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Short communication

Simultaneous quantitative determination of 9 active components in traditional Chinese medicinal preparation *ShuangDan* oral liquid by RP-HPLC coupled with photodiode array detection

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ABSTRACT

A simple, accurate and reliable method for the simultaneous separation and determination of 9 active components (danshensu, protocatechuic acid, protocatechuic aldehyde, caffeic acid, rosmarinic acid, salvianolic acid B, paeonol, paeoniflorin and gallic acid) in traditional Chinese medicinal preparation *ShuangDan* (SD) oral liquid was developed using reverse phase high-performance liquid chromatography (RP-HPLC) coupled with photodiode array (PDA) detection. The chromatographic separation was performed on a SinoChrom ODS-BP C_{18} column with gradient elution using methanol (A) and 3% glacial acetic acid aqueous solution (B) at a flow rate of 1.0 mL min^{-1}, and with a PDA detection. Good linear behaviors over the investigated concentration ranges were observed with the values of r^2 higher than 0.9992 for all the analytes. The recoveries and relative standard deviation (RSD), measured at three concentration levels, varied from 98.21% to 101.82% and 0.07% to 1.37%, respectively. The proposed method enables the simultaneous identification and determination of 9 active components in a single run for the quality control of *ShuangDan* oral liquid.

1. Introduction

Traditional Chinese medicines (TCMs) and their preparations have been used as the most important therapeutic treatment in China for more than 2000 years. TCMs are commonly prescribed in combination to obtain synergistic effects or diminish potential adverse reactions. The preparation usually consisted of dozens of chemical components from several herbs. It is universally accepted that the joint contribution of multi-components are responsible for the synergistic and therapeutic effect of TCM [1]. Conventional approaches generally use one or few mark components to control the quality of TCM, which is insufficient to reveal the synergistic effects and complex constituents of traditional medicines. Thus, a more comprehensive and global strategy, which could cover most of the active chemical constituents, is valuable for the quality control of traditional medicine [2].

ShuangDan (SD) oral liquid, composed of two traditional Chinese medicines (*Radix Salvia Miltiorrhizae* and *Cortex Moutan*), is an effective traditional Chinese medicinal preparation that has been officially recorded in Chinese Pharmacopoeia for the treatment of coronary heart disease [3]. Based on a large amount of pharmacological research, the phenolic constituents in *Radix Salviae Miltiorrhiae* such as danshensu (DSS), protocatechuic acid (PCA), protocatechuic aldehyde (PA), caffeic acid (CA), rosmarinic acid (RA) and salvianolic acid B (Sal B), marker constituents in *Cortex Moutan* such as paeonol (Pae), paeoniflorin (PF) and gallic acid (GA), were found to be responsible for the biological activities in the two TCMs and proved to be the active components contained in *ShuangDan* oral liquid [4–11]. Their chemical structures are shown in Fig. 1.

Currently, only a few analytical methods has been reported to determine the active components in *ShuangDan* oral liquid, including high-performance liquid chromatography-diode array detection–electrospray ionization tandem mass spectrometry (HPLC-DAD–ESI-MS) [12] and micellar electrokinetic capillary chromatography (MEKC) [13–15]. However, MEKC method developed by Yu et al. [13–15] can simultaneously determine at the most four components of SD in a single running, while He et al. [12] only focused on the qualitative analysis of chemical constituents in SD and lacked the information of quantitative determination for quality control. To date, the methods for the simultaneous separation and quantitative determination of multiple active components in *ShuangDan* oral liquid for quality control in a single running are not available. Therefore, an accurate and reliable method is needed

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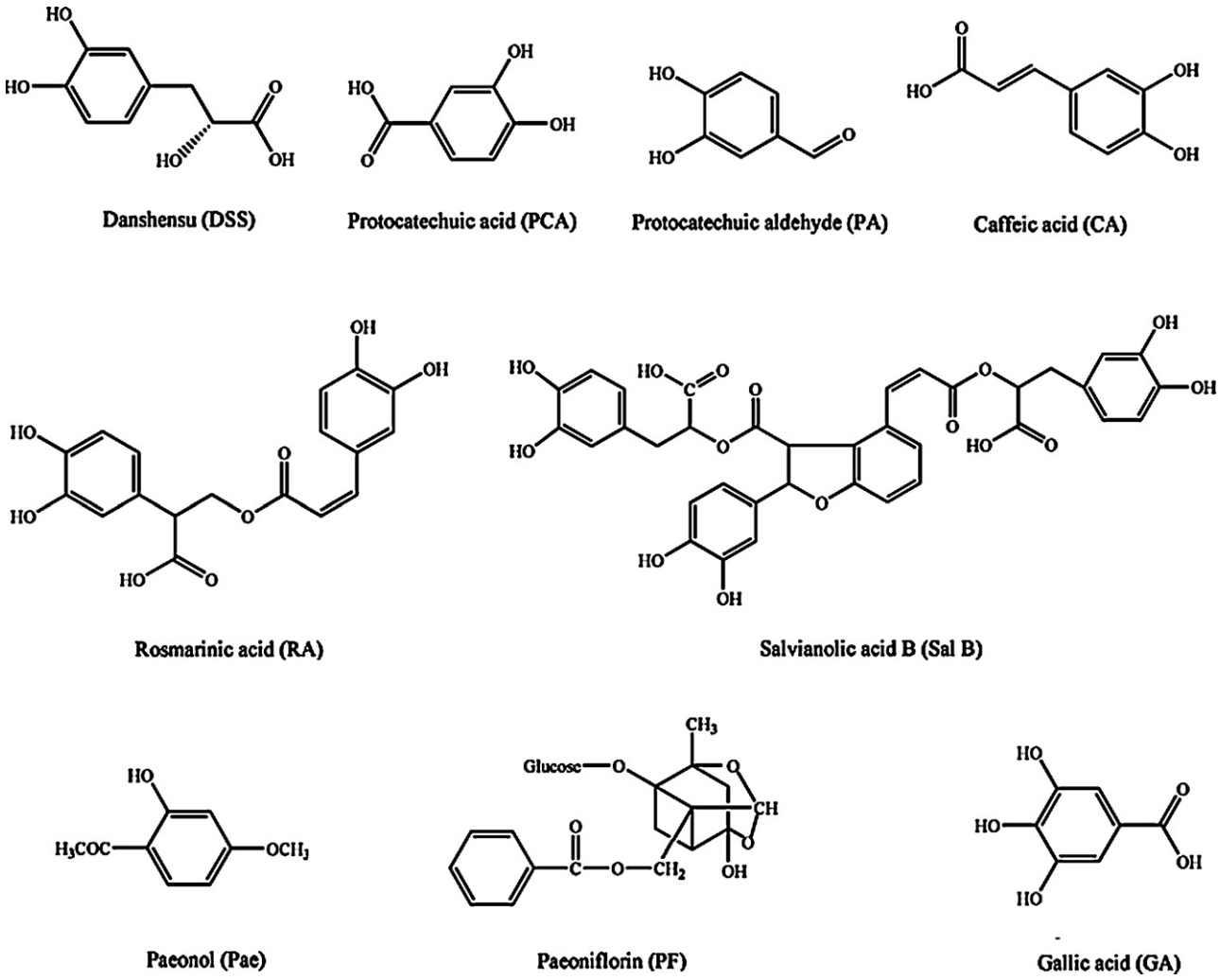

Fig. 1. Chemical structures of the 9 active components contained in *ShuangDan* oral liquid.

for the quality control of this famous traditional Chinese medicinal preparation.

In this study, a simple, accurate and reliable analytical method for the simultaneous quantitative determination of 9 active components (including DSS, PCA, PA, CA, RA, Sal B, Pae, PF and GA) contained in *ShuangDan* oral liquid was developed using reverse phase high-performance liquid chromatography (RP-HPLC) coupled with photodiode array (PDA) detection. The developed HPLC–PDA coupled method is very simple, particularly suitable for the routine analysis of *ShuangDan* oral liquid and its quality control.

2. Experimental

2.1. Chemicals and reagents

The standards of DSS, PCA, PA, CA, RA, Sal B, Pae, PF and GA were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China); Methanol (HPLC grade) was obtained from Honeywell (Muskegon, MI, USA). Glacial acetic acid (HPLC grade) was purchased from Mallinckrodt Baker (Phillipsburg, NJ, USA). The deionized water was prepared from Millipore water purification system (Milford, MA, USA) and filtered with a 0.22 μm membrane. Other reagents were all of analytical grade.

2.2. Apparatus and chromatographic conditions

An Waters 2695 Alliance HPLC system (Waters Corp., Milford, MA, USA), equipped with Empower™ Software and comprised of a quaternary solvent delivery system, an on-line degasser, an autosampler, a thermostated compartment and a 2996 photodiode array detection, was used for the chromatographic analysis. All separations were performed on a SinoChrom ODS-BP C_{18} column (250 mm × 4.6 mm, 5 μm, Yilite) and a C_{18} guard column was used before the analytical column. The mobile phase was composed of methanol (A) and 3% glacial acetic acid aqueous solution (B) with gradient elution (0–10 min, 8–15% A; 10–30 min, 15–25% A; 30–50 min, 25–35% A; 50–80 min, 35–50%). Re-equilibration duration was 5 min between individual runs. The flow rate of the mobile phase was 1.0 mL min^{-1}, and the temperature was maintained at 30 °C. The components were quantified based on peak areas at the maximum wavelength in their UV spectrum.

2.3. Preparation of standard solutions

A standard stock solution containing the 9 components (DSS 0.15 mg mL^{-1}, PCA 0.01 mg mL^{-1}, PA 0.10 mg mL^{-1}, CA 0.01 mg mL^{-1}, RA 0.03 mg mL^{-1}, Sal B 0.15 mg mL^{-1}, Pae 0.15 mg mL^{-1}, PF 0.02 mg mL^{-1} and GA 0.10 mg mL^{-1}) was prepared in 50% methanol aqueous solution and stored away from light at 4 °C. Working standard solutions containing the 9

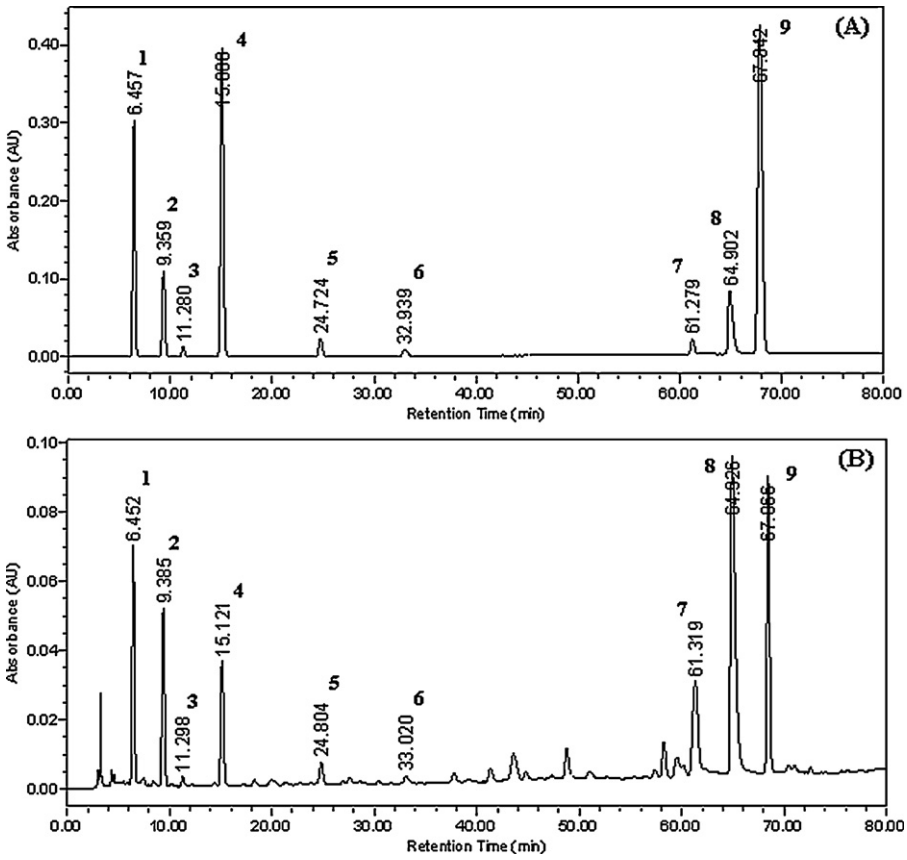

Fig. 2. Typical chromatograms of the standard mixture (A) and *ShuangDan* oral liquid (B) at 280 nm. (1) Gallic acid; (2) danshensu; (3) protocatechuic acid; (4) protocatechuic aldehyde; (5) caffeic acid; (6) paeoniflorin; (7) rosmarinic acid; (8) salvianolic acid B; (9) paeonol.

compounds were prepared by appropriate dilution of the stock solution.

2.4. Preparation of sample solutions

One milliliter of *ShuangDan* oral liquid was accurately diluted to 50 mL with 50% methanol aqueous solution. The solution was centrifuged at 12,000 × *g* for 15 min and was filtered with a 0.45 μm microporous membrane prior to analysis. Aliquot (20 μL) of sample solution was injected into the HPLC system for analysis.

3. Results and discussion

3.1. Optimization of HPLC conditions

In general, a suitable chromatographic column, mobile phase, elution mode and detection wavelength are critically important for good separation. In the present study, different columns packed with different materials, i.e. Yilite Hypersil BDS C_{18} column, Yilite SinoChrom ODS-BP C_{18} column and Phenomenex Luna 5u C_{18} column were employed. Various mobile phases consisting of acetonitrile–water and methanol–water with some modifiers including phosphoric acid, glacial acetic acid, formic acid, and formic acid solutions adjusted by ammonia or triethylamine with different pH values were investigated under different gradient elution modes, flow rate was also optimized. The detection wavelength was selected according to the maximum adsorption wavelengths of DSS, PCA, PA, CA, RA, Sal B, Pae, PF and GA at 281, 260, 280, 324, 331, 287, 276, 231 and 273 nm, respectively, shown in UV spectra with three dimension chromatograms of photodiode array detection. The desired components from *ShuangDan* oral liquid were identified by comparing both the retention times and UV spectra with those of the authentic standard. After many tests, SinoChrom ODS-BP C_{18} column with the methanol–3% glacial acetic acid solution system using gradient elution was found suitable for the simultaneous separation and determination. Excellent agreement between

Table 1
Regression equation, linear range and LODs of the developed method.

Constituent	Regression equation[a]	Correlation coefficient (r^2)	Linearity range (μg mL^{-1})	LOD (μg mL^{-1})
Gallic acid	$y = 59227x - 16660$	0.9996	5.0–100.0	0.13
Danshensu	$y = 15417x - 6724.3$	0.9997	7.5–150.0	0.36
Protocatechuic acid	$y = 63419x - 1162.9$	0.9995	0.5–10.0	0.1
Protocatechuic aldehyde	$y = 84891x + 2451.3$	0.9998	5.0–100.0	0.15
Caffeic acid	$y = 107704x - 5209.9$	0.9993	0.5–10.0	0.17
Paeoniflorin	$y = 22992x - 13377$	0.9992	1.0–20.0	0.45
Rosmarinic acid	$y = 30517x - 6621.6$	0.9997	1.5–30.0	0.38
Salvianolic acid B	$y = 20699x - 25146$	0.9999	7.5–150.0	0.63
Paeonol	$y = 97918x + 8373.2$	0.9997	7.5–150.0	0.17

[a] *y*: peak area of components; *x*: concentration of components.

Table 2
The precision data of the proposed HPLC method.

Components	Nominal concentration ($\mu g\,mL^{-1}$)	Precision			
		Intra-day ($n=5$)		Inter-day ($n=3$)	
		Mean ± SD ($\mu g\,mL^{-1}$)	RSD (%)	Mean ± SD ($\mu g\,mL^{-1}$)	RSD (%)
Gallic acid	10.00	10.00 ± 0.06	0.60	9.99 ± 0.05	0.47
	40.00	39.71 ± 0.06	0.16	39.73 ± 0.06	0.15
	80.00	79.72 ± 0.01	0.01	79.73 ± 0.01	0.01
Danshensu	15.00	14.98 ± 0.05	0.32	14.95 ± 0.05	0.34
	60.00	60.42 ± 0.23	0.34	60.37 ± 0.14	0.24
	120.00	119.06 ± 0.02	0.02	119.11 ± 0.07	0.06
Protocatechuic acid	1.00	0.99 ± 0.01	0.52	0.99 ± 0.01	0.64
	4.00	4.01 ± 0.00	0.06	4.01 ± 0.01	0.28
	8.00	8.02 ± 0.02	0.20	8.00 ± 0.03	0.32
Protocatechuic aldehyde	10.00	9.98 ± 0.02	0.17	9.97 ± 0.03	0.34
	40.00	39.94 ± 0.04	0.10	39.97 ± 0.06	0.14
	80.00	80.02 ± 0.04	0.05	80.02 ± 0.05	0.13
Caffeic acid	1.00	1.01 ± 0.00	0.10	1.00 ± 0.00	0.25
	4.00	4.01 ± 0.00	0.10	4.01 ± 0.00	0.11
	8.00	8.01 ± 0.00	0.13	8.00 ± 0.01	0.15
Paeoniflorin	2.00	2.01 ± 0.01	0.41	2.01 ± 0.01	0.54
	8.00	7.99 ± 0.02	0.26	8.00 ± 0.02	0.30
	16.00	16.11 ± 0.04	0.22	16.1 ± 0.04	0.22
Rosmarinic acid	3.00	3.00 ± 0.00	0.08	3.00 ± 0.01	0.20
	12.00	12.02 ± 0.03	0.24	12.02 ± 0.02	0.20
	24.00	23.98 ± 0.01	0.06	23.98 ± 0.02	0.07
Salvianolic acid B	15.00	14.05 ± 0.01	0.10	14.06 ± 0.02	0.16
	60.00	59.97 ± 0.01	0.01	60.00 ± 0.03	0.06
	120.00	119.98 ± 0.14	0.11	119.95 ± 0.11	0.10
Paeonol	15.00	14.06 ± 0.01	0.07	14.06 ± 0.01	0.06
	60.00	59.91 ± 0.01	0.02	59.92 ± 0.05	0.08
	120.00	119.97 ± 0.04	0.03	119.95 ± 0.05	0.04

standard and sample spectra was found in all analyzed samples, indicating that under the proposed analytical conditions, the 9 marker constituents were sufficiently resolved and successfully separated. Typical chromatograms of the authentic standards and *ShuangDan* oral liquid (batch number: 100506) are shown in Fig. 2.

3.2. Method validation

3.2.1. Calibration curves and the limit of detection

All calibration curves were plotted based on linear regression analysis of the integrated peak areas (y) versus concentrations (x, $\mu g\,mL^{-1}$) of the 9 marker constituents in the standard solution at seven different concentrations. The regression equations, correlation coefficients, and linear ranges for the analysis of the 9 marker constituents are shown in Table 1.

The limit of detection value (LOD) was calculated as the amount of the injected sample which gave a signal-to-noise ratio of 3 (S/N = 3). The LOD values of the method for the 9 components are also listed in Table 1.

3.2.2. Precision, accuracy and stability

The relative standard deviation (RSD) was taken as a measure of precision and accuracy. Intra- and inter-day precisions were determined by assaying standard solutions at three concentrations during a single day and on five consecutive days, respectively. As shown in Table 2, the overall intra- and inter-day variations was less than 1.67% for all 9 analytes. The accuracy tests were carried out using a recovery test as followings: three different quantities (low, medium and high) of authentic standards were added into samples. The resultant samples were processed and analyzed as described in Section 2.4. The quantity of each analyte was subsequently obtained from the corresponding calibration curve. Recovery of all 9 tested bioactive constituents was within the range of 98.21–101.82%, with an RSD of between 0.07% and 1.37% ($n=3$). From the results of precision test and recovery test, it was known that the method manifested good precision and accuracy.

For stability test, the same sample solution was analyzed every 12 h in 3 days at the room temperature. The RSD values of the peak area and retention times were no more than 5.9% and 2.0%, respectively. The solution was therefore considered to be stable within 72 h.

3.3. Sample analysis

The newly established method has been applied to the determination of the 9 marker constituents in two commercial production batches of *ShuangDan* oral liquid. As shown in Fig. 2 and Table 3, under the analytical conditions, the 9 marker constituents (dan-

Table 3
Content of the 9 active components in two batches of *ShuangDan* oral liquid.

Components	Content (mean ± SD, $\mu g\,mL^{-1}$)	
	100506 ($n=3$)	101021 ($n=3$)
Gallic acid	892.43 ± 17.96	951.47 ± 4.26
Danshensu	2663.52 ± 7.55	2805.41 ± 9.91
Protocatechuic acid	93.55 ± 0.98	92.62 ± 0.16
Protocatechuic aldehyde	365.76 ± 2.11	398.24 ± 0.27
Caffeic acid	100.96 ± 0.14	127.95 ± 0.50
Paeoniflorin	108.76 ± 0.92	91.64 ± 0.50
Rosmarinic acid	1333.12 ± 5.61	1465.80 ± 3.46
Salvianolic acid B	7412.12 ± 29.18	7472.59 ± 18.18
Paeonol	627.22 ± 2.61	634.79 ± 0.57

shensu, protocatechuic acid, protocatechuic aldehyde, caffeic acid, rosmarinic acid, salvianolic acid B, paeonol, paeoniflorin and gallic acid) in *ShuangDan* oral liquid can be sufficiently resolved and separated, which is suitable for the routine analysis and quality control of commercial *ShuangDan* oral liquid.

4. Conclusion

An accurate and reliable HPLC method to simultaneously determine multiple active components in traditional Chinese medicinal preparation *ShuangDan* oral liquid was developed. This is the first report for the simultaneous determination of 9 major active components in *ShuangDan* oral liquid by using reverse phase high-performance liquid chromatography coupled with photodiode array detection. The proposed method is promising to be the routine analysis for *ShuangDan* oral liquid and its quality control with high simplicity, precision, accuracy and reliability.

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