on linear regression analysis. The regression curves were obtained from six concentration levels and all the analytes had good linearity (R>0.999) in the investigated ranges (Table 1). Limit of quantification (LOQ) was defined as the lowest concentration level resulting in a peak height of 10 times the baseline noise (the signal-to-noise ratio (S/N) is 10). The minimum concentration, which could be calculated at S/N=3, was considered to be the LOD. The LOD values for seven target components were in the range of 0.008~0.160 μg/ml (Table 1), which were better than those (0.04~0.43 μg/ml) in a previous report (Wasser *et al*., 1998).

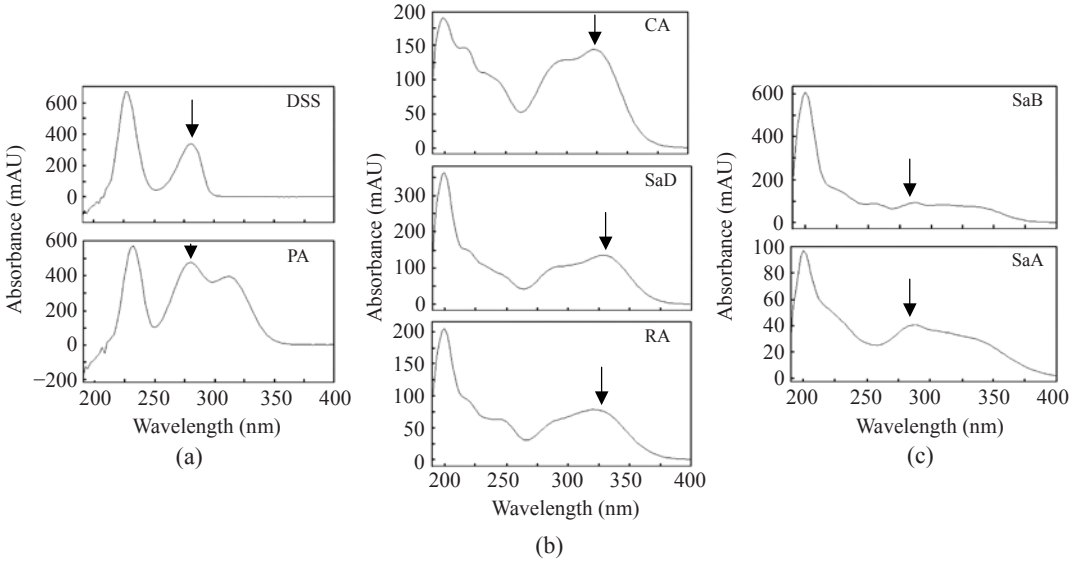

Fig.2 UV spectra of six target phenolic acids from DS. (a) 280 nm for DSS and PA, (b) 326 nm for CA, SaD and RA, and (c) 286 nm for SaB and SaA were selected as detective wavelengths

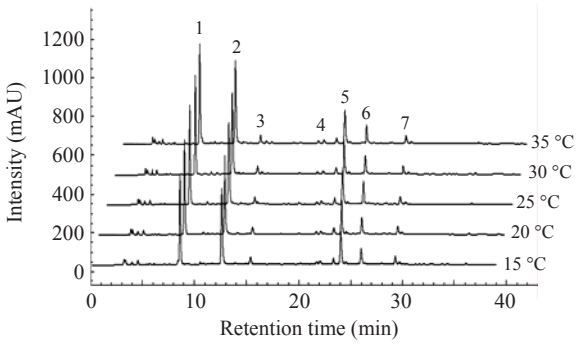

Fig.3 HPLC chromatograms (280 nm) obtained from using different column temperatures. The peaks marked with 1~7 were DSS, PA, CA, SaD, RA, SaB and SaA, respectively

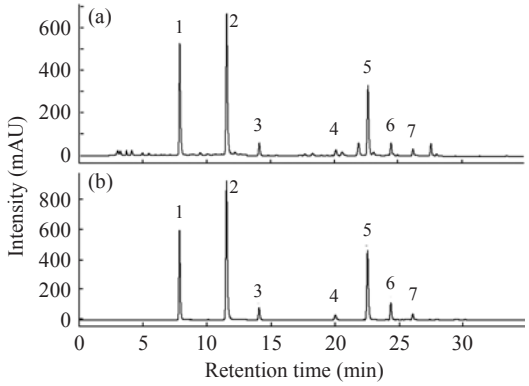

Fig.4 HPLC chromatograms at optimized conditions (280 nm in 0~13 min, 326 nm in 13~23.5 min, and 286 nm in 23.5~35 min, 1 ml/min, 30 °C). The peaks marked with 1~7 were DSS, PA, CA, SaD, RA, SaB and SaA, respectively. (a) Real sample solution; (b) Mixture of standard solution

Repeatability, precision and stability

The analysis repeatability was examined by using six duplicate samples from the same batch of DSI, treated according to the sample preparation procedure and analyzed with the established HPLC method. The stabilities of these seven compounds were tested at six time points (0, 2, 4, 8, 16 and 24 h) during 24 h. The result showed that DSS, PA, CA, SaD, RA and SaB were stable in the testing period, except for SaA with a stability of just 4 h (Table 2). According to this decomposing speed, it was almost certain that SaA in DSI became unstable just after opening the bottle of injections. After 4 h, the chromatographic peak of SaA in DSI had almost disappeared, while SaA in stock solution was found to be stable. It was thought to relate to their different pH values (about pH 7 in DSI and pH 3 in stock solution). The instability of SaA was thought to indicate that its relative standard deviation (*RSD*) value of repeatability analysis was more than 5% (Table 2).

The intra- and inter-day instrument precisions were determined by analyzing three different concentration solutions (low, medium and high) of authentic standards. The intra- and inter-day experiments were tested by injecting three times in a single day and three times on six successive days, respectively. The results are shown in Table 3. Due to its instability, the maximum *RSD* values in the precision test were obtained from SaA.

Table 1 Results of regression analysis on calibration curves and limits of detection (*n*=3)

Compounds	Retention time (min)	Test ranges (μg/ml)	Calibration curves	*R*	*LOQ* (μg/ml)	*LOD* (μg/ml)
DSS	7.85±0.05	40.50~607.50	Y=14.605X−31.357	0.9999	0.400	0.130
PA	11.50±0.05	10.20~183.60	Y=110.45X+95.782	0.9998	0.100	0.030
CA	14.05±0.05	1.00~15.00	Y=124.96X−0.2877	0.9990	0.025	0.008
SaD	19.96±0.05	3.45~34.50	Y=24.469X−1.8991	0.9994	0.160	0.050
RA	22.48±0.05	16.20~113.40	Y=80.06X−102.55	0.9993	0.150	0.050
SaB	24.31±0.05	10.00~90.00	Y=23.653X−11.357	0.9998	0.500	0.160
SaA	26.02±0.05	1.10~22.00	Y=62.356X−4.9326	0.9993	0.500	0.160

LOQ: Limit of quantification; *LOD*: Limit of defection

Table 2 Repeatability and stability of the target components (*n*=6)

Compounds	*RSD* for repeatability (%)	*RSD* for stability* (%)	Compounds	*RSD* for repeatability (%)	*RSD* for stability (%)
DSS	1.160	0.775	RA	0.712	0.763
PA	1.379	0.541	SaB	0.773	1.334
CA	0.757	1.806	SaA	9.178	5.743
SaD	2.476	3.401			

*Stability of SaA in 4 h and stability of others in 24 h; *RSD*: Relative standard deviation

Recovery test

In the experiment, three different quantities (low, medium and high) of authentic standards were added to sample solutions. Then the mixture solutions were analyzed using the developed HPLC-UV method mentioned above and the quantity of each component was subsequently calculated from the corresponding calibration curve. The result (Table 4) showed that the recovery of these six phenolic acids, except SaA, ranged from 94.32%~106.05%. In previous experiments, SaA was found to be unstable in DSI and relatively stable in phosphoric acid-methanol-water (stock solution). Due to this fact, the recoveries of SaA that were obtained were much higher than 100% at low concentration and lower than 100% at medium and high concentrations. These recoveries were tested from low concentration to high concentration.

Sample analysis

The developed method was applied to the simultaneous quantification of DSS, PA, CA, SaD, RA, SaB and SaA in DSIs from different batches. The result (Table 5) showed that as the unique target component for quality control of DSI, the content of PA approximated to 250 μg/ml in all of the samples, while contents of other components differed greatly among batches from the same company. The variety may result from the low level of quality standards. It is well known that the preparations of traditional Chinese medicines generally include multiple bioactive constituents while only one component (e.g. PA in DSI) was considered as the target for quality control. It meant that only the target compound was well monitored during the manufacturing process and other components were ignored.

Table 3 Analytical results of intra- and inter-day variabilities

Exp.	Comp.	Low concentration			Medium concentration			High concentration		
		Found (μg/ml)	*RSD* (%)	Accuracy* (%)	Found (μg/ml)	*RSD* (%)	Accuracy (%)	Found (μg/ml)	*RSD* (%)	Accuracy (%)
Intra-day (*n*=3)	DSS	41.97±0.016	0.038	103.62	203.06±0.115	0.057	100.28	322.84±0.153	0.047	88.57
	PA	9.76±0.003	0.032	95.71	52.48±0.046	0.087	102.91	93.25±0.089	0.096	101.58
	CA	0.96±0.092	0.097	95.59	4.36±0.004	0.094	109.00	7.087±0.008	0.113	101.24
	SaD	3.37±0.006	0.185	97.69	9.47±0.012	0.125	102.90	16.87±0.039	0.231	97.80
	RA	17.24±0.031	0.178	106.42	38.25±0.037	0.097	101.20	64.07±0.048	0.075	98.88
	SaB	10.08±0.066	0.656	100.84	29.38±0.049	0.166	97.94	52.43±0.015	0.029	104.85
	SaA	1.03±0.032	3.080	93.99	4.15±0.048	1.161	94.34	15.24±0.074	0.485	92.35
Inter-day (*n*=6)	DSS	42.12±0.182	0.432	104.02	203.34±0.919	0.452	100.42	321.24±1.485	0.462	88.13
	PA	9.81±0.068	0.689	96.19	52.76±0.416	0.789	103.45	93.55±0.687	0.734	101.91
	CA	0.96±0.007	0.742	96.15	4.39±0.037	0.844	109.79	7.10±0.041	0.578	101.44
	SaD	3.42±0.048	1.408	99.21	9.60±0.114	1.192	104.32	16.87±0.067	0.395	97.81
	RA	17.40±0.177	1.015	107.64	38.48±0.261	0.679	101.79	64.14±0.288	0.449	98.98
	SaB	10.32±0.220	2.130	113.42	29.62±0.204	0.688	98.74	52.50±0.365	0.695	105.00
	SaA	0.98±0.091	9.296	89.05	4.01±0.235	5.862	91.08	15.15±0.538	3.552	91.80

*Accuracy (%)=(mean of measured concentration)/(nominal concentration)×100

Table 4 Recovery test of developed method (*n*=3)

Comp.	Phenolic acid spiked (μg/ml)	Found (μg/ml)	*RSD* (%)	Recovery* (%)	Comp.	Phenolic acid spiked (μg/ml)	Found (μg/ml)	*RSD* (%)	Recovery (%)
DSS	114.82	120.52	0.361	104.97	RA	11.02	10.39	0.248	94.32
	143.17	150.21	0.395	104.92		13.50	13.46	0.081	99.70
	170.10	163.39	0.150	96.06		16.20	15.66	0.278	96.66
PA	22.03	23.05	0.112	104.62	SaB	6.50	6.28	0.871	96.61
	27.85	28.74	0.259	103.21		8.20	8.09	0.265	98.70
	33.15	32.59	0.087	98.32		9.80	9.31	0.718	94.97
CA	1.47	1.41	1.643	95.85	SaA	1.60	1.91	0.819	119.65
	1.85	1.96	1.474	106.05		1.98	1.64	1.610	82.92
	2.25	2.16	1.719	96.16		2.39	1.93	1.216	80.74
SaD	4.40	4.20	1.370	95.42					
	5.52	5.27	0.880	95.55					
	6.67	6.32	1.960	94.79					

* Recovery (%)=(amount found)/(amount spiked)×100

Table 5 Determination of seven phenolic acids in DSIs by the developed HPLC method

Batch No.	Content (μg/ml)						
	DSS	PA	CA	SaD	RA	SaB	SaA
0610261	1232.70	260.36	18.34	37.57	95.23	62.65	16.23
0607282	1448.54	288.97	18.57	76.14	173.64	86.94	31.11
0611291	1296.28	275.80	20.82	52.10	122.59	76.92	23.09
0611301	1245.27	254.05	16.37	54.92	133.73	100.60	28.11
0612011	1153.68	253.37	17.75	46.18	120.19	80.26	18.34
0612031	956.39	232.31	15.31	35.07	100.46	49.11	14.62
0610201	1352.65	263.70	16.94	54.18	163.86	70.07	21.15
0701051	1239.32	240.19	19.31	43.44	124.76	83.88	24.19
0612051	1066.27	256.42	19.28	32.96	110.59	55.54	14.02
0612061	1363.77	275.05	20.74	52.49	123.91	97.36	26.57
0703221	450.22	224.64	15.20	16.31	58.19	14.72	6.94
0612021	1352.92	263.58	22.40	50.21	129.32	87.37	23.37
0610251	1426.40	277.45	18.60	55.05	135.90	81.75	19.90
0610241	1446.65	225.00	16.25	65.85	171.05	94.10	24.20

CONCLUSION

In the present study, an HPLC method with ultraviolet detector has been developed for the simultaneous detection of seven phenolic acids of DSS, PA, CA, SaD, RA, SaB and SaA in DSI. This method was sensitive since the LOD values were improved to be in the range of 0.008~0.160 μg/ml. All of the target components were base-line separated during 27 min. This method was applied to analyze DSIs from different batches. Otherwise, SaA was found to be unstable in injections (pH 7). Therefore, the research into the transformation or degradation of SaA would be an interesting topic in future.

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