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Chemical fingerprint and metabolic fingerprint analysis of Danshen injection by HPLC–UV and HPLC–MS methods

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Abstract

HPLC–UV and HPLC–MS techniques were used in fingerprint analysis of Danshen injection and its raw materials (roots and rhizoma of *Salvia miltiorrhiza*). HPLC profiles of Danshen injections from a Chinese pharmaceutical factory and their raw materials were established as their characteristic fingerprint and employed to assess their consistency and difference. To develop the representative fingerprint of Danshen injection, 10 batches of samples were analyzed under the same HPLC conditions. The results showed that 10 batches of Danshen injections had very similar HPLC fingerprints. To characterize the major constituents of Danshen injection for quality control, 11 major chromatographic peaks were characterized by their MS spectra and comparison with the reference standards. Through comparison of the HPLC profiles of Danshen injection with its raw material, it was found that they are greatly different, which indicated the changes of major constituents in the course of preparation procedure. In addition, the rat's plasma was analyzed by HPLC–MS technique after intravenous administration of Danshen injection at different time intervals to explore the in vivo metabolism of the major active constituents. Except for protocatechuic aldehyde, the major phenolic acids in Danshen injection appeared in rat's plasma after intravenous administration, but quantity of each phenolic acids was very different from that in Danshen injection. With the administration time prolonged danshensu and salvianolic acid B disappeared quickly, salvianolic D, lithospermic acid and salvianolic A slowly decreased and maintained relatively high concentration after 30 min of intravenous administration. This indicated that polyphenolic acids were significant for biological acitivity of Danshen injection. It might be concluded that chemical fingerprint combined with metabolic fingerprint is a useful means to control the quality and to clarify the possible mechanism of action of herbal products.
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Keywords: Chemical fingerprint; Metabolic fingerprint; Danshen injection; *Salvia miltiorrhiza*; HPLC–MS technique

1. Introduction

The quality control has always been the key issue in the development of herbal medicines. Fingerprint technique is a powerful tool for the quality control of multi-component herbal medicines and have been widely accepted as a useful means for the evaluation and quality control of herbal materials and their finished products [1,2].

There have been a number of reports regarding the use of HPLC, CE, TLC, NIR, IR fingerprints on the quality assessment of some herbal medicines and their raw materials [3].

Danshen injection made from the aqueous extracts of *Salvia miltiorrhiza* Bunge is one of most widely used traditional Chinese preparation to treat coronary heart disease, heart-stroke and cerebrovascular diseases. It also has good clinical efficacy on hepatitis, hepatocirrhosis and chronic renal failure. The chemical constituents of *S. miltiorrhiza* cover two chemical types: diterpenoid quinones and water-soluble phenolic acids. Before 1970s, the studies were mainly focused on the lipophilic diterpenoid, which have been considered to be responsible for the clinical efficacy [4]. But due to the widely use of Danshen injection in clinical treat-

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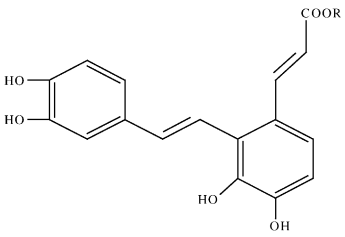
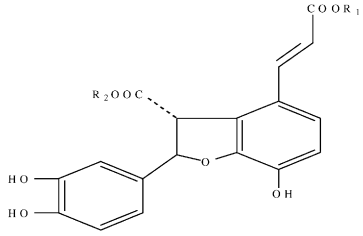


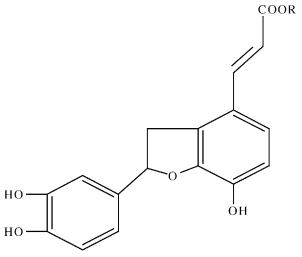
salvianolic acid A (M.W. 494), R = X



salvianolic acid B (M.W. 718), R_1 =R_2 = X

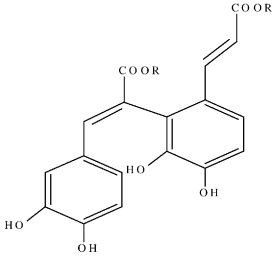
salvianolic acid F (M.W. 314), R = H

linthospermic acid(M.W. 538), R_1 = X, R_2 = H



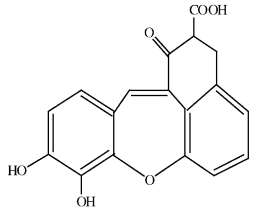
salvianolic acid C (M.W. 492)

R = X

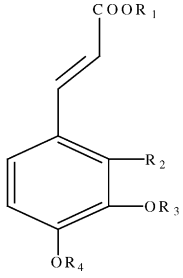


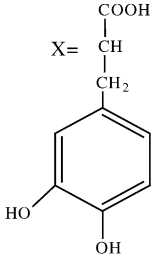
salvianolic acid E (M.W. 718)

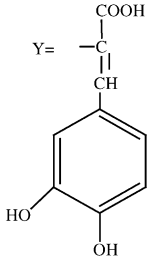
R = X

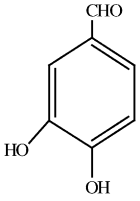


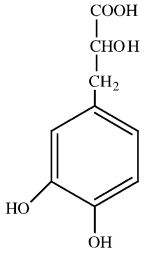
salvianolic acid G (M.W. 340)











salvianolic acid D (M.W. 418), R_1 = X, R_2 = CH2COOH, R_3 = R_4 = H

protocatechuic aldehyde

Danshensu

salvianolic acid I (M.W. 538), R_1 = X, R_2 = R_3 = H, R_4 = Y

salvianolic acid H (M.W. 538), R_1 = X, R_2 = R_4 = H, R_3 = Y

rosmarimic acid (M.W. 360), R_1 = X, R_2 = R_3 = R_4 = H

Fig. 1. Carbon skeletons of polyphenolic acids.

ment, the water-soluble constituents in *S. miltiorrhiza* were investigated and over 15 phenolic acids have been isolated and identified [5–7]. And through pharmacological and clinical investigation, these phenolic acids were found to be the real active principles other than the lipophilic diterpenoids as reported previously. The chemical structures and molecular weight of polyphenolic acids are shown in Fig. 1.

The pharmacological studies on the phenolic acids of *S. miltiorrhiza* showed that the polyphenolic acids such as salvianolic acid A and B possessed more potent biological activities than single phenolic acids such as danshensu, caffeic acid and protocatechuic aldehyde. But in the quality standard of Danshen injection, only danshensu and protocatechuic aldehyde were quantified and used as marker compounds for the quality control of preparation. Hence, this existed quality

control standard could not reflect the real and comprehensive active constituents of Danshen injection, and therefore inadequate to control the quality of Danshen injection [8,9].

The present study aimed at developing the HPLC–UV fingerprint of Danshen injection and characterizing the major active constituents of Danshen injection. Then the fingerprint model could accurately reflect the quality and guarantee clinical efficacy of Danshen injection. The metabolic HPLC fingerprint profiles were developed after intravenous administration of Danshen injection in rats at different time intervals to clarify the mechanism of clinical efficacy of Danshen injection. Chemical fingerprint combined with metabolic fingerprint would be potentially useful to establish the suitable quality control models to control the quality and to guarantee the clinical efficacy of Danshen injection.

2. Experimental

2.1. Chemicals

Acetonitrile was of HPLC grade from Caledon, Canada. Methanol was of GR grade from Peking Chemical Factory. Acetic acid and EtOAc were of analytical grade from Peking Chemical Factory. Physiological saline was purchased from Shijiazhuang Pharmaceutical Cooperation (Hebei province China). Protocatechuic aldehyde was purchased from the National Institute for the Control of Biological and Pharmaceutical Drugs (China). Danshensu was a gift from Shanghai Medical University. Salvianolic acid B was isolated from the roots of *S. miltiorrhiza* in the group, purity of which was over 97%. Danshen injection and its raw materials were provided by a Chinese Pharmaceutical Factory. Heparin sodium was from Shanghai Biochemical Reagent Co. Ltd. Pentobarbital sodium was obtained from Fuoshan Chemical Factory. Heparin sodium and Pentobarbital sodium were each dissolved in physiological saline at the concentration of 50 mg/ml and 200 μg/ml, respectively. Heperin solution was used to rinse the test tubes prior to blood collection for plasma.

2.2. Instrumentation and conditions

HPLC–UV analysis was carried out on a Agilent 1100 Series HPLC with diode-array detector using a 5 μm Zobax Extend RP-18 column (4.6 mm × 250 mm). The column was maintained at 30 °C. Detection wavelength was 288 nm. The flow rate was 0.8 ml/min. A gradient elution of A (acetonitrile) and B (1% aqueous acetic acid) was used starting and keeping with 12% A and 88% B for 5 min, then to reach 20% A and 80% B at 35 min and 23% A and 77% B at 45 min, finally to reach 30% A and 70% B at 55 min. Then the system was recovered to initial conditions after 10 min. HPLC–MS was performed with a Agilent 1100 Series HPLC and PE SCIEX QSTAR MASS. The HPLC conditions were the same as above. The mass spectra were recorded using ESI in the negative mode with ion spray voltage at 3300 eV, source temperature at 400 °C, gas spray 1 at 60 psi, Gas spray 2 at 40 psi, current gas at 40 psi, desolvent voltage 1 at 40 eV, desolvent voltage 2 at 15 eV, focus voltage at 215 eV and scanning from 100 to 1500 amu. A centrifuge made in Shanghai Medicinal Instrumentation Factory was used.

2.3. Animal and biological sample collection

Twelve male SD rats (200 ± 20 g body weight) were provided by Animal Center of Health Science Center of Peking University. Prior to intravenous administration of Danshen injection, 12 rats were fasted in metabolic cage for 24 h maintaining with physiological saline. After 5-min intravenous administration of pharmacological saline at the dose 0.8 ml/kg body weight, three rats were anaesthetized by pentobarbital sodium and blank blood was collected from abdominal artery in clean heparinized glass tubes. Blank blood was centrifuged at 3500 rpm to separate blank plasma. Other nine rats were intravenously administrated Danshen injection at the dose of 0.8 ml/kg body weight. Blood contained the chemical constituents from Danshen injection was collected as above workup after 5, 10 and 30 min of administration. The corresponding plasma contained chemical constituents from Danshen injection were obtained.

2.4. Sample preparation

Danshen injection was diluted with deionized water at the ratio of 1 to 3 and filtrated through 0.45 μm filter for HPLC–UV and HPLC–MS analysis. The total phenolic acids in roots of *S. miltiorrhiza* was obtained by following extraction method. The root powder of *S. miltiorrhiza* (0.5 g) was immersed in 30 ml deionized water overnight and extracted under thermal reflux for 2 h. The aqueous extracts were filtrated and the filtrate was adjusted to pH 2.5, then extracted with EtOAc of three-fold volume. The EtOAc was evaporated to dryness under reduced pressure at 30 °C. The residue was dissolved in methanol and filtrated through 0.45 μm filter for HPLC–UV and HPLC–MS analysis. Rat plasma was adjusted to pH 2.5 and extracted with equal volume EtOAc for three times. The EtOAc fractions were collected and evaporated to dryness under reduced pressure at 30 °C. The residue was dissolved in methanol and filtered through a 0.45 μm filter for HPLC–UV and HPLC–MS analysis. Protocatechuic aldehyde, danshensu and salvianolic acid B were all dissolved in methanol for HPLC–UV analysis.

3. Results and discussion

3.1. HPLC–UV fingerprint analysis of Danshen injection and the raw material

Danshen injection and its corresponding raw materials which were used to produce Danshen injection were analyzed under the established HPLC conditions. The HPLC–UV pro-

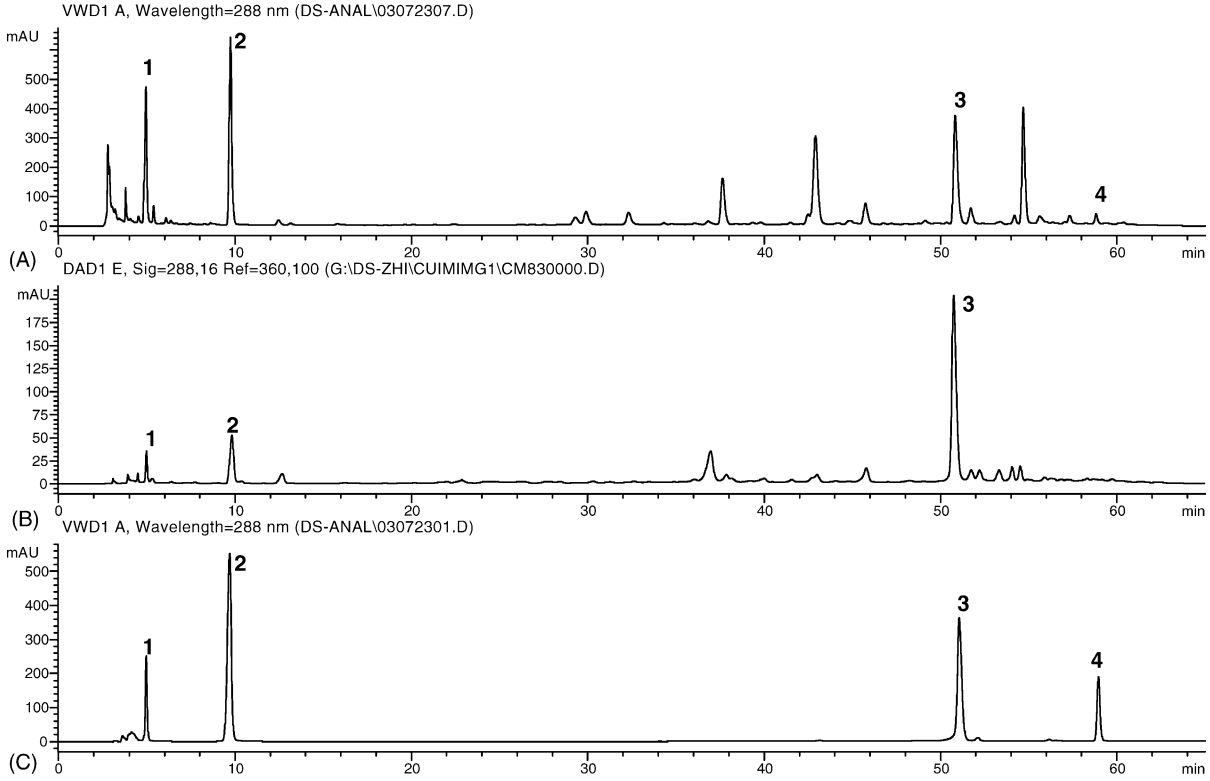


Fig. 2. HPLC–UV profiles of Danshen injection (A), its raw material (B) and standards (C). Peaks represented: 1, danshensu; 2, protocatechuic aldehyde; 3, salvianolic acid B; and 4, salvianolic acid C.

files of Danshen injection and the total phenolic acid in raw material were presented in Fig. 2A,B. The constituents in Danshen injection and raw material were well separated under the established HPLC conditions. Three main phenolic acids were recognized by comparing the retention times and UV spectra with standards of danshensu, protocatechuic aldehydrate and salvianolic acid B. The HPLC chromatogram of three reference standards was shown in Fig. 2C. In order to obtain stable and repeatable chromatographic fingerprint of Danshen injection for quality control, the method validation of HPLC fingerprint analysis was performed on the basis of the retention time and the peak area. The sample solution of Danshen injection was successively injected into HPLC system for six times. The precisions of retention times and peak areas of all peaks were not exceeding 0.05% and 3.7%, respectively. The stability test was performed with sample solution of Danshen injection for 12 h. The RSDs of peak areas were less than 6.3%, which indicated that the sample solution was stable within 12 h. To establish the representative chromatographic fingerprint, 10 batches of Danshen injec-

Table 1
Peak area ratio of common peaks from batch to batch

Common peak no.	Peak area ratio from batch to batch (%)									
1	7.69	8.60	7.90	8.20	10.20	7.95	8.64	8.79	8.91	8.31
2	10.22	11.85	12.24	12.05	13.53	12.52	13.22	9.65	9.08	13.33
3	2.36	2.67	1.72	1.73	3.16	1.72	3.11	2.66	2.56	1.79
4	2.26	2.49	1.72	1.75	2.99	1.81	2.95	2.52	2.37	1.85
5	6.30	5.53	4.72	4.18	6.22	4.77	5.93	7.46	7.43	5.35
6	14.14	17.91	10.77	11.45	16.56	11.95	13.11	15.13	14.25	12.33
7	1.51	1.61	1.3	1.01	1.09	0.94	1.31	1.02	0.95	0.64
8	2.84	2.57	2.70	2.40	2.02	2.68	3.56	2.71	3.77	2.67
9	14.29	12.08	11.76	9.94	6.71	11.62	11.40	14.54	19.78	11.89
10	2.09	3.01	1.92	1.67	1.31	1.93	1.81	3.62	3.79	1.99
11	1.26	1.22	1.01	0.98	0.92	1.04	1.20	1.38	1.65	1.11
12	14.36	8.48	11.77	12.37	10.89	9.58	10.80	4.24	2.13	7.15
13	0.75	1.29	1.59	1.31	0.55	1.64	1.25	1.14	1.17	1.74
14	0.90	1.24	1.25	0.978	1.45	1.51	0.70	1.47	1.00	1.31
15	0.56	1.48	0.71	0.76	1.38	1.04	1.15	1.68	2.08	1.60

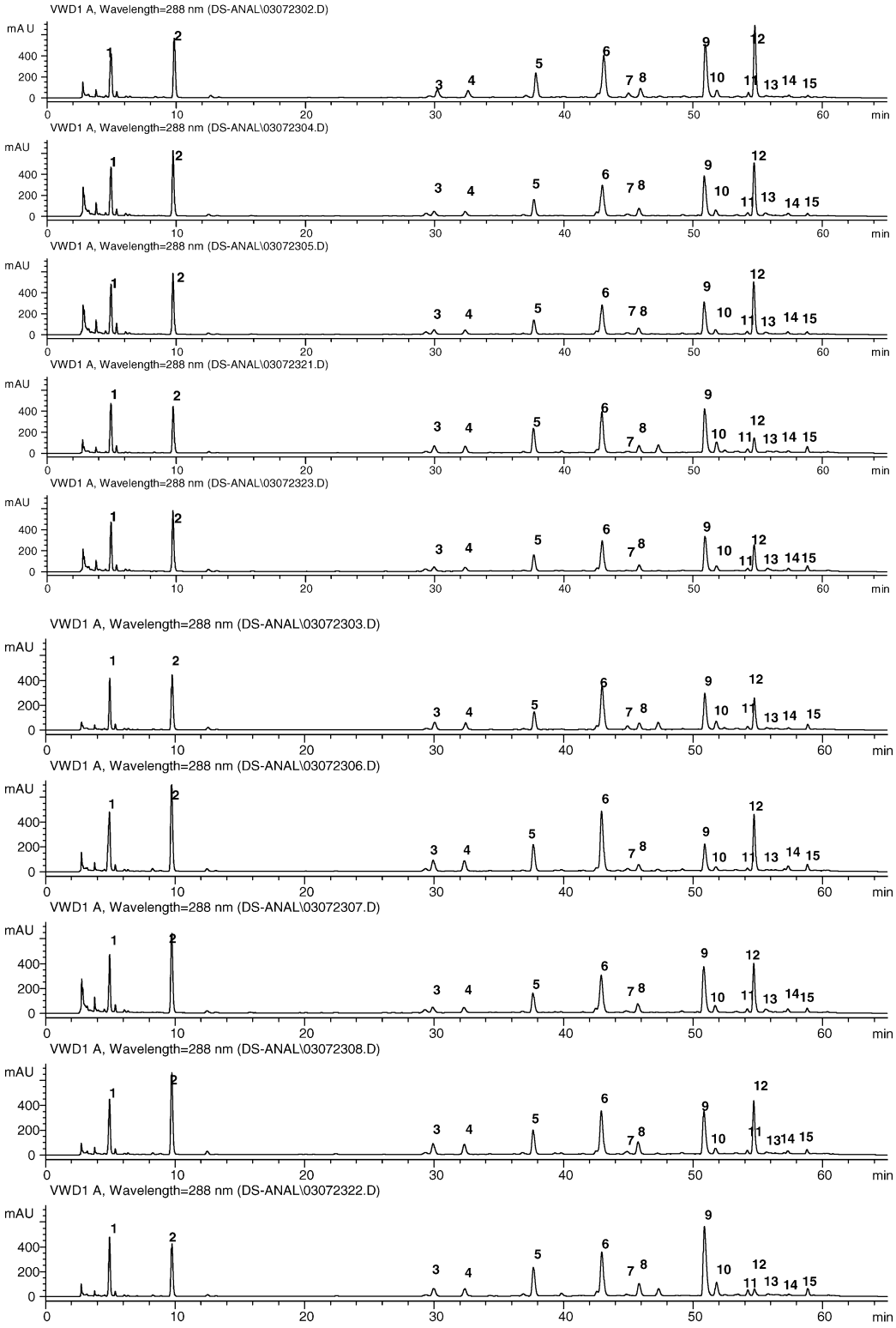


Fig. 3. HPLC fingerprint of 10 batches of Danshen injection marked peaks were common peaks of Danshen injection.

tion were analyzed under the established HPLC–UV method. Peaks that appeared in all HPLC profiles of 10 batches were assigned as “commom peak” which represented the characteristics of Danshen injection. There are 15 “commom peaks” in HPLC fingerprint (Fig. 3). The whole HPLC profiles and the area ratio of 15 “commom peaks”(Table 1) could be used to characterize the Danshen injection and assess the consistency from batch to batch.

Through comparing the HPLC chromatograms (Fig. 2A and B), it was found that the fingerprint profiles of Danshen injection was greatly different from that of the raw material. Danshensu, protocatechuic aldehyde and salvianolic acid B are the main constituents of Danshen injection, while the main constituent of raw material is salvianolic B, which indicated the changes of phenolic acids in the course of preparation. It is clear that salvianolic B was decomposed and produced other phenolic acids.

3.2. HPLC–MS analysis of Danshen injection

In order to identify the structures of main constituents in Danshen injection, the sample was analyzed by HPLC–MS techniques. ESI in both negative and positive mode were tried. The results showed that ESI in negative mode was sensitive to phenolic acids. Except for danshensu and protocatechuic aldehyde, other major constituents were well detected. The detected constituents all exhibited their quasi-molecular ions $[M-H]^-$. By careful studying on the mass spectra of these compounds and comparing with standards and reference data [5–7], 11 commom peaks in Danshen injection were designated and identified (Table 2). They were danshensu (peak 1), protocatechuic aldehyde (peak 2), salvianolic acid H or I (peak 3), salvianolic acid G (peak 4), salvianolic acid D (peak 5), rosmarinic acid (peak 6), lithospermic acid (peak 8), salvianolic acid B (peak 9), salvianolic acid E (peak 11), salvianolic acid A (peak 12) and salvianolic acid C (peak 15), respectively.

Table 2
HPLC–MS data and identification for Danshen injection

Common peak no.	TR (min)	$[M-1]^-$	MW	Identification
1	4.95	197	198	Danshensu
2	9.85	137	138	Protocatechuic aldehyde
3	27.40	537	538	Salvianolic acid H or I
4	34.88	339	340	Salvianolic acid G
5	35.78	417	418	Salvianolic acid D
6	41.03	359	360	Rosmarinic acid
7	43.90	493	494	Unknown compound
8	45.97	537	538	Lithospermic acid
9	49.18	717	718	Salvianolic acid B
11	53.28	717	718	Salvianolic acid E
12	53.55	493	494	Salvianolic acid A
15	57.92	491	492	Salvianolic acid C

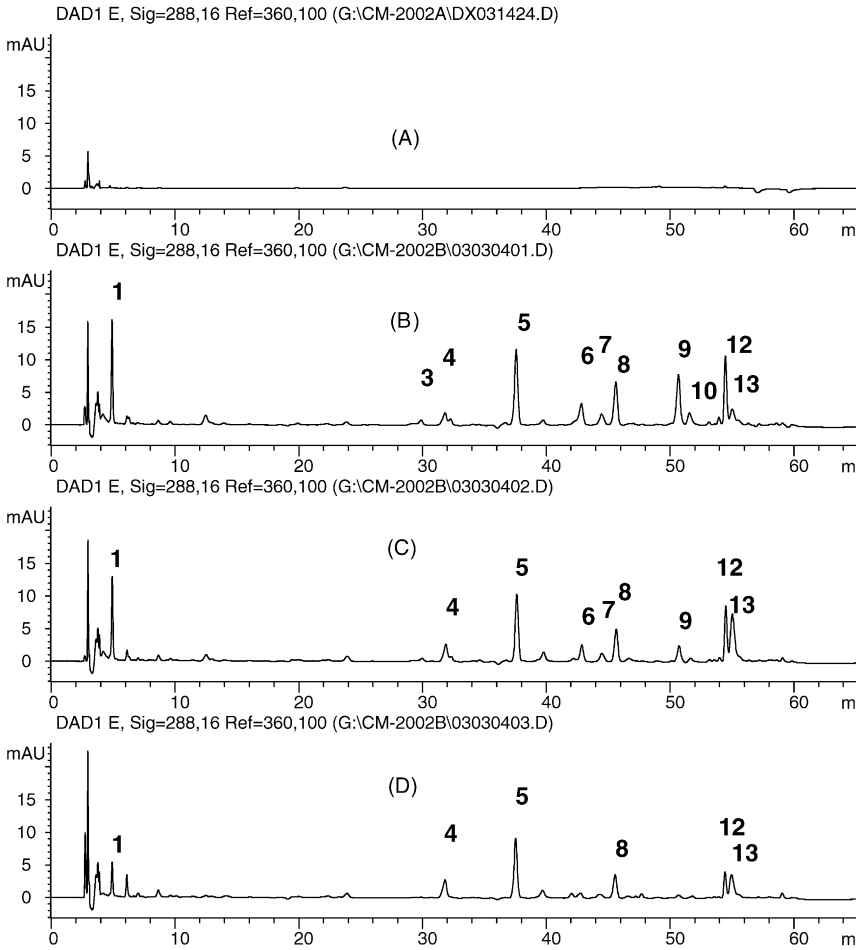


Fig. 4. HPLC profiles of blank rat's plasma (A) and plasma after 5 min (B), 10 min (C), and 30 min (D) administration, respectively. Peak number was consistent with the common peak number of Danshen injection.

3.3. Metabolic fingerprint analysis of Danshen injection

To clarify the active constituents responsible for the pharmacological action and guarantee the clinical efficacy of herbal medicines, it is necessary to know the metabolic changes and in vivo chemical constituent profile in biological systems. Therefore, the rat plasma after intravenous administration of Danshen injection at different time intervals were analyzed by the same HPLC–UV method as used for Danshen injection. The HPLC profiles were shown in Fig. 4. Comparing with HPLC profiles of blank plasma, there is no interference found around the main active constituents. It could be considered that the established plasma preparation method and HPLC analytical method for Danshen injection were suitable for the analysis of active constituents in rat blood. The plasma after 10-min intravenous administration of Danshen injection was analyzed by HPLC–MS under the same conditions and phenolic acids in rat plasma were also well separated and detected. The HPLC analytical results showed that the major phenolic acids of Danshen injection all appeared in rat plasma. But the ratio of each phenolic acid was greatly different from that in Danshen injection. This might be caused by either binding affinity difference of the phenolic acids with plasma protein or by the difference of metabolic rate in blood. Danshensu, salvianolic acid A, B, D and lithospermic acid were the major constituents in plasma after 5 min administration. With the administration time prolonged, danshensu and salvianolic B reduced remarkably. However, salvianolic A, D and lithospermic acid still existed in plasma at relatively high concentration after 30 min administration, which indicated that these salvianolic acids might be intermediates of other polyphenolic acid in the process of metabolism. These results suggested that salvianolic acids A, D and lithospermic acid might be closely related to the pharmacological activity of Danshen injection. It is worthy of studying the pharmacological activities of these constituents in depth. These results provided useful information regarding the active constituents of Danshen and marked components for quality control of Danshen injection. Based on this study, the indexes for fingerprint could be optimized to improve the quality control efficiency and the related preparation procedure could be designed and optimized to improve the quality of Danshen injection.

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