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Short communication

Solid-phase extraction and liquid chromatography–electrospray mass spectrometric analysis of saponins in a Chinese patent medicine of formulated *Salvia miltiorrhizae* and *Panax notoginseng*

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Abstract

In this paper, a method using solid-phase extraction (SPE) and HPLC/ESI-MS^n for the identification of the major saponins in "Danshen Dripping Pill", a Chinese patent medicine consisting of *Salvia miltiorrhizae* and *Panax notoginseng*, is described. Through solid-phase extraction process, the saponins in "Danshen Dripping Pill" were separated from the phenolic constituents of *S. miltiorrhizae* and, meanwhile, efficiently concentrated. Subsequently, these saponins were characterized by HPLC/ESI-MS^n analysis. Based on the studies of MS and MS^2 spectra and the comparison with reference compounds and literature data, a total of 19 saponins were identified.

Keywords: *Panax notoginseng*; *Salvia miltiorrhizae*; Saponin; Solid-phase extraction; HPLC/ESI-MS^n

1. Introduction

"Danshen Dripping Pill" (Chinese name Fufang-Danshen-Diwan), consisting of *Salvia miltiorrhizae* (Chinese name Danshen) and *Panax notoginseng* (Chinese name Sanqi), is a widely used drug for the prevention and treatment of coronary arteriosclerosis, angina pectoris and hyperlipaemia. Moreover, it has been well sold over a few years as diet supplement or drug in countries such as USA, Russia, Singapore, South Korea and UAE.

Nevertheless, the pharmaceutically active constituents in "Danshen Dripping Pill" are still not well understood. Therefore, the chemical studies on this drug to reveal its major constituents are primarily necessary for pharmacological studies and, nowadays, to some extent for the improvement the quality control of this drug, which is mainly based on the quantitative assay of 3,4-dihydroxyphenyllactic acid (Danshen su), a bioactive compound from *S. miltiorrhizae* [1].

Saponins, known as ginsenosides and notoginsenosides, are widely believed to be the main bioactive constituents in *P. notoginseng* [2–5] and theoretically, should have been in part responsible for the therapeutic activity of "Danshen Dripping Pill". However, up to now, only a few of saponins in this drug including ginsenosides Rg_1, Rb_1 and notoginsenosides R_1 were studied [6] and most of the saponins in this drug are still unknown.

So far, HPLC/MS coupling technique has been widely used for the qualitative studies on saponins, especially ginsenosides, in botanic extracts [7–9]. But for this drug, the serious interference coming from the water-soluble constituents from *S. miltiorrhizae*, mainly the phenolic acids, makes the direct HPLC/MS analysis for saponins difficult. In this paper, an HPLC/ESI-MS^n method for identification of saponins in "Danshen Dripping Pill" is developed with the solid-phase extraction (SPE) as sample pre-preparation.

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2. Experimental

2.1. Instrumentation

An Agilent 1100 series HPLC system coupled with G1315 DAD (Agilent Company, USA) was used for HPLC analysis. The UV spectra were recorded in the scale from 190 to 400 nm. Chromatographic separation was achieved on a Zorbax SB-C18 column (4.6 mm × 250 mm, 5 μm, Serial No. USCL 009296, Agilent Company) along with Agilent C18 pre-column (4 mm × 5 mm) at a flow rate of 0.8 mL/min. The mobile phase consisted of A (CH_3COOH:H_2O = 0.01:100) and B (CH_3COOH:CH_3CN = 0.01:100). Gradient programs at 30 °C were performed as follows: 20–35% B at 0–15 min, 35% B isocratic for 10 min, 35–43% B at 25–40 min, 43–46% B at 40–50 min, 46–58% B at 50–65 min and 58–75% B at 65–70 min.

HPLC–MS analysis was performed with Agilent LC-MSD/Trap System (Agilent Company) equipped with an electrospray interface. The MS spectra were acquired in negative ion mode. N_2 was used as both drying gas with a flow rate of 10 L/min and as nebulizing gas with a pressure of 60 psi. The nebulizer temperature was set at 350 °C and the capillary voltage was set at 3500 V. The mass spectra were recorded in the range of 400–1500 μm. A fragment amplification of 1.5 V was selected for MS^2 analysis.

2.2. Regents and chemicals

Reference compounds of notoginsenosides R_1, R_2, ginsenosides Rb_1, Rd, Re, Rg_1, Rg_2, 20(*S*)-Rg_3, Rh_1 were purchased from college of pharmaceutical science, Jilin University (Changchun, China). HPLC grade methanol, acetonitrile (Tedia Company, Fairfield, USA) and A.R. grade acetic acid (Hangzhou Reagent Company, Hangzhou, China) were used for the HPLC analysis. HPLC grade methanol (Tedia Company) was used for SPE preparation. Water for HPLC analysis and SPE preparation was purified by Milli-Q academic water purification system (Milford Company, MA, USA).

2.3. Solid-phase extraction

A 2 g weight of "Danshen Dripping Pill" was dissolved using 20 mL 4% ammonia in an ultrasonic bath at 25 °C for 15 min. After centrifugation at 5000 × *g* for 10 min, a certain volume of the supernatant fluid was loaded and drawn through by gravity on SPE cartridge (5 mL, packed with 250 mg of 40 μm octadecyl silica, Waters, USA), which was pretreated by passing through 5 mL of methanol followed by 5 mL water before loading, and drawn through by gravity. Then, the solid-phase cartridge was washed with 10.0 mL of water to elute the phenolic compounds entirely off. Finally, the cartridge was eluted with 1.0 mL methanol, in which fraction most of the saponins were concentrated. A 20 μL volume of the methanol eluent was injected into the HPLC system.

3. Results and discussion

3.1. SPE preparation of "Danshen Dripping Pill"

Due to the low content of saponin in "Danshen Dripping Pill" and the serious interference from phenolic constituents on UV detection of saponins, a SPE pre-treating procedure of "Danshen Dripping Pill" was developed in order to eliminate the negative impact from phenolic constituents and to acquire saponins concentrated enough for qualitative study by HPLC/MS^n analysis.

As described in Section 2.3, different volumes of "Danshen Dripping Pill" solution were loaded on the cartridge and then processed by SPE operation. Fig. 1 showed the HPLC chromatogram of different methanol eluents.

The phenolic constituents from *S. miltiorrhizae* with great absorbances at 280 nm were not detected (seen in Fig. 1a), which should have transformed into salt in 4% ammonia and been eluted out by 10 mL water. The HPLC analysis of water eluent was also performed and a number of peaks with strong abundance were detected at 280 nm, which verified the presence of phenolic compounds. From Fig. 1b, it could be seen that the chromatogram of methanol eluents with different loading volume of "Danshen Dripping Pill" solution exhibited the same chromatographic profiles but the peaks significantly enlarged when the loading volume increased from 1 to 20 mL (seen in Fig. 1b). These results indicated that saponins in "Danshen Dripping Pill" had been efficiently separated and also concentrated through SPE procedure.

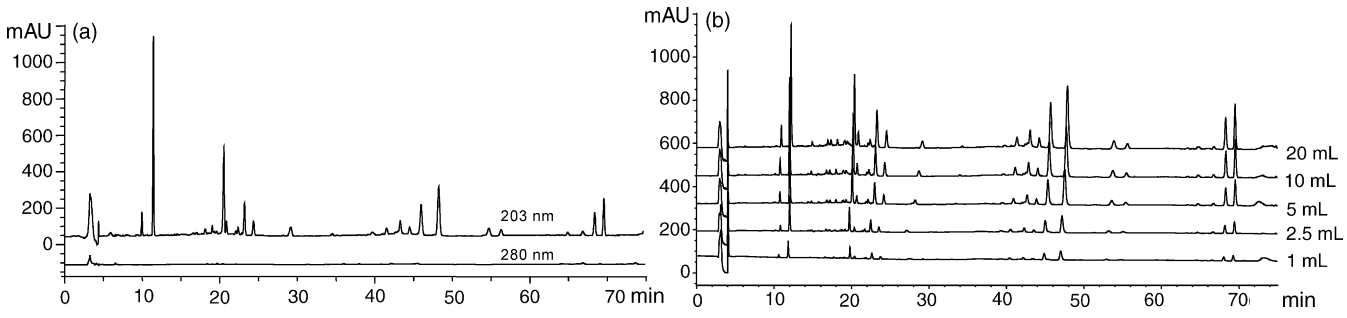


Fig. 1. (a) HPLC chromatograms of methanol eluents at 203 and 280 nm and (b) HPLC chromatograms (203 nm) of methanol eluents with different loading volume of "Danshen Dripping Pill" solution.

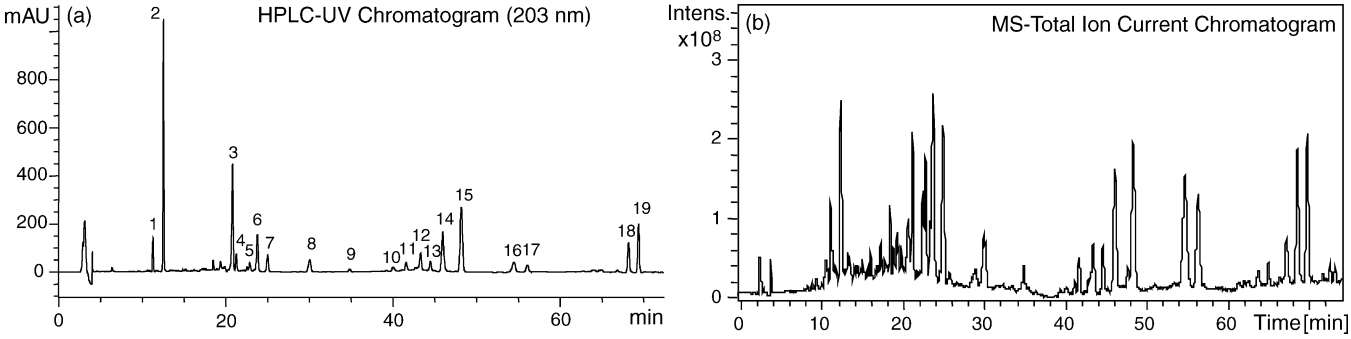


Fig. 2. HPLC chromatogram and MS-TIC chromatogram of SPE processed "Danshen Dripping Pill".

3.2. HPLC/MS^n analysis of saponins in "Danshen Dripping Pill"

Fig. 2 showed the HPLC (203 nm) and online MS-TIC chromatogram of saponins. The MS^n data of each compound are listed in Table 1. Comparing with the reference compounds, peaks 1–7, 9 and 17 were identified.

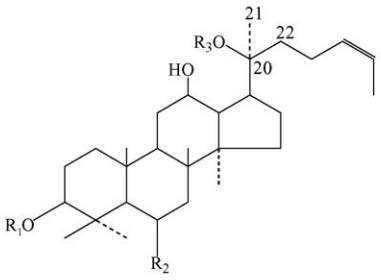
By CID experiments of reference compounds, the fragmentation pathways of ginsenosides' negative quasi-molecular ion, i.e. $[M–H]^-$, were concluded to be the successive loss of sugar and finally exhibited the deprotonted ion of aglycone, which was in accordance with literatures [7–9]. Thus, other peaks involving peaks 13–16 and 18–19 were identified by studies of their MS^2 spectra and by comparison with the literature data [9,10] (structures seen in Fig. 3). Some geometric isomers were distinguished by MS^2 spectra in terms of the aglycone variety and sugar unit sequences.

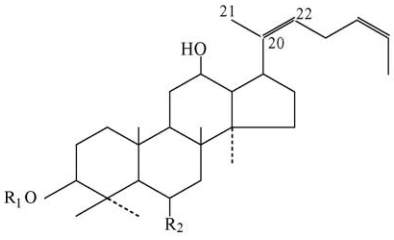
For an instance, peaks 13 and 18 (data seen in Table 1) both exhibited quasi-molecular ion at *m/z* 765 in the MS spectra. But from MS^2 spectra, peak 13 showed the successive loss of rhamnose and glucose and exhibited the deprotonated ion of aglycone at *m/z* 457, while peak 18 showed the loss of two glucoses and exhibited the deprotonated ion of aglycone at *m/z* 441. Thus, peak 13 was tentatively identified as Rg_6 or F_4 and peak 18 was tentatively identified as Rk_1 or Rg_5. For peaks 18 and 19, which were identified as Rk_1 or Rg_5, they were differentiated only on the dehydrated positions, i.e. C20–C21 bond or C20–C22 bond. Due to the lack of reference compounds and of stereo structure information from MS^2 spectra, they could not be exactly assigned to Rk_1 or Rg_5 in this study. For peaks 8 and 10–12, they were not found to be in consistent with reported compounds in notoginseng and ginseng. The MS and MS^2 spectra of peaks 8 and 10 were same as those of Rh_1 and Rd, respectively. It indicated that they were the isomers of Rh_1 and Rd, respectively. To our knowledge, such compounds have not been reported yet. In the same way, peaks 11 and 12 were found to be the isomers and they were not previously reported as well. Their aglycone varieties and sugar unit sequence were determined by MS^2 spectra of their deprotonated ion. The phytochemical study on these compounds needs to be carried out.

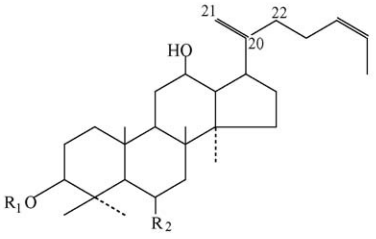
Table 1
HPLC/ESI-MS^n data and identification of saponins

Compound	Ret time	$[M–H]^-$	Fragment ion *m/z* (MS^2 spectra)	Identity
1	10.68	931	799$[M–H–Xyl]^-$; 637$[M–H–Xyl–Glc]^-$; 475$[M–H–Xyl–2Glc]^-$	R_1
2	12.38	945	799$[M–H–Rham]^-$; 783$[M–H–Glc]^-$; 637$[M–H–Rham–Glc]^-$; 475$[M–H–Rham–2Glc]^-$	Re
3	12.5	799	637$[M–H–Glc]^-$; 475$[M–H–2Glc]^-$	Rg_1
4	21.97	1107	945$[M–H–Glc]^-$; 783$[M–H–2Glc]^-$; 621$[M–H–3Glc]^-$; 459$[M–H–4Glc]^-$	Rb_1
5	22.52	769	637$[M–H–Xyl]^-$; 475$[M–H–Xyl–Glc]^-$	R_2
6	24.07	783	637$[M–H–Rham]^-$; 475$[M–H–Rham–Glc]^-$	Rg_2
7	25.02	637	475$[M–H–Glc]^-$	Rh_1
8	26.25	637	475$[M–H–Glc]^-$	Rh_1 iso.
9	31.05	945	783$[M–H–Glc]^-$; 621$[M–H–2Glc]^-$; 459$[M–H–3Glc]^-$	Rd
10	35.4	945	783$[M–H–Glc]^-$; 621$[M–H–2Glc]^-$; 459$[M–H–3Glc]^-$	Rd iso.
11	42.04	751	619$[M–H–Xyl]^-$	Not determined
12	43.72	751	619$[M–H–Xyl]^-$	Not determined
13	44.89	765	619$[M–H–Rham]^-$; 457$[M–H–Rham–Glc]^-$	Rg_6 or F_4
14	46.43	619		Rk_3 or Rh_4
15	48.68	619		Rk_3 or Rh_4
16	54.97	783	621$[M–H–Glc]^-$; 459$[M–H–2Glc]^-$	20(*R*)Rg_3
17	56.48	783	621$[M–H–Glc]^-$; 459$[M–H–2Glc]^-$	20(*S*)Rg_3
18	68.35	765	603$[M–H–Glc]^-$; 441$[M–H–2Glc]^-$	Rk_1 or Rg_5
19	69.53	765	603$[M–H–Glc]^-$; 441$[M–H–2Glc]^-$	Rk_1 or Rg_5

	R_1	R_2	R_3	MW
Rb_1	Glc-Glc	H	Glc-Glc	1108
Rc	Glc-Glc	H	Glc-Ara(f)	1078
Rd	Glc-Glc	H	Glc	946
Re	H	O-Glc-Rha	Glc	946
Rg_1	H	O-Glc	Glc	800
Rg_2	H	O-Glc-Rha	H	784
Rg_3	Glc-Glc	H	H	784
Rh_1	H	O-Glc	H	638
Noto-R_1	H	O-Glc-Xyl	Glc	962
Noto-R_2	H	O-Glc-Xyl	H	770







	R_1	R_2	MW
Rg_5	Glc-Glc-	H	766
F_4	H	O-Glc-Rha	766
Rh_4	H	O-Glc	620

	R_1	R_2	MW
Rk_1	Glc-Glc	H	766
Rg_6	H	O-Glc-Rha	766
Rk_3	H	O-Glc	620

Glc:β-D-glucose; **Rha:**α-L-rhamnose; **Ara(p):**α-L-arabinose(pyranose); **Araf:**α-L-arabinose(furanose)
Xyl:β-D-xylose

Fig. 3. Structures of saponins in "Danshen Dripping Pill".

4. Conclusion

In this study, solid-phase extraction was demonstrated to be very efficient for separation and concentration of saponins in Chinese patent drug "Danshen Dripping Pill", which is consisted of *S. miltiorrhizae* and *P. notoginseng*. By HPLC–MS^n analysis, a total of 19 saponins were identified, 15 of which were reported and other 4 were supposed to be new ginsenosides that have not been reported before. The results would be helpful to improve quality control of "Danshen Dripping Pill". Moreover, the method developed in this study could be introduced into the analysis of other Chinese patent drugs consisting of *S. miltiorrhizae* and *P. notoginseng*.

Acknowledgement

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