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Identification and analysis of absorbed and metabolic components in rat plasma after oral administration of 'Shuangdan' granule by HPLC–DAD–ESI-MS/MS

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Abstract

A valid method using liquid chromatography–diode array detection–electrospray ionization (ESI) ion trap mass spectrometry was established for the study of the absorbed and metabolic components of a Chinese medicine 'Shuangdan' granule in rat plasma after oral administration. The plasma samples were acidified with 1 M hydrochloric acid and extracted with ethyl acetate (EtOAc) of two-fold volume for three times. The chromatographic separation was carried out on a Zorbax SB-C_{18} column with a linear gradient whereas 0.1% acetic acid/water/acetonitrile was used as mobile phase. Mass spectra were acquired in both negative and positive modes. More than 20 components including 16 components from 'Shuangdan' granule and 5 metabolites were simultaneously identified by comparing their mass spectra and retention behavior with reference compounds or literature data. The results proved that the established method could be used to identify the structure of active components responsible for the pharmacological effects of 'Shuangdan' granule.

Keywords: High performance liquid chromatography/ion trap mass spectrometry; Absorbed and metabolic components; 'Shuangdan' granule; Rat plasma

1. Introduction

'Shuangdan' granule, made from the aqueous extracts of *Radix Salvia Miltiorrhizae* and *Cortex Moutan*, is one of the most commonly used traditional Chinese medicines (TCM). It has been authorized to sell by SFDA of China (No. Z10960044) for the treatment of cardiovascular disease, especially acute heart ischemia [1–3]. Phenolic acids and diterpenoid quinones were regarded as the active components for the therapeutic effects of *Salvia miltiorrhiza Bunge* [4,5], while paeoniflorin and paeonol were reported to be the main bioactive components of *Cortex Moutan* [3,6].

Traditional Chinese medicine commonly consisted of dozens of compounds from several herbs. The active components responsible for the pharmacological action are usually unclear. Conventional approaches generally use one or few mark components to control the quality and study the pharmacokinetics of TCM. It is not sufficient to reflect the overall efficacy of TCM, because multiple components are responsible for its therapeutic effects and these may play synergistic roles. Thus, it is important to establish rapid and reliable methods for screening and analysis of effective components in TCM. Homma et al. [7] proposed serum pharmacological screening strategy based on the hypothesis that active compounds should appear in blood after administration of TCM. This strategy can be subdivided into serum pharmacology and serum pharmacochemistry. Huang et al. [8] indicated that the method of serum pharmacochemistry was very straightforward and helpful to recognize the real active components in TCM.

Recently, electrospray ionization tandem mass spectrometry (ESI-MS^n) has demonstrated its great advantages for structural analysis of components in herb extracts with high sensitivity, short time and low consumption of samples. In addition, liquid chromatography-mass spectrometry (LC-MS) and liquid chromatography coupled with sequential mass spectrometry (LC–MS^n) have been extensively applied to the on-line structure elucidation of herb components [9–11]. Tandem mass spectrometry techniques have been playing an important role in

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metabolism, as well, such as the structural elucidation of drug metabolites via biotransformation [12,13].

Over 100 compounds have been isolated and identified in *Radix Salviae Miltiorrhizae* and *Cortex Moutan*, but only a small portion of compounds were reported to be responsible for their bioactive effects. Up to now, *in vivo* studies of *Radix Salviae Miltiorrhizae* and *Cortex Moutan* mainly focused on pharmacokinetics of one or few bioactive components [14–16]. In the present study, we applied serum pharmacochemistry strategy in screening and analysis of the multiple effective components of 'Shuangdan' granule. Hyphenated liquid chromatography and mass spectrometric method was adopted to simultaneously separate the effective components from interfering ingredients and determine the structure of them in rat plasma. The structure of absorbed components and metabolites in rat plasma was elucidated based on the UV and mass spectra. This investigation provided a basis for evaluating the bioactivities of components in 'Shuangdan' granule.

2. Experiment

2.1. Chemicals and reagents

The reference compounds of protocatechuic aldehyde, paeoniflorin, paeonol, salvianolic acid B, dihydrotanshinone I and cryptotanshinone were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Danshensu (purities higher than 99%) was provided by Shanghai Medical College of Fudan University (Shanghai, China). Gallic acid of chemical grade was purchased from Shanghai Chemical Reagent Corporation of China Medicine (Group) (Shanghai, China). Acetonitrile and methanol (HPLC grade) were obtained from Merck (Darmstadt, Germany) while glacial acetic acid (HPLC grade) was from Tedia (Fairfield, OH, USA). The deionized water was prepared from Millipore water purification system and was filtered with 0.22 μm membrane. Analytical-grade hydrochloric acid and ethyl acetate (EtOAc) were purchased from Hangzhou Reagent Company (Hangzhou, China). 'Shuangdan' granule was provided by a Chinese pharmaceutical company.

2.2. Instrumentation and conditions

HPLC-UV analysis was carried out on an Agilent 1100 Series HPLC system (Agilent, Waldbronn, Germany) with diode array detector using a Zorbax SB-C_{18} column (4.6 mm × 250 mm, 5 μm, Agilent). The temperature of column was maintained at 35 °C. UV spectra were recorded from 190 to 400 nm and the detection wavelength was set at 288 and 230 nm. The flow rate was 0.5 ml/min, and a C_{18} guard column was used before the analytical column. A gradient elution of A (0.1% aqueous acetic acid) and B (acetonitrile containing 0.1% acetic acid) was used. The gradient was as follows: started at 95% A and 5% B, then to 77% A and 23% B at 25 min, 70% A and 30% B at 45 min, 30% A and 70% B at 55 min, kept with 5% A and 95% B from 65 to 75 min. After that the system was restored to initial conditions in 15 min.

HPLC/MS^n analysis was performed with an Agilent 1100 Series HPLC and Finnigan LCQ Deca XP^{plus} ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an ESI source, with Xcalibur 1.3 controlling software. Nitrogen (N_2) was used as the sheath and auxiliary gas, and helium (He) was used as the damping and collision gas. HPLC conditions were the same as described above. Mass spectra were acquired in both negative and positive modes with ion spray voltage at 4.5 kV, capillary temperature at 350, capillary voltage at 19 V, sheath gas flow at 30 (arbitrary units), auxiliary gas at 10 (arbitrary units) and tube lens offset at 25 V.

2.3. Animals, drug administration and blood sampling

Nine male Sprague–Dawley (SD) rats (200–230 g body weight) were obtained from the Laboratory Animal Center of Zhejiang Province (Hangzhou, China) and fasted for 12 h with free access to water, prior to the experiments. 'Shuangdan' granule was dissolved in adequate volume of water and the mixture was sonicated in an ultrasonic bath for 30 min to prepare the decoction. The decoction was orally administered to six rats at a total dose of 3 g 'Shuangdan' granule/100 g body weight within four times a day. Physiological saline was orally administered to other three rats. The rat was decapitated 30 min after last administration and blood was collected in heparinized microcentrifuge tube and centrifuged at 553.9 × *g* for 10 min to separate plasma. Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication 85–23, revised edition 1985).

2.4. Sample preparation

A 0.05 g aliquot of 'Shuangdan' granule was dissolved in 1 ml methanol and the mixture was sonicated in an ultrasonic bath for 30 min, followed by centrifugation for 10 min at 6154.5 × *g* for HPLC/MS/MS analysis.

The plasma sample (500 μl) was added into 5 ml polypropylene test tube, 500 μl deionized water and 200 μl 1 M hydrochloric acid were added using an Eppendorf repeater pipette. The mixture was extracted with EtOAc at the ratio of 1–2 (*v*/*v*) for three times. Extraction was performed by vortex, mixing the tubes for 2 min, followed by centrifugation for 10 min at 6154.5 × *g*. The EtOAc fractions were transferred into a glass tube and dried at 30 °C under a stream of compressed air. The residue was dissolved in 100 μl reconstitution solvent (methanol–water–acetic acid; 50:50:0.1, *v*/*v*/*v*). After centrifugation at 6154.5 × *g* for 10 min, a volume of 20 μl was injected into the HPLC/MS^n system.

3. Results and discussion

3.1. LC–DAD–ESI-MS analysis of 'Shuangdan' granule and plasma samples

Various sample preparation methods were tested to select an efficient clean up of the plasma sample for obtaining a better recovery of the target compounds. The methods included

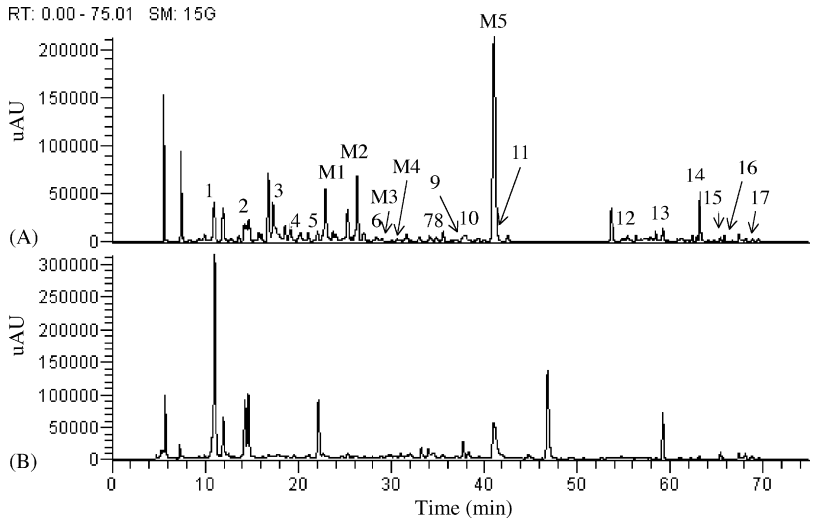

Fig. 1. UV chromatograms of rat plasma sample and 'Shuangdan' granule (detection at 288 nm): (A) rat plasma sample collected after oral administration of 'Shuangdan' granule and (B) 'Shuangdan' granule. Peak numbers were consistent with those shown in Table 1.

liquid–liquid extraction with ether and EtOAc, and solid-phase extraction with various sorbents such as YMC C_{18} cartridges (Kyoto, Japan) or Waters Oasis HLB cartridges (Milford, MA, USA). Finally, liquid–liquid extraction with ethyl acetate was chosen because it not only ensures the simultaneous extraction of all target compounds, but also causes less interference from the co-eluted endogenous matrixes.

Both methanol/water and acetonitrile/water mobile phase systems were tested for the chromatographic separation. The results demonstrated that the latter had higher elution efficiency. Therefore, in this study acetonitrile-based gradient was used as mobile phase. As to obtain good resolution, the LC conditions were optimized and long gradient LC runs were needed.

Table 1
HPLC/MS^n data and identification of herb components in rat plasma

Peak no.	t_R (min)	Ionization mode	MS (*m*/*z*)	MS^2 (*m*/*z*)	MS^3 (*m*/*z*)	Identification
1	10.99	Negative	169 $[M-H]^-$	125 $[M-H–CO_2]^-$	81 $[M-H–2\times CO_2]^-$	Gallic acid[a]
2	14.22	Negative	197 $[M-H]^-$	179 $[M-H–H_2O]^-$	135 $[M-H–H_2O–CO_2]^-$	Danshensu[a]
3	17.52	Negative	182 $[M-H]^-$	163		Unknown compound
4	20.26	Negative	179 $[M-H]^-$	135 $[M-H–CO_2]^-$		Caffeic acid
5	22.11	Negative	137 $[M-H]^-$	109 $[M-H–CO]^-$		Protocatechuic aldehyde[a]
6	28.90	Negative	479 $[M-H]^-$	449 $[M-H–CH_2O]^-$	327 $[M-H–CH_2O–$(benzoic acid)$]^-$	Paeoniflorin[a]
7	34.08	Negative	417 $[M-H]^-$	373 $[M-H–CO_2]^-$	175 $[M-H–CO_2–$(danshensu)$]^-$	Salvianolic acid D
8	34.64	Negative	339 $[M-H]^-$	321 $[M-H–H_2O]^-$		Salvianolic acid G
9	37.78	Negative	359 $[M-H]^-$	161 $[M-H–$(danshensu)$]^-$ 179 $[M-H–$(caffic acid)$]^-$		Rosmarimic acid
10	38.26	Negative	537 $[M-H]^-$	493 $[M-H–CO_2]^-$	295 $[M-H–CO_2–$(danshensu)$]^-$	Lithospermic acid
11	41.67	Negative	717 $[M-H]^-$	519 $[M-H–$(danshensu)$]^-$	321 $[M-H–2\times$(danshensu)$]^-$	Salvianolic acid B[a]
12	55.00	Negative	491 $[M-H]^-$	293 $[M-H–$(danshensu)$]^-$	265 $[M-H–$(danshensu)–CO$]^-$	Salvianolic acid C
13	60.56	Positive	167 $[M+H]^+$	149 $[M+H–H_2O]^+$	121 $[M+H–H_2O–CO]^+$	Paeonol[a]
14	63.26	Positive	315 $[M+H]^+$	297 $[M+H–H_2O]^+$	253 $[M+H–H_2O–CO_2]^+$	Neocryptotanshinone
15	65.50	Positive	279 $[M+H]^+$	261 $[M+H–H_2O]^+$	233 $[M+H–H_2O–CO]^+$	Dihydrotanshinone I[a]
16	65.84	Positive	315 $[M+H]^+$	297 $[M+H–H_2O]^+$	279 $[M+H–2\times H_2O]^+$	Tanshinone V
17	68.70	Positive	297 $[M+H]^+$	279 $[M+H–H_2O]^+$	251 $[M+H–H_2O–CO]^+$	Cryptotanshinone[a]

[a] Identified by comparison with reference compounds.

Table 2
HPLC/MS^n data and identification of metabolites in rat plasma

Metabolite	t_R (min)	MS (*m*/*z*)	MS^2 (*m*/*z*)	Identification
M1	22.90	327 $[M-H]^-$	151 $[M-H–$glucose$]^-$	2-Hydroxyacetophenone-4-*O*-glucuronide or 4-hydroxyacetophenone-2-*O*-glucuronide
M2	26.52	341 $[M-H]^-$	165 $[M-H–$glucose$]^-$	2-Methoxyacetophenone-4-*O*-glucuronide or paeonol-2-*O*-glucuronide
M3	28.27	247 $[M-H]^-$	167 $[M-H–SO_3]^-$	2,4-Dihydroxyacetophenone-5-*O*-sulfate
M4	30.68	261 $[M-H]^-$	181 $[M-H–SO_3]^-$	2-Hydroxy-4-methoxyacetophenone-5-*O*-sulfate
M5	41.43	151 $[M-H]^-$	109 $[M-H–CH_2CO]^-$	Resacetophenone

The flow rate was 0.5 ml/min, which was a little high for ESI source, especially at the beginning of the gradient. The LC column effluent was splitted via a T-piece directly in front of the ESI-probe to feed the ESI source with a reduced flow. This flow was adjusted by selecting capillary tubing of appropriate internal diameter and length for the waste line. The flow was reduced from 500 to 100 μl/min for the analysis and the signal intensity was significantly increased.

Under the established LC conditions, LC chromatograms (288 nm) of 'Shuangdan' granule and rat plasma sample were shown in Fig. 1. Through comparing the LC chromatograms of 'Shuangdan' granule and rat plasma sample, it was found

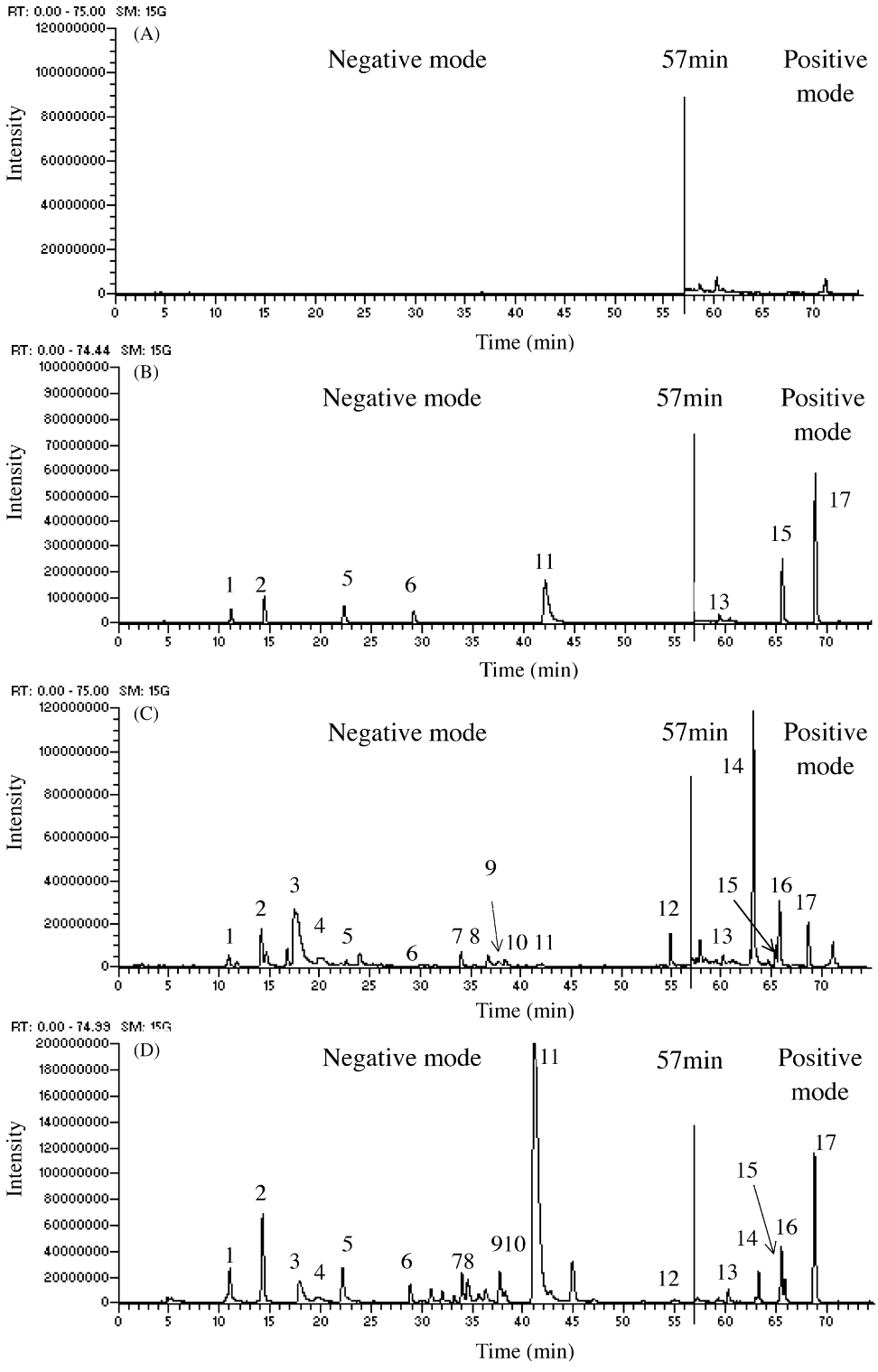

Fig. 2. Extracted ion chromatograms of the 17 components: (A) blank rat plasma, (B) standards in reconstitution solvent, (C) rat plasma sample collected after oral administration of 'Shuangdan' granule and (D) 'Shuangdan' granule. Peak numbers were consistent with those shown in Table 1.

that the profile of rat plasma sample was greatly different from that of 'Shuangdan' granule. This illustrated the changes of herb components in the course of physiological disposition.

In order to screen absorbed and metabolic components of 'Shuangdan' granule in rat plasma, 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 more sensitive in our study, except for paeonol and some tanshinones. The spectra were recorded in the *m*/*z* range of 100–800. For the purpose of obtaining the highest signal intensity in one analysis turn, negative scan mode was adopted from 0 to 57 min and positive mode was applied at the remaining time. Absorbed and metabolic components in rat plasma could be primarily identified by comparing the MS spectra with that of literature data. The structure of them should further be confirmed by more information, for example, MS^n spectra.

3.2. Identification of herb components in rat plasma

As to obtain fragmentation patterns of constituents from 'Shuangdan' granule and plasma samples, MS^n spectra of eight reference compounds were firstly analyzed by direct infusion. MS/MS and MS^n data were obtained by collision-induced dissociation (CID). The fragmentation patterns were proposed and it was very helpful for the constituents' structure identification in 'Shuangdan' granule and plasma samples that had the similar framework.

In order to identify the structure of herb components in plasma sample, 'Shuangdan' granule, rat plasma sample obtained 30 min after administration of 'Shuangdan' granule and blank rat plasma sample were analyzed under the established HPLC/MS^n method. On the basis of full-scan results, specified precursor ions in each MS scan were selected in turn and subjected to tandem mass spectrometry (MS^n, $n = 2$–3) analyses. The relative collision energy for CID varied from 15 to 45% of

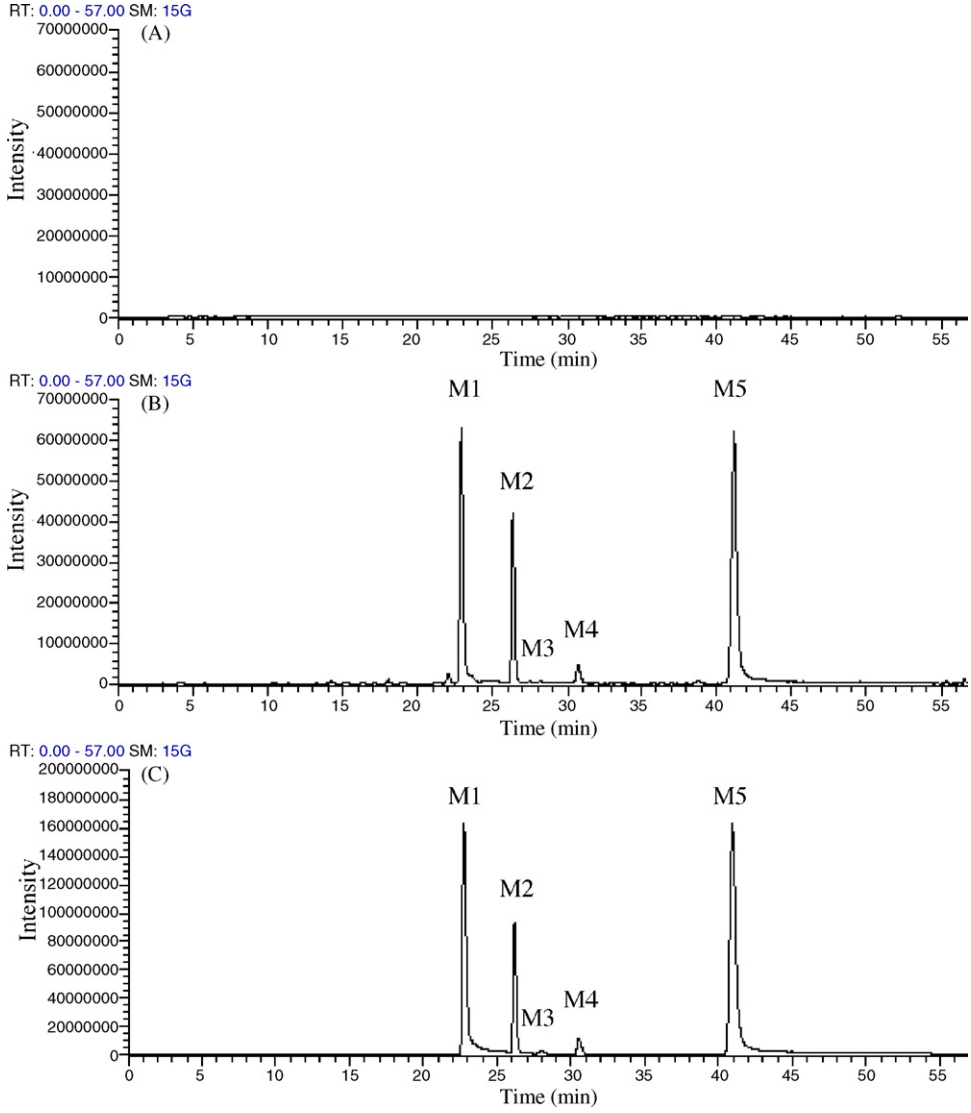

Fig. 3. Extracted ion chromatograms of the metabolites: (A) blank rat plasma, (B) rat plasma sample after oral administration of 'Shuangdan' granule and (C) rat plasma sample after oral administration of paeonol.

maximum to produce optimum yields of product ions, and the isolation width of precursor ions was 3.0 Th. Under the aforementioned conditions 17 main components were detected in both plasma samples and 'Shuangdan' granule. Table 1 illustrated the HPLC/MS^n data of the 17 herb components in rat plasma.

Peaks 1, 2, 5, 6, 11, 13, 15 and 17 were designated to be gallic acid, danshensu, protocatechuic aldehyde, paeoniflorin, salvianolic acid B, paeonol, dihydrotanshinone, cryptotanshinone, respectively, by comparing the retention time and mass spectra with those of reference compounds. Because of the lack of reference compounds, peaks 4, 7, 8, 9, 10, 12, 14 and 16 were tentatively characterized by comparing MS and MS^n spectra with that of literature data [17–20]. The results showed that the ESI-MS–MS spectra of salvianolic acids displayed a characteristic fragmentation pattern. The MS^n spectra of all salvianolic acids gave the fragment ions $[M-H-198]^-$ derived from neutral loss of one molecule of danshensu from the molecular ion. The fragment ion $[M-44-H]^-$ corresponding to the loss of CO_2 molecule was also observed in the spectra of most of the phenolics due to the presence of –COOH group in all of these compounds. The abundance of the de-carboxyl fragment ion $[M-44-H]^-$ at *m/z* 493 in the HPLC–ESI-MS–MS spectrum

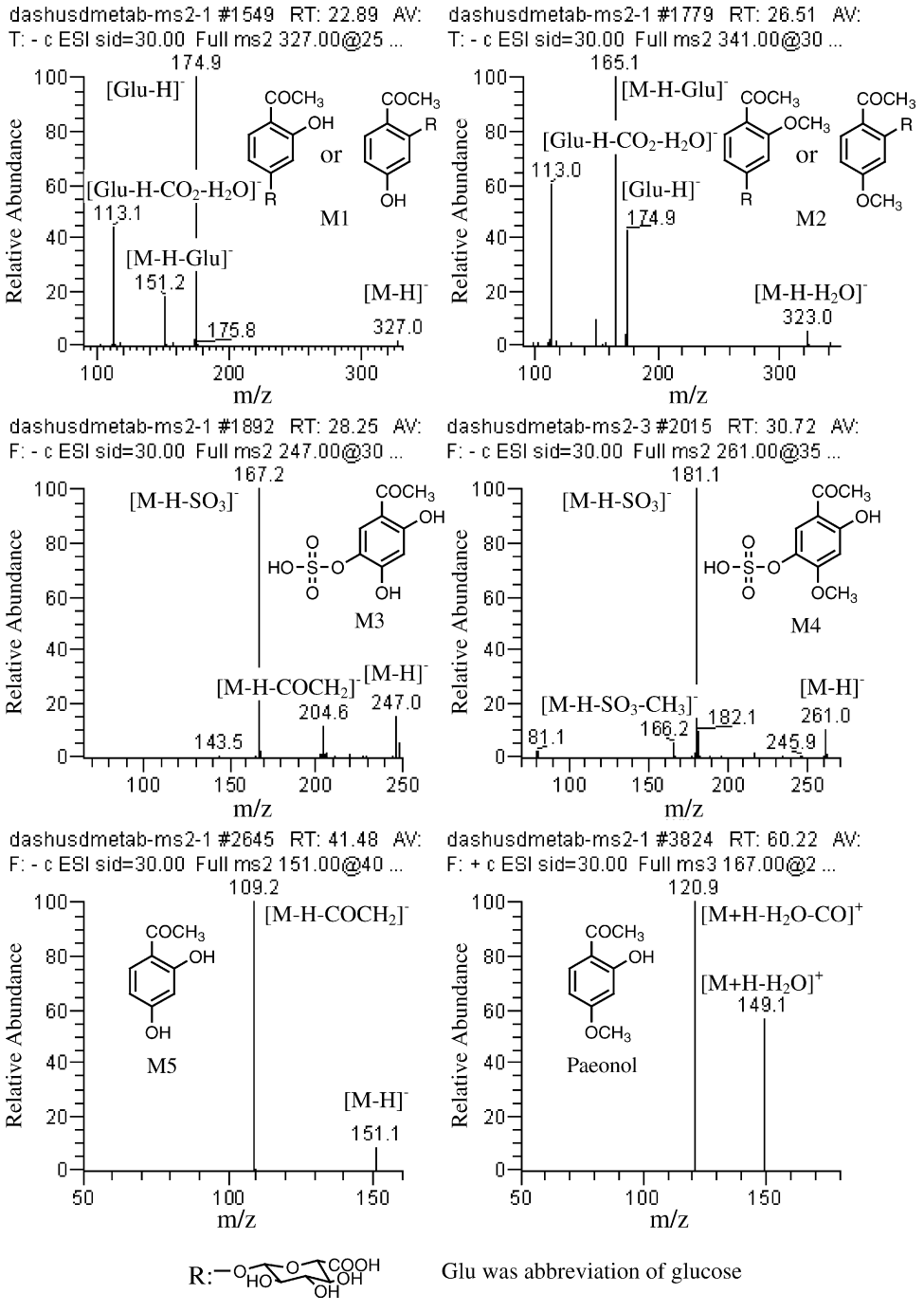

Fig. 4. MS spectra and structures of paeonol and the metabolites.

of peak 10 (lithospermic acid) was much higher than that of its quasi-molecular peak $[M-H]^-$ at *m/z* 537. The ESI-MS fragmentation of tanshinones mainly involved in the loss of a molecule of H_2O $[M+H-18]^+$ due to enolic rearrangement of a ketone group [19,20].

Because some of the components occurred at trace levels, the signals of them were masked by other components at high concentration in the total ion chromatograms of full-scan. Quasi-molecular ions of the 17 components were simultaneously extracted from the total ion chromatogram of full-scan to form an extracted ion chromatogram. The extracted ion chromatograms of blank rat plasma, standards in solvent, rat plasma sample collected 30 min after oral administration of 'Shuangdan' granule and 'Shuangdan' granule were shown in Fig. 2. The results showed that the pattern of components in rat plasma (Fig. 2C) was significantly different from that in 'Shuangdan' granule (Fig. 2D). Water-soluble phenolics, especially salvianolic acids B (peak 11) presented in low concentration in plasma while nonpolar tanshinones (peak 14–17) exhibited relatively higher abundance. Differences of components in absorption, metabolic rate or binding with plasma proteins may have contributed to the observed change of patterns.

3.3. Identification of metabolites in rat plasma

Compounds M1–M5 were only found in plasma samples. The UV spectrum of M5 was found to be very similar to that of paeonol, indicating that they maybe come from a similar structure. The molecular weight of M5 was 152, which was 14 less than that of paeonol. Therefore, M5 was supposed to be demethylated metabolite of paeonol. Meanwhile earlier publication had reported that paeonol undergone rapid absorption and metabolism after its oral administration to rats. Resacetophenone, 2,4-dihydroxyacetophenone-5-*O*-sulfate, and 2-hydroxy-4-methoxyacetophenone-5-*O*-sulfate were designated to be metabolites of paeonol [21].

In order to further confirm the origin and then to identify the structure of the metabolites, reference compound of paeonol was orally administered to rats. The results showed that M1–M5 were also found in plasma obtained from rat after administration of paeonol and they had the same retention time and fragmentation behavior as those in plasma of rat after administration of 'Shuangdan' granule (see Fig. 3). As a result, M1–M5 were identified to be metabolites of paeonol.

HPLC/MS^n data and MS spectra of metabolites M1–M5 were listed in Table 2 and Fig. 4, respectively. Quasi-molecular ion $[M-1]^-$ of M5 was 151, which was identified to be resacetophenone. Quasi-molecular ion $[M-1]^-$ of M1 was 327, and the product ion was 175, 113, 151, showing that M1 was the glucuronide conjugates of M5 (175 and 113 were product ion of glucuronide [22]). Quasi-molecular ion $[M-1]^-$ of M2 was 341, indicating that M2 was the addition of a methyl group to M1. M3 and M4 were determined to be 2,4-dihydroxyacetophenone-5-*O*-sulfate and 2-hydroxy-4-methoxyacetophenone-5-*O*-sulfate, respectively [21]. The structure of paeonol and the proposed structures of the metabolites and paeonol were shown in Fig. 4.

M1, M2 and M5 were detected in high concentration after oral administration of 'Shuangdan' granule, whereas M3 and M4 were found in relatively lower concentration. As a result, the signal of M3 and M4 could easily be masked by that of other components in total ion chromatogram of full-scan. The quasi-molecular ions of the five metabolites were simultaneously extracted from the full-scan chromatogram to form an extracted ion chromatogram. The extracted ion chromatograms of blank plasma, rat plasma sample collected after administered 'Shuangdan' granule and rat plasma collected after administered paeonol were shown in Fig. 3. The results showed that the ratio of metabolites in rat plasma after administration of 'Shuangdan' granule was very similar with that after administration of paeonol, which further confirmed that M1–M5 were metabolites of paeonol.

4. Conclusion

An HPLC–ESI-MS/MS method was applied for analyzing absorbed and metabolic components in rat plasma samples after administration of 'Shuangdan' granule. Over 20 components including 16 components from 'Shuangdan' granule and 5 metabolites were simultaneously identified. Furthermore, the origin of the metabolites was confirmed by comparing the mass spectra and chromatographic behavior with that of rat plasma sample obtained after administration of reference compound. The proposed method was simple, reliable and sensitive, which revealed that it was appropriate for rapid screening and structural characterization of absorbed and metabolic components of 'Shuangdan' granule. The study offered scientific data to clarify active components responsible for the pharmacological effects of 'Shuangdan' granule. It was also helpful to better understand the *in vivo* metabolism of components in 'Shuangdan' granule.

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