Supplementary materials for

A strategy for integrated pharmacokinetic study of cardiovascular herbal medicines based on chemiluminescence and HPLC-MS/MS assays: A case using Danshen injection

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Simultaneous determination of five active phenolic compounds in Danshen Injection by HPLC−UV method

MATERIALS AND METHODS

Materials and Reagent

Sodium danshensu (DSSN), protocatechuic acid (PCA), protocatechuic acid, caffeic acid, protocatechuic aldehyde (PCAL), caffeic acid (CA), rosmarinic acid (RA), salvianolic acid A (SAA) and salvianolic acid B (SAB) were purchased from Shanghai Ronghe Medicine Technology Development Co. Ltd. (Shanghai, China). The purities of all the compounds were determined to be above 98% by HPLC analysis. Danshen freeze-dried powder injections (batch no. 1501720) were obtained from local hospital in Fuzhou.

Acetonitrile and methanol were of chromatographic grade (Merk KGaA, Darmstadt, Germany). Double distilled water for HPLC analysis was prepared in our lab. Glacial acetic acid was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Chromatographic Conditions

Analyses were performed using an Agilent-1260 series HPLC instrument (Agilent Technologies, USA) equipped with a low pressure quaternionic pump, an auto-sampler, a column compartment and diode-array detection (DAD). All separations were carried out on an Ultimate^{TM} XB-C_{18} column (4.6 mm × 50 mm, 3.5 μm). The mobile phase consisted of acetic acid in water (0.5%, v/v) (A) and acetonitrile (B). A gradient program was carried out as follows: 0–1 min, start with 15% B, then linearly

increase to 20% B; 1–2.5 min, linearly increase to 22% B; 2.5–4.5 min, linearly increase to 28% B; 4.5–7.5 min, linearly increase to 38% B; 7.6–8.5 min, 95% B; then decrease to 15% B at 8.6–10 min. The flow rate was 0.6 mL/min^{-1}, and the temperature of column oven was 30 ◦C. The detection wavelength was set at 288 nm.

Standard Solutions Preparation

Standard stock solutions of danshensu (3.465 mg/ml), protocatechuic aldehyde (1.850 mg/ml), rosmarinic acid (2.250 mg/ml), salvianolic acid A (2.35 mg/ml) and salvianolic acid B (2.15 mg/ml) were prepared in 2 mL volumetric flasks, respectively, and diluted with methanol to obtain appropriate concentrations for the establishment of calibration curves. All solutions were stored in a refrigerator (4 ◦C) until analysis.

Sample preparation

50 mg of freeze-dried powder injection was weighed accurately and dissolved to 50 ml with methanol. The sample solutions were filtered through a 0.45 μm membrane filter before HPLC analysis.

RESULTS

Validation of the Method

The methods were validated by assessing linearity of the calibration curve, precision, repeatability, stability and recovery. The typical Chromatograms are shown in Figure 1.

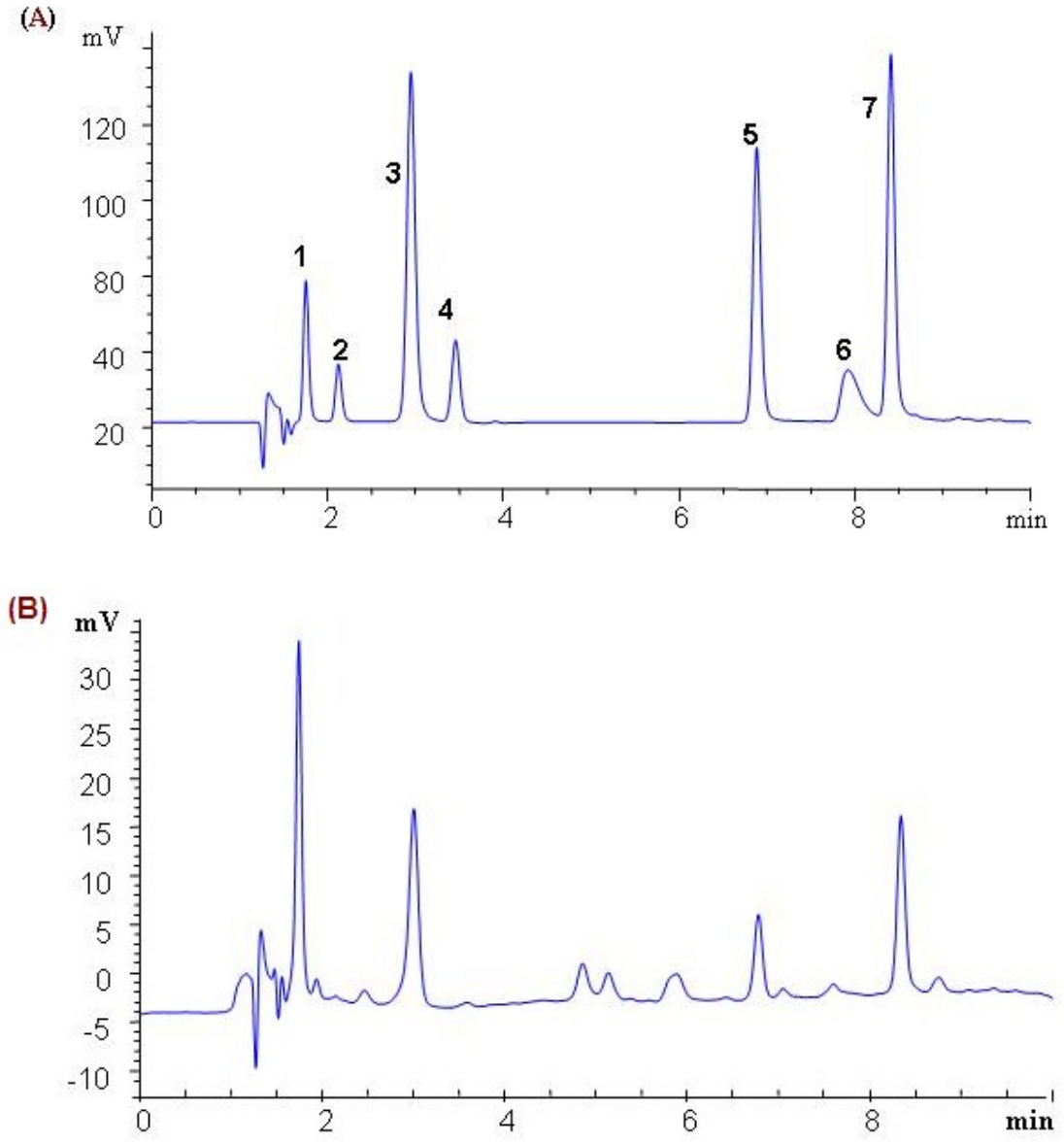

Figure 1. Chromatograms of reference substances (A) and Danshen freeze-dried powder injection (B). Peaks 1-7 represent danshensu, protocatechuic acid, protocatechuic aldehyde, caffeic acid, rosmarinci acid, salvianolic acid B and salvianolic acid A, respectively.

The calibration curve for each component was established with at least eight different concentrations by plotting the peak area versus concentration. All the compounds showed a good linearity (R^2 > 0.9982) in the relatively wide concentration range (Table 1). LOD was in the range of 0.44–1.68 μg/ml, and LOQ was in the range

of 0.92–3.56 μg/ml.

Table 1. Calibration curves, linear range, LOD, and LOQ of five components

Analytes	Calibration curves[a]	Linear range (μg/mL)	R^2 (n = 8)	LOD[b] (μg/mL)	LOQ[c] (μg/mL)
DSSN	$y = 1.9237\,x + 2.4152$	1.35–346.5	0.9998	0.68	1.35
PCAL	$y = 14.22\,x + 8.1883$	1.44–185	1	0.72	1.44
RA	$y = 8.5943\,x + 3.372$	0.88–225	0.9999	0.44	0.88
SAB	$y = 4.0941\,x - 5.9047$	3.56–215	0.9996	1.68	3.56
SAA	$y = 10.105\,x + 20.597$	0.92–235	0.9982	0.46	0.92

[a] y: peak area of analyte; x: concentration of analyte (μg/mL).

[b] Limits of detection were evaluated at signal-to-noise ratios (S/N) of 3.

[c] Limits of quantification were evaluated at signal-to-noise ratios (S/N) of 10.

The precision was tested by assaying the mixed authentic standard solution samples within 1 day in six times. The relative standard deviation (R.S.D.) was taken as a measure of precision. The results were in the range of 1.82 – 5.70% (Table 2).

Table 2. Precision, repeatability and stability of five components (n = 6)

Analytes	R.S.D. for precision (%)	R.S.D. for repeatability (%)	R.S.D. for stability (%)
DSSN	3.97	0.74	3.45
PCAL	2.04	2.42	0.76
RA	1.82	1.63	9.78
SAB	3.35	5.70	6.76
SAA	5.70	1.06	8.30

To evaluate the repeatability of the developed assay, six samples from the same batch of Danshen injection were treated according to the sample preparation procedure and analyzed with the established method. The R.S.D. of each compound ranged from 0.74 to 5.7% (Table 2).

The stability was confirmed with a sample of diluted Danshen injection at room

temperature and analyzed at 0, 2, 4, 8 and 12 h within 1 day. The R.S.D. values were all less than 10% (Table 2).

The recovery was determined by spiking accurately known amounts of the authentic standard solution to the diluted Danshen injection samples, and then the mixture solutions were analyzed with the described method. The content of each component was calibrated from the corresponding calibration curve. The recovery was in the range of 92.1 – 103.6%, with R.S.D. less than 2.7%.

Sample Analysis

The optimized method was applied to the simultaneous quantification of five components in Danshen freeze-dried powder injection. The contents (n = 3) of the five compounds analyzed were listed in Table 3.

Table 3. Contents of five components in Danshen freeze-dried powder injection (*n* = 3)

Analytes	Danshen freeze-dried powder injection (mg/g)	Content (%) in Danshen freeze-dried powder
Danshensu sodium	554.13	55.41
protocatechuic aldehyde	40.55	4.06
Rosmarinic acid	21.66	2.17
Salvianolic acid B	1.67	0.17
Salvianolic acid A	68.60	6.86
Total	686.61	68.66