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Short communication

Identification of major constituents in the traditional Chinese medicine "QI-SHEN-YI-QI" dropping pill by high-performance liquid chromatography coupled with diode array detection-electrospray ionization tandem mass spectrometry

Li Yunfei, Qu Haibin *, Cheng Yiyu

Department of Chinese Medicine Science & Engineering, Zhejiang University, Hangzhou 310058, People's Republic of China

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Abstract

An HPLC–DAD–ESI-MS^n method was developed for simultaneous analysis of major chemical constituents in "QI-SHEN-YI-QI" dropping pill, a traditional Chinese medicine (TCM) widely used for treating cardiovascular diseases. The chromatographic separation was performed on an intertsil ODS-3 C_{18} column (4.6 mm × 250 mm, 5 μm), whilst water with 0.05% acetic acid and acetonitrile were used as mobile phase. On the basis of the characteristic UV absorption profile, the information of molecular weight, and structure provided by ESI-MS^n, 31 constituents derived from *Astragalus membranaceus*, Radix Salviae Miltiorrhizae, and *Panax notoginseng*, were detected and 20 of them were identified in this study. The proposed method contributes to the quality control of "QI-SHEN-YI-QI" dropping pill.

Keywords: "QI-SHEN-YI-QI" dropping pill; Components analysis; High-performance liquid chromatography–diode array detection-electrospray ionization tandem; Traditional Chinese medicine

1. Introduction

There is a worldwide trend of increasing use of alternative medicines. Traditional Chinese medicine (TCM), considered alternative medicine in the West, is usually made by several medicinal plants containing hundreds of compounds. The extreme complexity in TCM preparations causes tremendous difficulty in their quality control. In order to discern the chemical compositions of TCM preparation, many efforts to date have been made to develop specific analytical methods for comprehensively describing and identifying phytochemical components of TCM [1–5].

'QI-SHEN-YI-QI' dropping pill is one of the most widely used TCM prescription, in China for treating chronic heart failure, myocarditis, myocardial infarction and myocardial fibrosis. 'QI-SHEN-YI-QI' dropping pill is made by four commonly used Chinese herbs, i.e. *Astragalus membranaceus*, Radix Salviae Miltiorrhizae, *Panax notoginseng* and *Dalbergia odorifera*. Up to now, some researchers have reported their works on the analysis of bioactive constituents in *A. membranaceus* [6,7], Radix Salviae Miltiorrhizae [8–11], *P. notoginseng* [12,13] and *D. odorifera* [14,15]. However, to the best of our knowledge, the analytical method to identify multi-constituents in 'QI-SHEN-YI-QI' dropping pill was not yet explored. In order to provide valuable information for quality control, new studies are urgently needed to analyze the constituents in 'QI-SHEN-YI-QI' dropping pill.

As a powerful analytical tool, liquid chromatography coupled electrospray ionization tandem mass spectrometry (LC–ESI-MS^n) has been widely applied for the identification of the known compounds and helping the elucidation of unknown compounds in the botanic extracts and TCM preparation [16–20]. For example, using the fragmentation patterns and the comparison of the UV, MS data with the data from authentic compounds and literature, even the unknown constituents might be interpreted. In this paper, an HPLC–DAD–ESI-MS^n method was developed

* Corresponding author. Tel.: +86 571 88208428; fax: +86 571 88208428.
E-mail address: quhb@zju.edu.cn (Q. Haibin).

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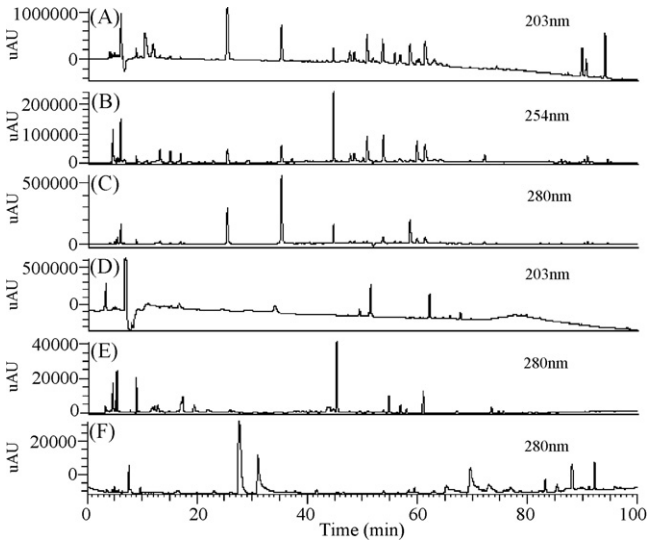

Fig. 1. HPLC–UV chromatogram of 'QI-SHEN-YI-QI' dropping pill (A–C), extracts of *Astragalus membranaceus* (D), Radix Salviae Miltiorrhizae (E) and *Panax notoginseng* (F).

to identify the main constitutes in 'QI-SHEN-YI-QI' dropping pill.

2. Experimental

2.1. Solvents and chemicals

The HPLC grade acetonitrile from Merck (Darmstadt, Germany) was used for chromatography. Analytical-grade methanol and acetic acid were purchased from Hangzhou Reagent Company (Hangzhou, China). Water was purified by Milli-Q academic water purification system (Millipore, France).

Reference compounds, danshensu, protocatechuic aldehyde, salvianolic acid B, notoginsenside R_1, ginsenside Rg_1, ginsenside Rg_3, and astragaloside IV were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The purity of these compounds were determined to be more than 98% by normalization of the peak areas detected by HPLC, and showed to be very stable in methanol solution.

"QI-SHEN-YI-QI" dropping pill and its raw materials including *A. membranaceus*, Radix Salviae Miltiorrhizae, and *P. notoginseng* were provided by TASLY Pharmaceutical Co. Ltd. (Tianjin, China). The voucher specimens were deposited in our laboratory.

2.2. Sample preparation

Ten grams of "QI-SHEN-YI-QI" dropping pill was ultrasonically extracted with 50 mL of methanol for 30 min. The filtrate was centrifuged at 10,000 rpm for 10 min to remove particles, evaporated to dryness in vacuum at 50 °C and the residue was dissolved in 5 mL of methanol. The dry Radix *A. membranaceus*, Radix Salviae Miltiorrhizae and *P. notoginseng* were ground into powder. Each crude material (5 g) were extracted with 50 mL water at 100 °C for 1 h, and then followed by 50 mL 70% alcohol aqueous solution. The operations were repeated for two times, and the total extracts were combined together. The solvents were removed at 60 °C under vacuum by Büchi rotavapor B-490. The residues were then dissolved in 100 mL water. All the samples were filtered through a 0.45 μm film before HPLC analysis.

2.3. HPLC–DAD–MS system

High-performance liquid chromatographic analysis was carried out using an Agilent 1100 series HPLC system (Waldbronn, Germany) equipped with a quaternary pump with on-line degasser, autosampler, column oven and diode array detector (DAD). An intertsil ODS-3 C_{18} column (4.6 mm × 250 mm, 5 μm, Dikma) was used, sample injection volume was 10 μL, the temperature of column oven was set at 30 °C, flow rate was 0.5 mL min^{-1}, mobile phases consisted of water with 0.05% formic acid (A) and acetonitrile (B). A gradient was performed according to the following profile: 0–20 min, 2–10% B; 20–70 min linear increase to 50% B; 70–90 min linear increase to 95% B; 90–100 min hold on 95% B. UV spectra were recorded from 190 nm to 400 nm, and the monitored wavelengths were set at 202 nm, 230 nm, 254 nm and 280 nm. An LCQ DECA XP^{plus} mass spectrometer (Thermo Finnigan, San Jose, USA) equipped with an ESI interface and an ion trap mass analyzer was used for carrying out the MS and MS^n analysis. Data were acquired and processed by Thermo Finnigan Xcalibur1.3 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 L min^{-1}; auxiliary gas (N_2) flow rate, 10.0 L min^{-1}. 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 widths. The collision energy values were automatically selected.

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

Reference compounds	MS (*m/z*)	MS^2 (*m/z*)	MS^3 (*m/z*)
Danshensu	197 $[M-H]^-$	179 $[M-H-H_2O]^-$	135 $[M-H-H_2O-CO_2]^-$
Protocatechuic aldehyde	137 $[M-H]^-$	109 $[M-H-CO]^-$	–
Salvianolic acid B	717 $[M-H]^-$	519 $[M-H-(danshensu)]^-$	321 $[M-H-2\times(danshensu)]^-$
Notoginsenoside R_1	991 $[M+CH_2COOH]^-$	977 $[M+COOH]^-$	931 $[M-H]^-$
Ginsenoside Rg_1	859 $[M+CH_2COOH]^-$	799 $[M-H]^-$	637 $[M-H-Xyl]^-$
Ginsenoside Rg_3	819 $[M+COOH]^-$	783 $[M-H]^-$	621 $[M-H-Xyl]^-$
Astragaloside IV	843 $[M+CH_2COOH]^-$	783 $[M-H]^-$	621 $[M-H-Xyl]^-$

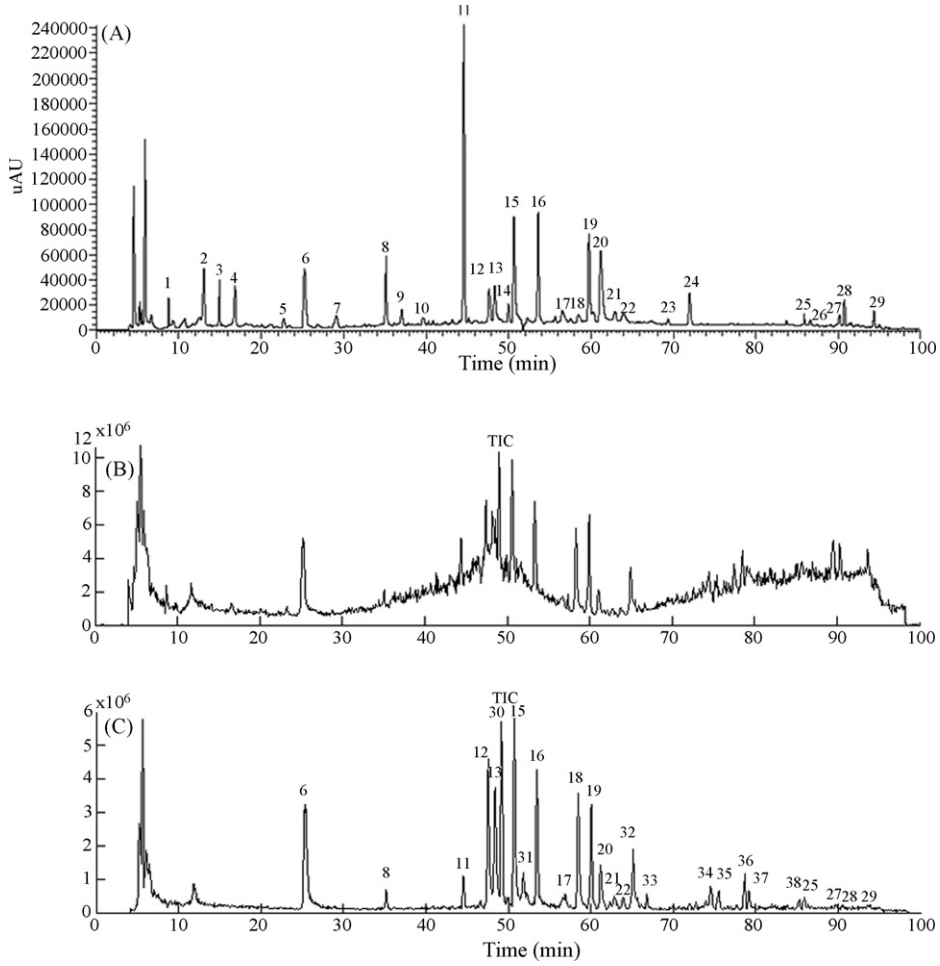

Fig. 2. HPLC–UV chromatogram (254 nm) of 'QI-SHEN-YI-QI' dropping pill (A), total ion current (TIC) chromatogram of 'QI-SHEN-YI-QI' dropping pill (B) and total ion current chromatogram after denoising of 'QI-SHEN-YI-QI' dropping pill (C).

2.4. Data processing

An in-house program that reduces the noise of LC–MS data was written in Matlab 7.0 (Mathworks Inc. Natick, USA). The computer configuration was a Pentium 4, 2.8 GHz with 256 MB of RAM.

3. Results and discussion

3.1. HPLC–DAD analysis of "QI-SHEN-YI-QI" dropping pill

The main constituents of "QI-SHEN-YI-QI" dropping pill were water-soluble compounds from *A. membranaceus*, Radix Salviae Miltiorrhizae and *P. notoginseng* and essential oils from *D. odorifera*. Our preliminary study further indicated that the essential oils were rarely detected and the main constituents of "QI-SHEN-YI-QI" dropping pill were saponins, flavones, and phenolic acids. According to the characteristic UV profile of these major constituents, the approximate maximum UV absorption wavelengths of triterpene saponins were at 203 nm; phenolic acids were at 220 nm and 280 nm; flavones were at 230 nm and

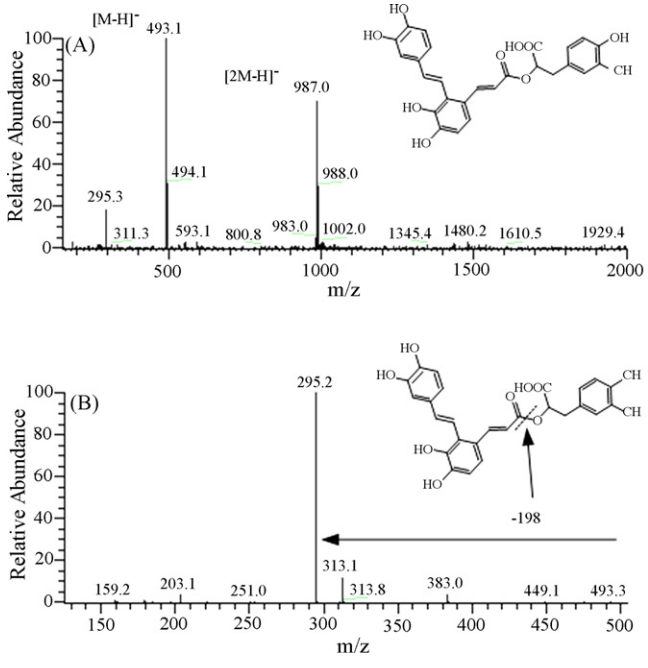

Fig. 3. ESI spectra of peak 18: (A) negative ion ESI mass spectrum of peak 18 (assigned as Salvianolic acid A) and (B) MS^2 spectra of peak 18.

Table 2
HPLC–DAD–ESI-MS identification

Peak no.	RT (min)	UV (nm)	Negative ion	Chemical	Crude herb[a]
1	8.76	192	539, 863, 701	–	1
2	13.09	206, 262	289, 387	–	1
3	14.91	208, 258	269	–	1
4	16.73	254	611	Ethyllithospermate	2
5	23.27	234, 260	479, 433	–	–
6	25.39	204, 280	197, 395	danshensu	2
7	29.10	234, 254	–	–	–
8	35.14	230, 280, 312	275	Protocatechuic aldehyde	2
9	37.02	236, 258	510	–	–
10	39.55	234, 266	727	–	1
11	44.56	255, 248, 284	937, 491, 283	Calycosin-7-*O*-β-D-glucopyranoside	1
12	47.59	236, 324	537, 1075	Salvianolic acid H or I	2
13	48.28	236, 324	537, 1075	Salvianolic acid H or I	2
14	49.88	236, 296, 322	537	Linthospermic acid	2
15	51.12	224, 248, 320	417	Salvianolic acid D	2
16	53.44	222, 232	359, 719	Rosmarinic acid	2
17	56.35	232, 284	717, 991, 509	Linthospermic acid B	2
18	58.60	228, 288	493, 987	Salvianolic acid A	2
19	59.74	238, 290	283, 567	Calycosin-7-*O*-β-D-glucopyranoside-6″-*O*-malonate	1
20	61.19	230, 254, 280	717, 739	Salvianolic acid B	2
21	62.87	232, 254, 286	717, 519, 327	Salvianolic acid E	2
22	64.37	238, 284, 314	537	–	2
23	69.26	248, 270	961, 1231	–	1
24	71.99	248, 299	267	Formononetin	1
25	85.90	246	825	–	–
26	86.46	248	–	–	–
27	89.47	264	295	–	2
28	90.71	246, 266	417	–	2
29	93.81	268	687	–	2
30	49.04	–	977, 931	Notoginsenoside R_1	3
31	51.12	–	417, 845, 859	Ginsenoside Rg_1	3
32	64.79	–	829	Astragaloside IV	1
33	65.23	–	991	Notoginsenoside k	3
34	74.48	–	987, 977	–	1
35	75.63	–	987, 977	–	1
36	78.24	–	1183	–	–
37	79.25	–	829	Ginsenoside Rg_3	3
38	85.12	–	811	–	–

[a] 1, *Astragalus membranaceus* Bge.; 2, Radix Salviae Miltiorrhizae; 3, *Panax notoginseng*.

275 nm. The framework type of compounds could be rapidly identified based on their characteristic UV profiles.

Fig. 1 displayed the HPLC–UV chromatogram of "QI-SHEN-YI-QI" dropping pill, extracts of *A. membranaceus*, Radix Salviae Miltiorrhizae and *P. notoginseng*. As revealed in Fig. 1A–C, the main constituents in "QI-SHEN-YI-QI" dropping pill were well separated on reversed-phase column with gradient elution. Comparing the chromatograms of "QI-SHEN-YI-QI" dropping pill (Fig. 1A–C) with those of extracts of *A. membranaceus* (Fig. 1D), Radix Salviae Miltiorrhizae (Fig. 1E) and *P. notoginseng* (Fig. 1F), the major constituents in "QI-SHEN-YI-QI" dropping pill were assigned to the crude plants.

3.2. HPLC–MS analysis of authentic compounds

To obtain fragmentation patterns of constituents in "QI-SHEN-YI-QI" dropping pill, MS^n spectra of seven reference compounds, i.e. danshensu, protocatechuic aldehyde, salvianolic acid B, notoginsenside R_1, ginsenside Rg_1, ginsenside Rg_3, and astragaloside IV, 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 "QI-SHEN-YI-QI" dropping pill that had the similar framework. All authentic compounds exhibited quasi-molecular ion $[M-H]^-$ and adducted ions ($[M+Cl]^-$ and $[M+AcO]^-$). The retention time (RT), UV λ max, *m/z* values of ions, MS data and their main fragment in MS^n spectra of the seven reference compounds are summarized in Table 1.

3.3. HPLC–DAD–MS^n analysis of "QI-SHEN-YI-QI" dropping pill

Fig. 2 showed the HPLC–UV chromatogram at 254 nm (Fig. 2A) and total ion chromatogram of the "QI-SHEN-YI-QI" dropping pill (Fig. 2B). As we can see, due to the poor signal-to-noise ratio in mass data, it was difficult to compare the components or peaks in TIC chromatograms. Here, we

employed a commonly used method, called CODA [21], to denoise the MS data. Fig. 2C showed the total ion chromatogram after denoising. A total of 29 major peaks were detected by DAD and 18 of them have the corresponding peaks in the MS chromatogram. Except these 29 peaks detected by DAD, 9 other peaks with very low UV absorption were also detected in the MS chromatogram. By comparing HPLC–UV and HPLC–MS chromatograms of "QI-SHEN-YI-QI" dropping pill with those obtained from the extracts of crude herbs, most peaks in the chromatogram of "QI-SHEN-YI-QI" dropping pill were assigned to the corresponding crude herb. The chemical identities corresponding to peaks 6, 8, 20, 30, 31, 32 and 37 were identified by comparing the RT, UV spectra and *m*/*z* value with those of the reference compounds. The results are listed in Table 2.

By comparing the UV spectra, MS and MS^2 spectra with fragmentation patterns of aforementioned reference data and

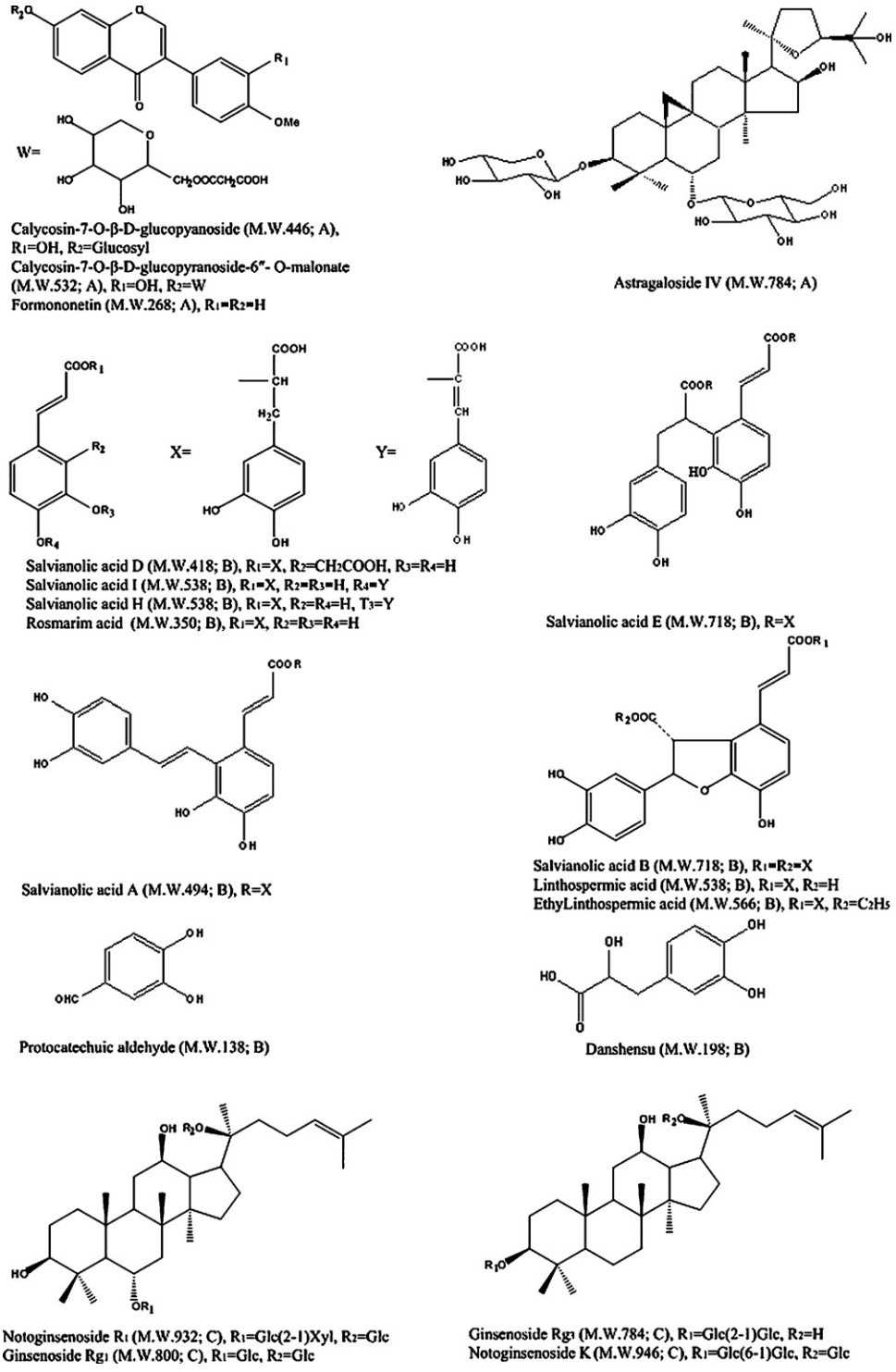

Fig. 4. Structure of the identified compounds (A, *Astragalus membranaceus*; B, Radix Salviae Miltiorrhizae; C, *Panax notoginseng*).

the literature [11,22,23], peaks 4, 11, 14, 15, 16, 17, 18, 19, 21, 24 and 33 were identified as compounds ethyllithospermate, calycosin-7-*O*-β-D-glucopyranoside, linthospermic, salvianolic acid D, rosmarinic acid, linthospermic acid B, salvianolic acid A, calycosin-7-*O*-β-D-glucopyranoside-6″-*O*-malonate, salvianolic acid E, formononetin, notoginsenoside k, respectively (Table 2). For instance, peak 18 exhibited a quasi-molecular ion $[M-H]^-$ of *m/z* 493. Its MS^2 spectra gave rise to fragment ions of *m/z* 295 (Fig. 3), corresponding to the loss of a molecule of danshensu. The maximum UV absorption wavelengths of peak 18 were at 228 nm and 288 nm. These data are consistent with those in the literature [23]. Therefore, peak 18 was tentatively identified as salvianolic acid A. Peaks 11 and 19 exhibited adducted ions $[M+Cl]^-$ of *m/z* 577 and *m/z* 481 respectively. Their MS^2 spectra gave the fragment ions of *m/z* 283, which was a molecule of calycosin. Therefore, peaks 11 and 19 were tentatively identified as calycosin-7-*O*-β-D-glucopyranoside and calycosin-7-*O*-β-D-glucopyranoside-6″-*O*-malonate [22]. For peak 24, a quasi-molecular ion $[M-H]^-$ of 267 was observed. The maximum UV absorption wavelengths of peak 24 were at 248 nm and 299 nm. According to these data, peak 24 was tentatively identified as formononetin [22].

Several peaks, such as 5, 7, 9, 25, 26, 36 and 38, are failed to be assigned to any crude plant. One of the most likely reasons is that they are the intermediates of manufacture or sample preparation. For peaks 1, 2, 3, 10, 22, 23, 27, 28, 29, 34, and 35, the MS^2 spectra were not observed, probably because of the low abundance of the precursor ions. Therefore, they were characterized by the *m/z* values, UV spectra and corresponding crude plant in Table 2. The data of individual peaks is summarized in Table 2 and the structures of the identified compounds are shown in Fig. 4.

4. Conclusions

In this study, the chemical constituents in "QI-SHEN-YI-QI" dropping pill were analyzed by HPLC–DAD–ESI-MS^n. Based on retention time, UV spectra, and MS information, a total of 20 compounds were successfully identified. This study demonstrates that HPLC–DAD–ESI-MS^n is a powerful technique to analyze the multi-ingredients medicine formulae, e.g. TCM preparation. The results of this study would be helpful to discover the biologically active compounds in "QI-SHEN-YI-QI" dropping pill. More importantly, it provided valuable information for development and improvement of the quality control of 'QI-SHEN-YI-QI' dropping pill.

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