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Analysis of 'SHUANGDAN' granules by high-performance liquid chromatography–diode array detection–electrospray ionization tandem mass spectrometry

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Abstract

An HPLC–DAD–ESI–MS^n method was developed for simultaneous analysis of the chemical constituents in 'SHUANGDAN' granules, a newly developed drug widely used for treating cardiovascular disease. The chromatographic separation were performed on a Zorbax Stable Bond C_{18} column (4.6 mm × 250 mm, 5 μm) with water with 0.5% acetic acid (A) and acetonitrile (B) as mobile phase. According to the characteristic UV absorption profile, the information of molecular weight and structure provided by ESI–MS^n, 29 constituents which attributed to Radix Salviae Miltiorrhizae and Cortex Moutan, respectively, were detected and 28 constituents including 14 phenolic acids, 6 diterpenoid quinones, 6 monoterpenoids and 2 other components were identified, while some isomers were distinguished based on the MS^n spectra. This method was rapid and reliable for identification of constituents in complex chemical system, 'SHUANGDAN' granules, and the newly fragmentation patterns proposed could be extended to the compounds elucidation with similar framework.

Keywords: 'SHUANGDAN' granules; Chinese compounds prescription; HPLC–DAD–ESI–MS^n; Constituent identification

1. Introduction

Chinese compounds prescription is playing an important role due to its effectiveness in Chinese public life for thousands years. Now, along with more modern theories and techniques applied to the further studies on the type, quantities, and pharmacological affection of active compounds in Chinese compounds prescription [1,2], the remarkable efficacy of these traditional drugs is increasingly being understood and accepted by more and more people in the world. However, Chinese compounds prescription that generally contains several Traditional Chinese Medicines is a very complex chemical system. Establishment of rapid and reliable analytical methods for multi-constituents in Chinese compounds prescription catches the researchers' attention increasingly now.

'SHUANGDAN' granules, made from Radix Salviae Miltiorrhizae and Cortex Moutan, is one of the widely used Chinese compounds prescription and was authorized to sell by SFDA of China (No. Z10960044) for treating coronary heart disease, miocardial infarction, angina and atherosclerosis. There were lots of reports about the pharmacological effect of the bioactive constituents in Radix Salviae Miltiorrhizae and Cortex Moutan. The active constituents were focused on the phenolic acids, diterpenoid quinones [3] in Radix Salviae Miltiorrhizae and the monoterpenoids [4] in Cortex Moutan. Although there were various analytical researches on the compounds in Radix Salviae Miltiorrhizae [5–7] and Cortex Moutan [8,9], respectively, the analytical method on the multi-constituents in this Chinese compounds prescription, 'SHUANGDAN' granules was not reported. In order to ensure the safety, efficiency and stability in clinical use, it is important to analyze the constituents in 'SHUANGDAN' granules exactly.

Liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI–MS^n) had been shown to be significant analytical tool for confirming the identification of the known compounds and helping the elucidation of unknown compounds. Undoubtedly ESI was one of soft ionization technique suitable for molecular weight determination, and CID was performed to enhance fragmentation of the selected

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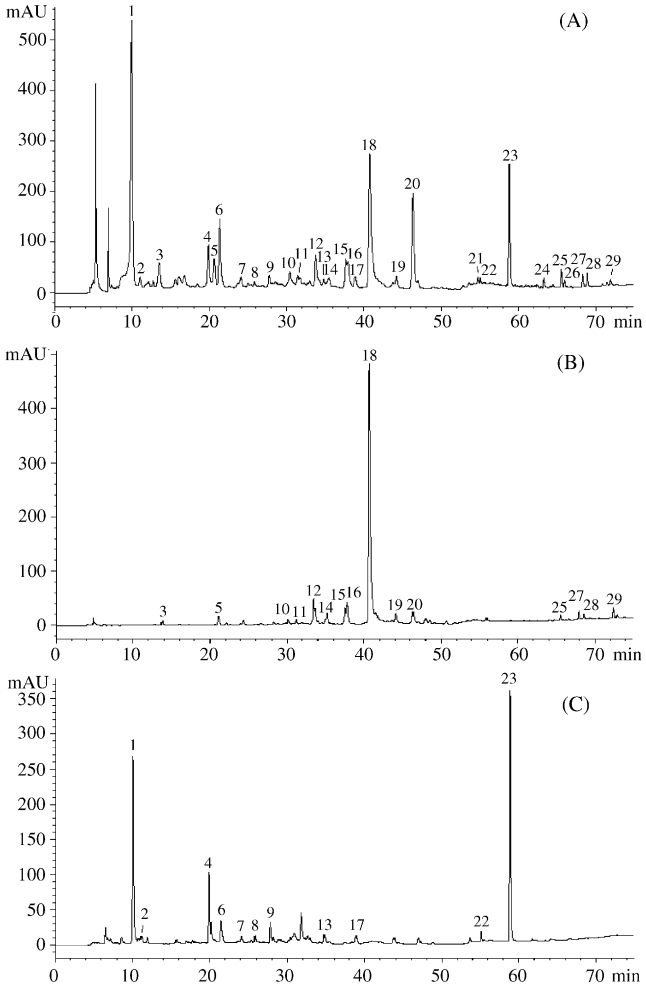


Fig. 1. HPLC-UV (254 nm) chromatograms of 'SHUANGDAN' granules (A), extracts of Radix Salviae miltiorrhizae (B) and Cortex Moutan (C).

precursor ions. Fragmentation information was obtained by the analysis of MS and MS^n data and the compounds' fragmentation patterns were proposed. Based on fragmentation patterns, some unknown compounds or isomers in the complex mixtures could be identified rapidly [10–14]. Although there were a few reports on the several compounds in Radix Salviae Miltiorrhizae and Cortex Moutan by LC–MS [8,15–18], the identification of compounds was only based on the molecular weight provided by MS and the results was trustless especially to the isomers. It was necessary to establish novel analytical method for the complex chemical group rapidly and reliably.

In this paper, we developed an HPLC–DAD–ESI–MS^n method to study on the complex chemical constituents in 'SHUANGDAN' granules. Based on the fragmentation patterns and the comparison of the UV, MS data with the literature data of authentic compounds' published data, the constituents in 'SHUANGDAN' granules were identified rapidly.

2. Experimental

2.1. Solvents and chemicals

The HPLC grade Acetonitrile from Merck (Darmstadt, Germany) was used for experiments. Analytical-grade methanol and acetic acid were purchased from Hangzhou Reagent Company (Hangzhou, China). Water for HPLC analysis was purified by a Milli-Q academic water purification system (Millipore, American). Reference compounds, Danshensu, Protocatechuic aldehyde, Salvianolic acid A, Salvianolic acid B, Paeoniflorin, Oxypaeoniflorin, Paeonol, Tanshinone I, Tanshinone IIA, Cryptotashinone and Dihydrotanshinone I were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The purity of these compound were determined to be more than 98% by normalization of the peak areas detected by HPLC, and showed very stable in methanol solution.

'SHUANGDAN' granules were provided by a Chinese pharmaceutical company. These raw materials, Radix Salviae Miltiorrhizae and Cortex Moutan, were purchased from Zhang-Tong-Tai Pharmaceutical Company (Hangzhou, China) and were identified by Dr. He.

2.2. Sample preparation

Five grams 'SHUANGDAN' granules was ultrasonically extracted with 50 ml of methanol for 30 min. The filtrate was centrifuged at 12,000 rpm for 15 min to remove particles, concentrated to dryness in vacuum at 50 °C and the residue was dissolved in 5 ml of methanol. The dry Radix Salviae Miltiorrhizae and Cortex Moutan was ground into powder. A 2 g pulverized herbal sample was ultrasonically extracted with 50 ml of methanol for 30 min also. After removed the solvent in vacuum, the residue was dissolved in 5 ml of methanol. All the

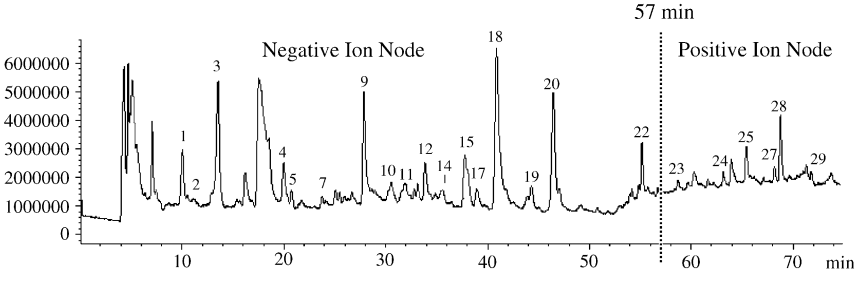
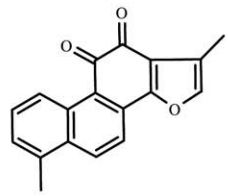
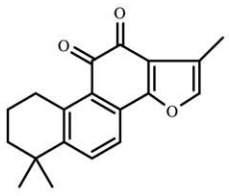


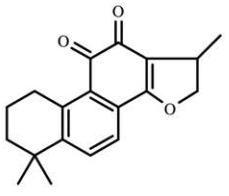
Fig. 2. TIC chromatogram of 'SHUANGDAN' granules (0–57 min, negative mode; 57–75 min positive mode).



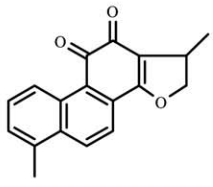
Tanshinone I (M.W.276; A)



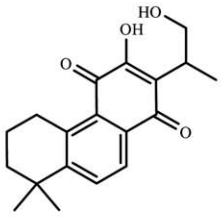
Tanshinone IIA (M.W.294; A)



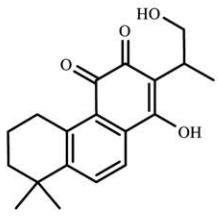
Cryptotashinone (M.W.296; A)



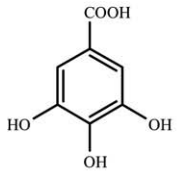
Dihydrotanshinone I (M.W.278; A)



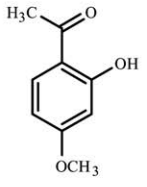
Neocryptotanshinone (M.W.314; A)



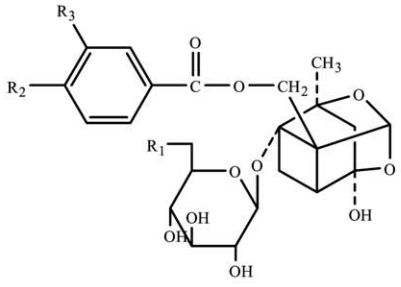
Tanshinone V (M.W.314; A)



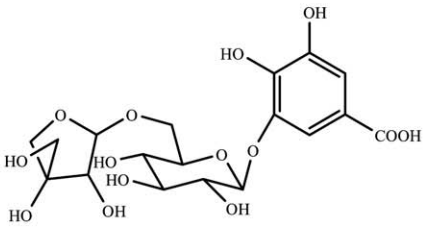
Gallic acid (M.W.170; B)



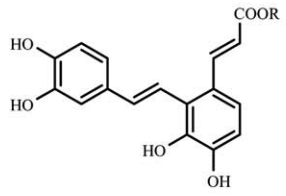
Paeonol (M.W.166; B)



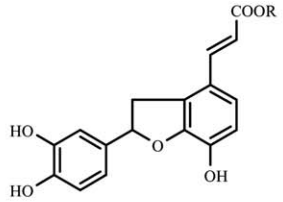
Paeoniflorin (M.W.480; B), R_1 = OH, R_2 = H, R_3 = H
Oxypaeoniflorin (M.W.496; B), R_1 = OH, R_2 = OH, R_3 = H
Galloyloxypaeoniflorin (M.W.648; B), R_1 = Galloyl, R_2 = OH, R_3 = H
Benzoylpaeoniflorin (M.W.584; B), R_1 = $OCOC_6H_5$, R_2 = H, R_3 = H
Mudanpioside E (M.W.526; B), R_1 = H, R_2 = H, R_3 = OCH_3
Mudanpioside H (M.W.616; B), R_1 = $OCOC_6H_4OH(p)$, R_2 = $OCOC_6H_4OH(p)$, R_3 = H



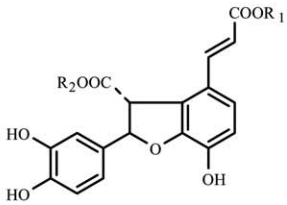
Mudanoside B (M.W.464; B)



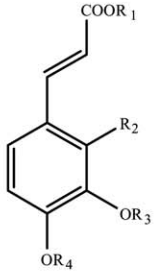
Salvianolic acid A (M.W.494; A), R = X

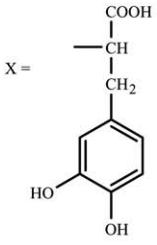


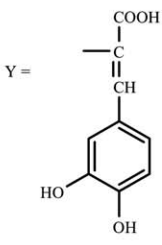
Salvianolic acid C (M.W.492; A), R = X



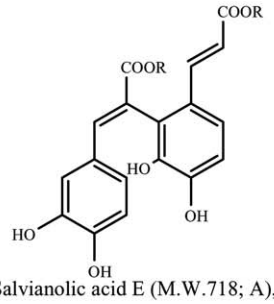
Salvianolic acid B (M.W.718; A), R_1 = R_2 = X
Linthospermic acid (M.W.538; A), R_1 = X, R_2 = H



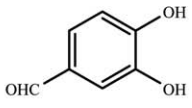




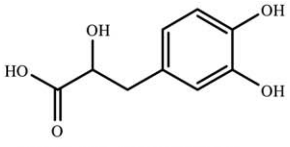
Salvianolic acid D (M.W.418; A), R_1 = X ,R_2 = CH_2COOH, R_3 = R_4 = H
Salvianolic acid I (M.W.538; A), R_1 =X, R_2 = R_3= H, R_4 = Y
Salvianolic acid H (M.W.538; A), R_1 =X, R_2 = R_4= H, R_3 = Y
Rosmarimic acid (M.W.360; A), R_1 =X, R_2 = R_3 = R_4= H



Salvianolic acid E (M.W.718; A), R = X



Protocatechuic aldehyde (M.W.138; A)



Danshensu (M.W.198; A)

Fig. 3. Structures of identified compounds: (A) Radix Salviae Miltiorrhizae and (B) Cortex Moutan.

samples were filtered through a 0.45 μm of PTFE membrane before HPLC analysis.

2.3. HPLC–DAD–MS system

High performance liquid chromatographic analysis was carried out using a Agilent 1100 series HPLC system (Waldbronn, Germany) equipped with a quaternary pump with on-line degasser, autosampler, column oven and diode array detector (DAD). The chromatographic conditions were: Zorbax Stable Bond C_{18} column (4.6 mm × 250 mm, 5 μm, Agilent), sample injection volume, 10 μl, the temperature of column oven, 35 °C, flow rate, 0.5 ml/min, mobile phases, water with 0.5% acetic acid (A) and acetonitrile (B). A gradient programmer was used according the following profile: 0–25 min, 5–23% B; 25–45 min linear increase to 30% B; 45–55 min linear increase to 70% B; 55–65 min linear increase to 95% B; 65–75 min hold on 95% B. UV spectra were recorded from 190 nm to 400 nm, and the monitor wavelengths were 230 nm, 254 nm and 280 nm, respectively.

A LCQ DECA XP ^{plus} mass spectrometer (Thermo Finnigan, San Jose, USA) that equipped with an ESI interface and an ion trap mass analyzer was used for carrying out the MS and MS^n analyses. Data were acquired and processed by Thermo Finnigan $Xcalibur_{1.3}$ workstation. The operating conditions for the ESI interface were as follows: positive/negative ionization mode; temperature of the capillary, 350 °C; spray voltage, 3.0 kV; capillary voltage, 20 V; sheath gas (N_2) flow rate, 30 A.U.; auxiliary gas (N_2) flow rate, 10 A.U. Full scan data acquisition was performed from *m/z* 100 to 1500 in MS scan mode. MS^n experiments were performed by colliding the precursor ions with Helium gas at 2.0 mass isolation width. The collision energy values were automatically selected.

3. Results and discussion

3.1. HPLC–DAD analysis

The main constituents of 'SHUANGDAN' granules were phenolic acids; diterpenoid quinones and monoterpenoids. According to studying on the characteristic UV profile of these main constituents, the approximate maximum UV absorption wavelengths of phenolic acids were at 220 nm and 280 nm; diterpenoid quinones were at 230 nm, 260 nm and 290 nm and monoterpenoids were at 230 nm and 275 nm. The framework type of compounds could be rapidly identified according to their characteristic UV profiles. 'SHUANGDAN' granules, extracts of Radix Salviae Miltiorrhizae and Cortex Moutan were presented in Fig. 1.The constituents in 'SHUANGDAN' granules were well separated on reversed-phase column with the gradient elution. Comparing the chromatograms of 'SHUANGDAN' granules with those of extracts of Radix Salviae Miltiorrhizae and Cortex Moutan, the main constituents in 'SHUANGDAN' granules were found in Radix Salviae Miltiorrhizae and Cortex Moutan, respectively. However, the quantities of the constituents among them were obvious difference, especially the

Table 1
The precursor ion and main fragment ions of the reference compounds in MS and MS^n analysis

Reference compounds	Ionization mode	MS ion (*m/z*)	MS^2 (*m/z*)	MS^3 ion (*m/z*)	MS^4 ion (*m/z*)	MS^5 ion (*m/z*)
Danshensu (SD 1)	Negative	197.1 $[M-H]^-$	179.0 $[M-H-H_2O]^-$	135.2 $[M-H-H_2O-CO_2]^-$	107.2 $[M-H-H_2O-CO_2-C_2H_4]^-$	–
Salvianolic acid A (SD 2)	Negative	493.1 $[M-H]^-$	295.2 $[M-H-(C_9H_{10}O_5)]^-$	159.1 $[M-H-(C_9H_{10}O_5)-C_8H_8O_2]^-$	–	–
Salvianolic acid B (SD 3)	Negative	717.3 $[M-H]^-$	519.2 $[M-H-(C_9H_{10}O_5)]^-$	321.2 $[M-H-2\times(C_9H_{10}O_5)]^-$	279.3 $[M-H-2$ $(C_9H_{10}O_5)$–$CH_2CO]^-$	251.1 $[M-H-2$ $(C_9H_{10}O_5)$–CH_2CO–$CO]^-$
Paeciflorin (SD 4)	Negative	479.4 $[M-H]^-$	449.2 $[M-H-CH_2O]^-$	327.2 $[M-H-CH_2O$–(benzoic acid)$]^-$	165.1 $[M-H-CH_2O$–(benzoic acid)–glucose$]^-$	137.1 $[M-H-CH_2O$–(benzoic acid)–glucose–$CO]^-$
Qxypaeoni florin (SD 5)	Negative	495.3 $[M-H]^-$	465.2 $[M-H-CH_2O]$	327.0 $[M-H-CH_2O$–(*p*-hydroxy-bezoic acid$)]^-$	165.2 $[M-H-CH_2O$–(*p*-hydroxy-bezoic acid)–glucose$]^-$	137.1 $[M-H-CH_2O$–(*p*-hydroxy-bezoic acid)–glucose–CO]
Tanshinone IIA (SD 6)	Positive	295.3 $[M+H]^+$	277.2 $[M+H-H_2O]^+$	249.1 $[M+H-H_2O-CO]^+$	205.2, 191.1	–
Dihydrctanshinone I. (SD 7)	Positive	278.9 $[M+H]^+$	261.0 $[M+H-H_2O]^+$	23 2.9 $[M+H-H_2O-CO]^+$	205.0 $[M+H-H_2O-2\times CO]^+$	
Cryptotanshincne (SD 8)	Positive	297.2 $[M+H]^+$	279.0 $[M+H-H_2O]^+$	251.3 $[M+H-H_2O-CO]+$	223.2 $[M+H-H_2O-2\times CO]^+$	206.9 $[M+H-H_2O-2\times CO-CH_4]^+$
Tanshinone I. (SD 9)	Positive	277.2 $[M+H]^+$	248 8 $[M+H-CO]^+$	221.0 $[M+H-2\times CO]^+$	193.2 $[M+H-3\times CO]^+$	–
Paeoiol (SD 10)	Positive	167.1 $[M+H]^+$	149.2 $[M+H-H_2O]^+$	121.1 $[M+H-H_2O-CO]^+$	–	–

less polar constituents that was due to the special technical process.

3.2. MS analysis

Through comparing the TIC chromatograms of 'SHUANGDAN' granules in different ionization modes, it was found that signal response was sensitive to large polar constituents in negative mode, while sensitive to less polar constituents in positive mode. For the high ionization efficiency and sensitive signal response, the ESI scan mode was set as follows: 0–57 min, negative mode, 57–75 min positive mode. The TIC chromatogram of 'SHUANGDAN' granules was shown in Fig. 2.

In order to obtain fragmentation patterns of constituents from 'SHUANGDAN' granules, MS^n spectra of 10 reference compounds (SD 1–10) were firstly analyzed by direct infusion. MS/MS and MS^n data were obtained by CID. The fragmentation patterns were proposed and it was very helpful for the constituents' structure identification in 'SHUANGDAN' granules that had the similar framework.

Compounds SD 1–5 were analyzed in negative mode and SD 6–10 in positive mode for obtaining more sensitive signal. In the full scan mass spectra, SD 1–5 exhibited the $[M-H]^-$ quasi-molecular ions as base peak easily, While SD 6–10 gave the $[M+H]^+$ adduct ions as base peak.

When subjected to MS^n analysis, the monoterpenoids dissociated at low CID energy and gave the fragment $[M-H–CH_2O]^-$ firstly, then lost Benzoic acid, glucose, CO in succession in MS^n spectra. The phenolic acids generally dissociated and gave the quasi-molecular ions after lost a fragment of $C_9H_{10}O_5$ (198 Da), CO and H_2O in succession in MS^n spectra, while diterpenoid quinones were tended to lose H_2O and CO. The MS data of the ten reference compounds and their main fragment in MS^n spectra was summarized in Table 1. It was obvious that the similar compounds displayed some common features and the fragmentation patterns were helpful for constituents' structure identification in 'SHAUNGDAN' granules.

3.3. HPLC–DAD–MS^n analysis

In HPLC–DAD chromatogram, 29 peaks were detected and each peak had its corresponding peak in total ion chromatogram, which was shown in Fig. 2. All the identified constituents were summarized in Table 2 and their structures were listed in Fig. 3.

Compared the retention time, UV characteristic profile and MS spectra of sample with those of reference compounds, peaks 3, 4, 5, 9, 18, 20, 23, 25, 27–29 were attributed to Danshensu,

Table 2
HPLC–DAD–MS data and identification of constituents from 'SHUANGDAN' granules

Peak	t_R (min)	$[M+H]^+$	$[M-H]^-$	λ_{max} (nm)	Plant material	Identification
1	10.14		169.2	224, 272	Cortex Moutan	Gallic acid
2	11.20		463.0	298	Cortex Moutan	Mudanoside B
3	13.68		197.1	224, 282	Radix Salviae Miltiorrhizae	Danshensu[a]
4	19.92		495.3	258	Cortex Moutan	Oxypaeoniflorin[a]
5	20.78		137.1	232, 280, 310	Radix Salviae	Protocatechuic aldehyde[a]
6	21.46		525.3	256	Miltiorrhizae	Mudanpioside E
7	24.16		495.2	262, 290, 320	Cortex Moutan	Oxypaeoniflorin isomer [19]
8	25.82		647.4	268	Cortex Moutan	Galloyloxypaeoniflorin
9	27.74		479.4	232, 274	Cortex Moutan	Paeoniflorin[a]
10	30.40		537.1	236, 292, 324	Radix Salviae Miltiorrhizae	Salvianolic acid H or I
11	31.42		537.2	232, 290, 326	Radix Salviae Miltiorrhizae	Salvianolic acid H or I
12	33.72		417.0	224, 248, 290, 320	Radix Salviae Miltiorrhizae	Salvianolic acid D
13	34.79		615.3	258	Cortex Moutan	Mudanpioside H
14	35.41		717.4	234, 250, 290, 330	Radix Salviae Miltiorrhizae	Linthospermic acid B
15	37.64		359.1	222, 246, 290, 328	Radix Salviae Miltiorrhizae	Rosmarimic acid
16	37.87		537.2	224, 254, 290, 312	Radix Salviae Miltiorrhizae	Linthospermic acid
17	38.99		265.2	230, 274	Cortex Moutan	Unknown
18	40.70		717.2	224, 254, 286, 308	Radix Salviae Miltiorrhizae	Salvianolic acid B[a]
19	44.10		717.4	220, 252, 288, 306	Radix Salviae Miltiorrhizae	Salvianolic acid E
20	46.29		493.1	224, 288, 310	Radix Salviae Miltiorrhizae	Salvianolic acid A[a]
21	54.70		491.2	218, 288, 314, 364	Radix Salviae Miltiorrhizae	Salvianolic acid C
22	55.02		583.2	230, 276	Cortex Moutan	Benzoylpaeoniflorin
23	60.26	167.1		230, 274, 312	Cortex Moutan	Paeonol[a]
24	64.15	315.0		238, 264, 308	Radix Salviae Miltiorrhizae	Neocryptotanshinone
25	66.30	278.9		244, 266, 290, 332	Radix Salviae Miltiorrhizae	Dihydrotanshinone I[a]
26	66.70	315.2		256, 292, 362	Radix Salviae Miltiorrhizae	Tanshinone V
27	68.84	277.0		246, 268, 292, 326	Radix Salviae Miltiorrhizae	Tanshinone I[a]
28	69.46	297.1		220, 264, 272, 290	Radix Salviae Miltiorrhizae	Cryptotanshinone[a]
29	71.78	295.1		226, 252, 270	Radix Salviae Miltiorrhizae	Tanshinone IIA[a]

[a] Identified by comparison with authentic compounds.

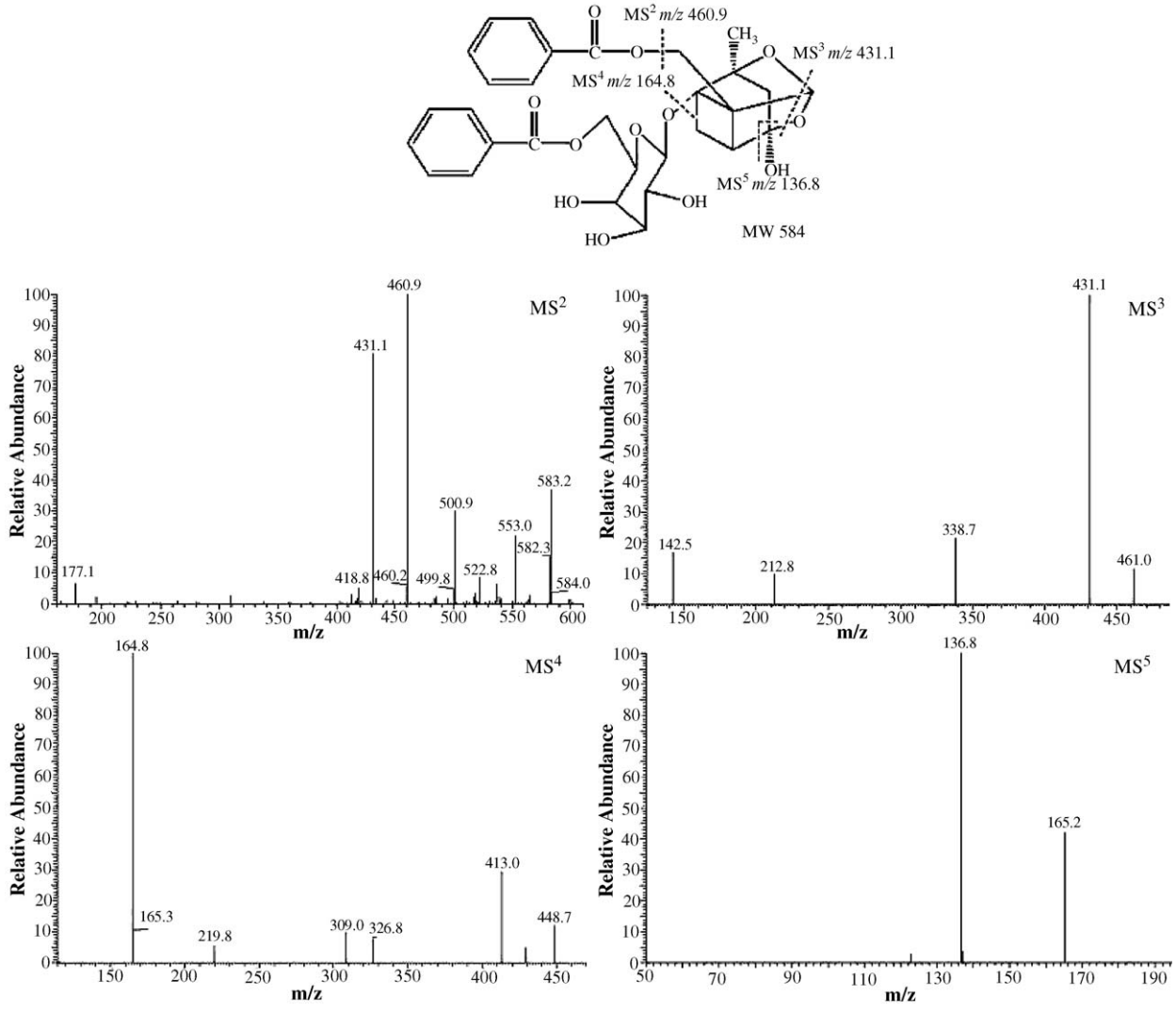


Fig. 4. The characteristic fragmentation and MS^n spectra of Benzoylpaeoniflorin.

Oxypaeoniflorin, Protocatechuic aldehyde, Paeoniflorin, Salvianolic acid B, Salvianolic acid A, Paeonol, Dihydrotanshinone I, Tanshinone I and Cryptotashinone, Tanshinone IIA, respectively.

Due to lack of reference compounds, structures of peaks 1, 2, 7, 8, 12, 13, 15, 21, 22 were deduced from their UV characteristic profile, MS and MS^n spectra by comparison with fragmentation patterns proposed above and the literature data [5,8,11]. For example, peak 22 exhibited quasi-molecular ion $[M-H]^-$ at *m/z* 583.2 and its maximum UV absorption wavelengths were at 230 and 276 nm that was similar with monoterpenoids in Cortex Moutan. The base peak *m/z* 460.9 in MS^2 spectra was $[M-H–(\text{benzoic acid})]^-$, then dissociated formaldehyde and gave fragment ion *m/z* 431.1 in MS^3 spectra. The fragment at *m/z* 164.8 in MS^4 spectra was due to the disconnection of glycoside and next lost CO to give the fragment ion at *m/z* 136.8 in MS^5 spectra. The fragmentation pattern of peak 22 was same as those of monoterpenoids in Cortex Moutan such as Paeoniflorin and Oxypaeoniflorin. After compared with the published data, it was identified as Benzoylpaeoniflorin. The characteristic fragmentation and MS^n spectra were shown in Fig. 4.

Several isomers were also detected and identified based on their fragmentation patterns. For example, three phenolic acids isomers (peak 10, 11, 16) showed the same quasi-molecular ion $[M-H]^-$ at *m/z* 537.1, and lost characteristic neutral fragment at *m/z* 44 assigned to CO_2 and *m/z* 198 assigned to Denshensu in succession, respectively, exhibited the same fragment ions $[M-H–CO_2–C_9H_{10}O_5]^-$ at *m/z* 295.2 (100%) in MS^3 spectra [18]. However, under the same collision energy values, the MS^4 fingerprints of fragment ion at *m/z* 295.2 from peak 10, 11 were different from that of peak 16 but were similar with the MS^3 fingerprint of Salvianolic acid A (Fig. 5). Major fragment ions of peak 10, 11 in MS^4 spectra were at *m/z* 277.1, 267.3, 185.1, 159.2 (100%), 131.0 and 109.2. The precursor ion (*m/z* 295.2) dissociated $C_8H_8O_2$, gave the fragment ion *m/z* 159.2 and had the same fragmentation pattern as that of Salvianolic acid A. So it was deduced that peaks 10, 11 were Salvianolic acid H and I,

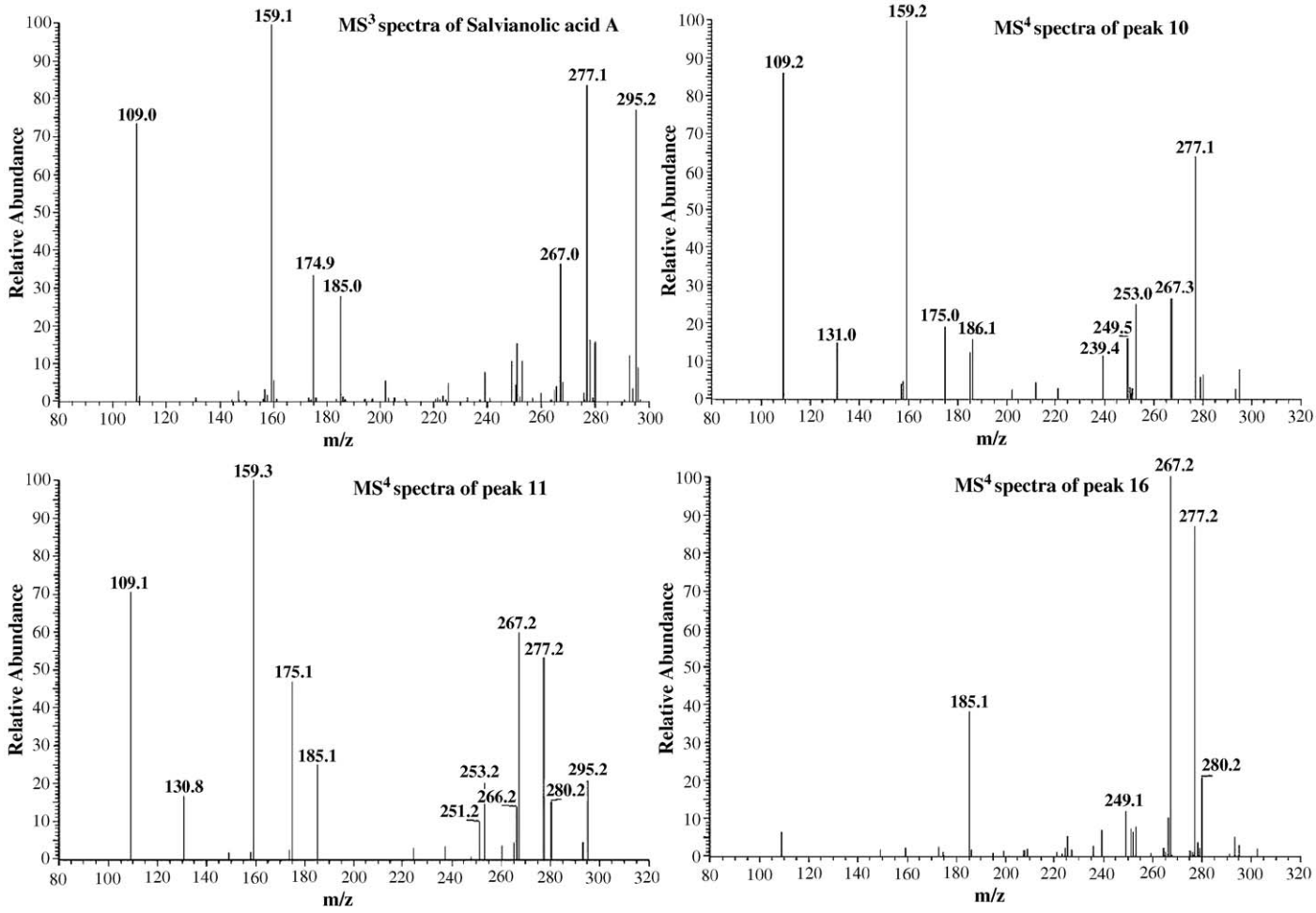


Fig. 5. Fingerprint of Salvianolic acid A in MS^3 spectra and fingerprints of peaks 10, 11, 16 in MS^4 spectra.

a couple of geometric isomer (*cis* and *trans*), while peak 16 was identified as Linthospermic acid with different framework from Salvianolic acid A.

According to the similar method, another couple isomers (peak 25, 28) were identified as Neocryptotanshinone and Tanshinone V by comparing their fragmentation patterns with that of Tanshinone IIA and the literature data, respectively [20].

Based on the retention time, UV and MS spectra, it was confirmed that peak 17 was from Cortex Moutan. But the chemical constituent with same molecular weight was not reported, and its fragmentation pattern was different from above. So it should be a novel constituent and its elucidation of structure needs more information such as nuclear magnetic resonance (NMR) spectrometry, etc.

4. Conclusions

Combining the HPLC separation, the characteristic UV absorption pattern and the information of molecular weight and structure provided by ESI–MS^n, HPLC–DAD–ESI–MS^n was proved to be an effective tool for the study of complex chemical system. This technique showed the superiority and became a key strategy in analyzing Chinese compounds prescription.

In this study, the chemical constituents in 'SHUANGDAN' granules were analyzed by HPLC–DAD–ESI–MS^n. Analyzed by direct infusion, the fragmentation patterns of reference compounds were proposed. Based on the fragmentation patterns and the comparison of the UV, MS data of sample with the published literature data, the constituents in 'SHUANGDAN' granules were identified rapidly. Twenty-nine constituents that attributed to Radix Salviae Miltiorrhizae and Cortex Moutan, respectively, were detected and 28 constituents including 14 phenolic acids, 6 diterpenoid quinones, 6 monoterpenoids and two other components were identified, while some isomers were distinguished. The newly fragmentation patterns proposed could be extended to the compounds elucidation with similar framework. These results would be helpful to ensure the safety and efficiency, optimize the quality control of 'SHUANGDAN' granules.

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