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Short communication

Simultaneous determination of abietane-type diterpenes, flavonolignans and phenolic compounds in compound preparations of *Silybum marianum* and *Salvia miltiorrhiza* by HPLC-DAD-ESI MS

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Abstract

A gradient HPLC-DAD-ESI MS method has been developed for simultaneous determination of multiple bioactive compounds such as abietane-type diterpenes, flavonolignans and phenolic compounds in compound preparations of *Silybum marianum* and *Salvia miltiorrhiza*. The separation was completed on an ODS column using 0.5% (v/v) formic acid aqueous solution and methanol as gradient mobiles. Fourteen components can be identified by ESI MS working on ESI− and ESI+ switching mode, respectively. Ten components can be quantified by using external standard method with UV detecting at 254 and 280 nm, respectively. The correlation coefficients of all the calibration curves were found to be higher than 0.992. The recoveries of the standards were about 96–101%. Besides quantification of the components, the chromatograms acquired by this method can be used as the bioactive components fingerprints for the quality control of compound preparations of *S. marianum* and *S. miltiorrhiza*.
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Keywords: Compound preparations; *Silybum marianum*; *Salvia miltiorrhiza*; HPLC-DAD-ESI MS

1. Introduction

Salvia miltiorrhiza Bunge, named 'Danshen' in Chinese, and *Silybum marianum*, a plant of milk thistle, have similar therapeutic effects on liver disease. And so far, many single plant preparations for protecting liver have been developed. Nevertheless, the pharmacological mechanisms of these two herbs are different. For example, *S. miltiorrhiza Bunge* can resist hepatic fibrosis by preventing liver cells from death [1], while *S. marianum* can have this therapeutic effect by restraining the activity of reactive oxygen species (ROS) [2]. It is likely to gain more satisfying therapeutic effect by mixing two herbs in one compound preparation. At present, there have already been some compound preparations containing these two herbs, such as Gantaikang and Yiganxintai and the like in Chinese market.

According to the chemical structures, the major bioactive constituents in *S. miltiorrhiza Bunge* can be classified into two groups: phenolic compounds such as danshensu, protocatechualdehyde, etc. and abietane-type diterpenes such as tanshinone I, tanshinone IIA, isotanshinone IIA, dihydrotanshinone I and cryptotanshinone, etc. In *S. marianum*, the major bioactive components are flavonolignans, including silybin A (SB_A), silybin B (SB_B), isosilybin A (ISB_A), isosilybin B (ISB_B), silydianin (SD) and silychristin (SC), etc.

So, the components of compound preparations of the two herbs will be more complex than that of the single herb preparations. It is valuable to develop a method for simultaneously determining the bioactive constituents of the two herbs in compound preparations for further study of the pharmacological effects and controlling the quality of the preparations. Up to now, there are many methods, such as capillary electrophoresis [3], HPLC equipped with UV, electrochemical or MS detection [3–6], etc., for the determination of flavonolig-

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nans in *S. marianum* and phenolic compounds or abietane-type diterpenes in *S. miltiorrhiza Bunge*, respectively. However, the simultaneous determination method of the bioactive constituents in compound preparations of the two herbs was seldom reported.

In this paper, a gradient HPLC-DAD-ESI MS method has been developed for simultaneous determination of multiple bioactive compounds such as abietane-type diterpenes, flavonolignans and phenolic compounds in compound preparations of *S. marianum* and *S. miltiorrhiza*. The chemical structures of analytes are shown in Fig. 1. Besides quantification of the components, the chromatograms acquired by this method can be used as the bioactive components fingerprints for the quality control of the compound preparations.

2. Experimental section

2.1. Chemicals and materials

Standards of silybin, danshensu, protocatechualdehyde, tanshinone I, crytotanshinone, dihydrotanshinone I and tanshinone II A were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing,

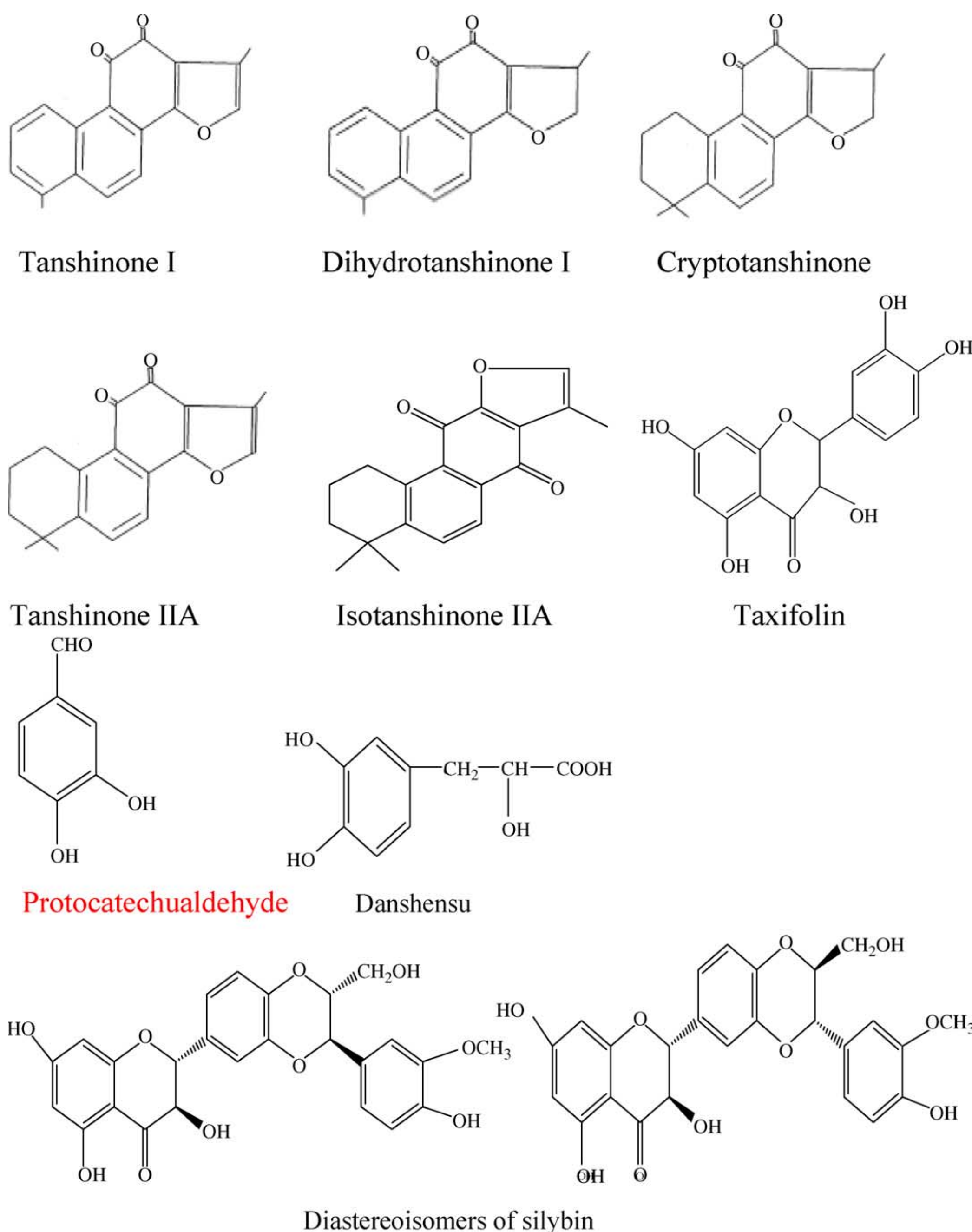

Fig. 1. Structures of the active constituents in *S. marianum* and *S. miltiorrhiza*.

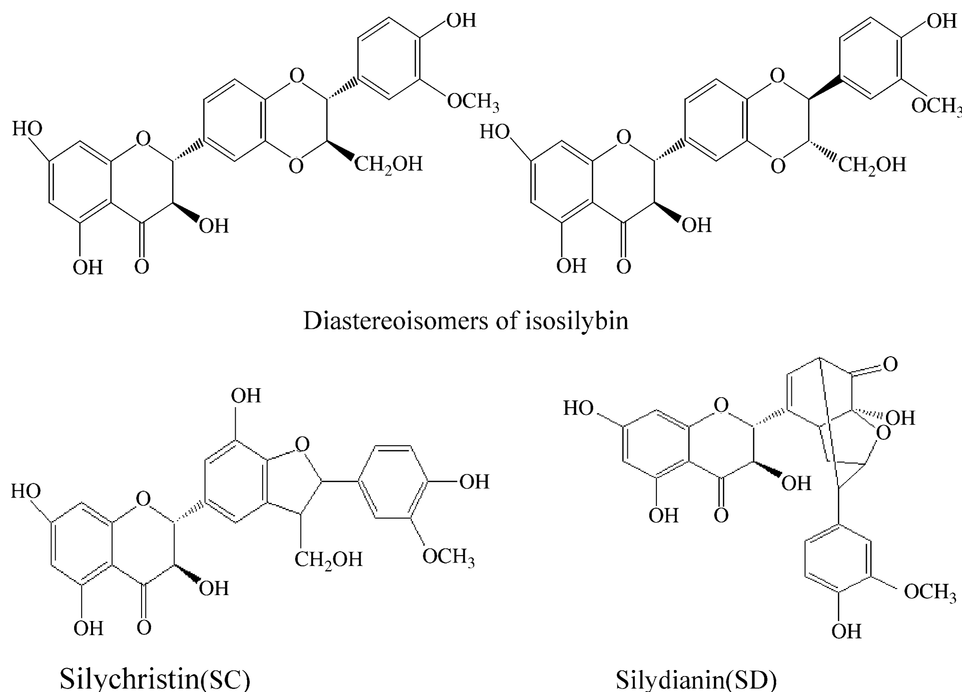

Fig. 1. (*Continued*).

China). Standards of silychristin, silydianin, isosilybin and the extracts of *S. marianum* and *S. miltiorrhiza* were obtained from Hunan phytoway TCM Co. China. Methanol (HPLC grade), formic acid (AR grade), and methanol (AR grade) were purchased from Shanghai Ludu Chemical Supply (Shanghai, China). Ultra-pure water was prepared using a Millipore Milli-Q purification system (Millipore Corp., Bedford, MA, USA).

2.2. Instrumentation

The HPLC-DAD MS system was from Waters (Milford, MA, USA), consisting of alliance 2695 liquid chromatography system equipped with two pumps, a thermostatically controlled column apartment, an autosampler with a 250 μl loop, a 996 photo diode array detector (DAD) and a Micromass ZQ 2000 mass spectrometer (Manchester, UK) equipped with an ESI source and a quadrupole analyzer. All the operations and the acquisitions of data were controlled by Masslynx$^{3.5}$ software.

2.3. HPLC-DAD-ESI MS conditions

Separations were carried out on a Shim-pack VP-ODS column (150 × 4.6 mm, i.d. 5 μm, Shimadzu, Japan). The binary gradient employed the methanol (A) and 0.5% formic acid aqueous solution (B) according to Table 1. The flow rate was 1.2 ml/min with the column kept at 30 °C. UV spectra were recorded over the range of 200–400 nm, and the quantification wavelength of these chromatograms was set at 254 and 280 nm. The outlet of the DAD was split, and only 0.2 ml/min portion of the column effluent was delivered into the ion source of MS. The injection volume was 10 μl.

The electrospray ionization source was operated at 105 °C in positive mode to produce $[M+H]^+$ or $[M+Na]^+$ and in negative mode to generate $[M-H]^-$ ions. The desolvation temperature was set at 200 °C, extract voltage was 4 V, desolvation gas and cone gas was set at 200 and 50 l/h, respectively. The full-scan mass spectra were acquired over the range 100–600 amu. Capillary voltages were 4.5 kV in ESI+, 4.0 kV in ESI− and cone voltages were 30 V in ESI+, 35 V in ESI−.

2.4. Preparations of the standard solutions and sample solutions

Stock standard solutions of silybin, silychristin, silydianin, isosilybin, tanshinone IIA, tanshinone I, dihydrotanshinone I, cryptotanshinone, protocatechualdehyde and danshensu were prepared by dissolving weighted quantities of

Table 1
The mobile phase gradient program

Time	A%	B%
0.01	0	100
5.00	12	88
10.00	22	78
28.00	38	62
30.00	50	50
35.00	65	35
45.00	68	32
55.00	80	20
70.00	90	10

standard compounds into methanol, respectively. The concentrations of them ranged from 0.5 to 1.5 mg/ml. By using the stock solutions, a series of mixed working standard solutions were prepared with the concentrations of 10.0–200 μg/ml of silybin, silychristin, silydianin, isosilybin, tanshinone IIA, dihydrotanshinone, cryptotanshinone and tanshinone I and 5–100 μg/ml of protocatechualdehyde and danshensu. All the solutions were stored under refrigeration.

An amount of 0.2 g of sample of the simulative compound preparations (containing 0.1 g extract of *S. marianum* and 0.1 g extract of *S. miltiorrhiza*) was extracted in an ultrasound bath with methanol (20 ml) for 15 min, and then centrifuged at 3000 rpm for 5 min. The extracting process was repeated. The extraction solutions were combined in a 50 ml volumetric flask and diluted to volume with methanol. A volume of 2 ml of the solution was filtered through a 0.45 μm filter into an HPLC sample vial before analyzed.

Because Gantaikang and Yiganxintai were prepared with extracts of the two herbs, and amylum was used as assistant material. And the amylum did not interfere in the analysis of the target analytes. So, treatment methods of the samples were the same as that of the simulative compound preparations. But the amount of samples was 0.5 g.

3. Results and discussion

3.1. Optimization of the HPLC conditions

Fig. 2 shows typical HPLC profiles of the abietane-type diterpenes, flavonolignans and phenolic compounds in one run under the above conditions and the peaks numbered from 1 to 18 were completely separated. The wavelength for determining abietane-type diterpenes compounds was 254 nm, and that for determining flavonolignans and phenolic compounds was 280 nm.

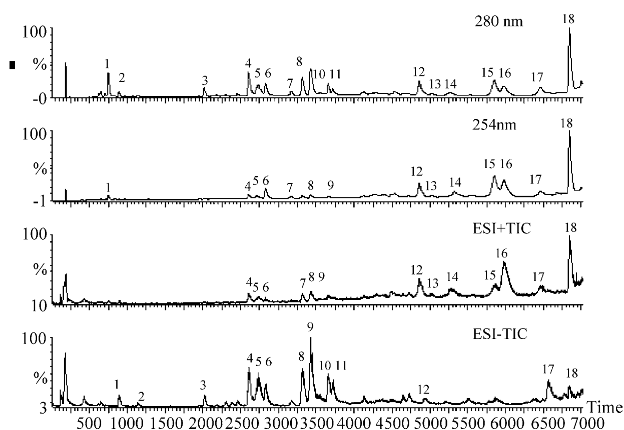

Fig. 2. HPLC-UV-ESI MS–total ion current (TIC) chromatograms of *silybum marianun* and *S. miltiorrhiza* mixture, with a 1:5 post-column stream splitting. The peak identification is given in Table 2.

Many HPLC methods have been reported for determination of one or several active constituents in the two herbs, respectively [3–9]. But the mobiles in those methods were not suitable for MS because of the presence of involatile salts such as phosphate, etc. In this method, formic acid was used as a mobile phase modifier. To obtain the optimal elution conditions for the separation and determination of the active constituents, various linear gradients of 0.5% formic acid aqueous solution and methanol at a flow rate of 1.2 ml/min were investigated. By the gradient program shown in Table 1, the peaks 1–18 could be well separated; meanwhile, the broadening and overlapping of target peaks were also restrained.

3.2. Optimization of the MS conditions

The satisfying MS response of abietane-type diterpenes was obtained in ESI+ mode, and that of flavonolignans and phenolic compounds was obtained in ESI− mode. So, 14 components can be identified by ESI MS working on ESI− and ESI+ switching mode, respectively.

The intensity of the $[M+H]^+$ and $[M-H]^-$ of compounds mainly depends on collision induced dissociation (CID) fragmentation voltage, so the cone voltage is the key factor which can influence sensitivity. Different cone voltages from 20 to 50 V were investigated with standard solutions. As the cone voltages increased, the peak areas varied obviously; the intensity of the pseudo molecule ions $[M-H]^-$ of abietane-type diterpenes arrived at the maximum at cone voltage 30 V, and the intensity of the pseudo molecule ions $[M-H]^-$ of flavonolignans and phenolic compounds achieved the maximum at cone voltage 35 V, see Fig. 3. When the cone voltage was higher than the optimum value, the intensity of pseudo molecule ions decreased because of producing more fragments. So the 30 and 35 V cone voltage were applied in ESI+ and ESI− mode, respectively.

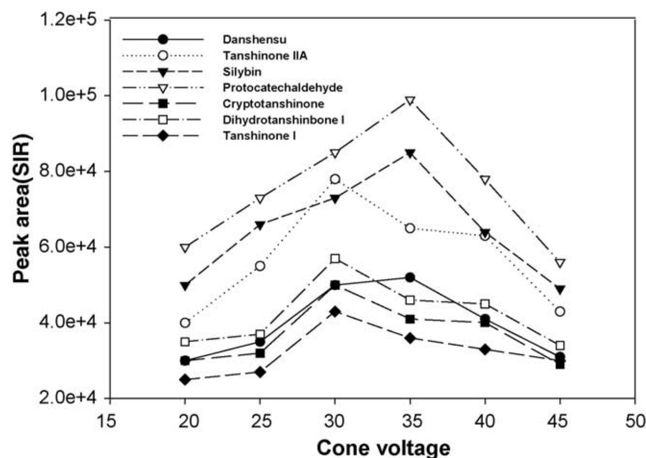

Fig. 3. The effect of cone voltage on SIR signal intensity of seven mix standards.

Table 2
The retention time, molecular ion of the active constituents

Peak no. (Fig. 2)	t_R (min)	m/z $[M-H]^-$	m/z $[M+H]^+$	m/z $[M+Na]^+$	UV λ_{max} (nm)	m/z $[2M+Na]^+$	Compound (Fig. 1)
1	7.49	197.1	–	–	279	–	Danshensu
2	8.48	137.0	–	–	282	–	Protocatechaldehyde
3	20.17	303.1	–	–	286	–	Taxifolin
4	26.11	481.1	–	–	288	–	Silybin A
5	27.34	481.1	–	–	288	–	Silybin B
8	33.14	481.1	–	–	288	–	Isosilybin A
9	34.25	481.1		–	288	–	Isosilybin B
10	36.74	481.1	–	–	288	–	Silychristin
11	37.49	481.1	–	–	288	–	Silydianin
12	48.64	–	279.2	–	252	579.2	Dihydrotanshinone I
14	52.84	–	295.2	–	260	–	Isotanshinone IIA
15	58.53	–	277.1	299.1	245	574.7	Tanshinone I
16	59.78	–	297.2	319.1	263	–	Cryptotanshinone
18	68.47	–	295.2	–	252	–	Tanshinone IIA

3.3. Identifications of the constituents in the mixed herbs

Table 2 lists the retention time (t_R), λ_{max}, pseudo molecular ions and assignment of HPLC peaks. These peaks were identified according to the information of the on-line UV, MS, literature data and standard samples.

Peaks 1 and 2 were identified as danshensu and protocatechualdehyde, correspondingly, according to the retention order identical to the standard samples. Peak 3 was assigned to taxifolin, which is confirmed by HPLC report [9] and on-line MS information. Peak 3 shows the pseudo molecule ion $[M-H]^-$ at m/z 303 and fragment ion $[M-H-H_2O]^-$ at m/z 285 (Fig. 4). Retention order of peaks 4, 5 and 8–11 were in agreement with the reported HPLC profile [3,6], so these peaks are identified as silycristin, silydianin, silybin A, silybin B, isosilybin A, and isosilybin B in turn. Silycristin, silydianin, silybin A, silybin B, isosilybin A and isosilybin B are isomeric pairs, which have the similar MS spectra, so only the MS spectra of silybin A is shown in Fig. 4. Peaks 12, 14, 15, 16, 18 were tanshinone for the retention order is in agreement with standards and the literatures [7,8]. Fig. 4 shows the MS spectra of m/z 279, 277, 297 and 295 in the positive ion mode, which were corresponding pseudo molecule ions $[M+H]^+$ of dihydrotanshinone I, isotanshinone IIA, tanshinone I, cryptotanshinone and tanshinone IIA. And the m/z 299 and 319 which were adduct ions $[M+Na]^+$ of tanshinone I and crytotanshinone can also be found in the MS spectra. Peaks 15 and 16 also showed the sodiated dimmer ion $[2M+Na]^+$ at the m/z 575, 579, respectively. As isomeric pairs, tanshinone IIA and isotanshinone IIA had the same mass spectrum; peak 15 was in agreement with the tanshinone IIA standard, so peak 15 was identified as tanshinone IIA. Peaks 6, 7, 13, and 17 were not identified, because there were no dominant ions or ions could not be matched with the known compounds in this herbal couple.

3.4. Validation of method

Fig. 2 shows typical HPLC profiles of the abietane-type diterpenes, flavonolignans and phenolic compounds in one run under the above conditions and the peaks numbered from 1 to 18 were completely separated. The wavelength for determining abietane-type diterpenes compounds was 254 nm, and that for determining flavonolignans and phenolic compounds

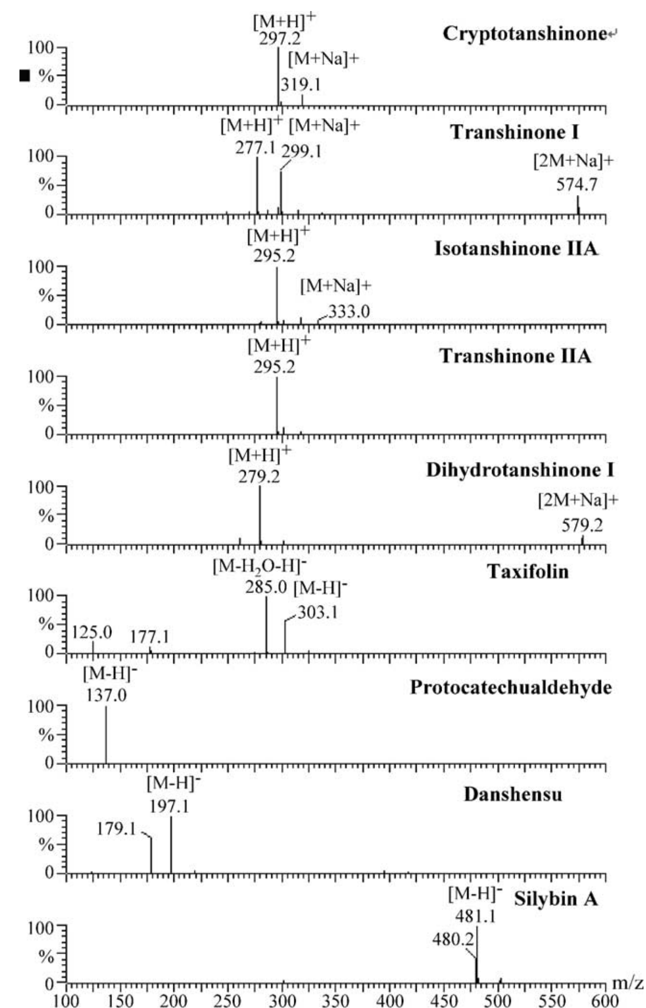

Fig. 4. MS spectrum of the active constituents.

Table 3
Results of analyses of Gantaikang and Yiganxintai (%) ($n = 3$)

Sample	Tanshinone I	Tanshinone IIA	Crytotanshinone	Dihydrotanshinone I	Danshensu	Protocatechaldehyde	Silybin	Isosilybin	Silydianin	Silychristin
Gantaikang batch no.026	2.01	3.86	5.62	2.51	12.32	1.52	19.36	15.96	11.23	10.24
Gantaikang batch no.032	2.09	3.76	5.85	2.43	12.62	1.33	18.69	15.36	11.65	10.75
Gantaikang batch no.028	2.12	3.98	5.96	2.32	12.50	1.59	18.29	15.29	11.38	10.58
Yiganxintaibatch no.008	1.03	1.83	2.70	1.20	5.20	0.78	11.23	7.69	6.93	5.32
Yiganxintaibatch no.010	0.91	1.60	2.89	1.12	5.39	0.70	11.69	7.52	6.81	5.21
Yiganxintaibatch no.012	0.95	1.69	2.95	1.10	5.45	0.82	11.98	7.99	6.62	5.52

was 280 nm. Ten components were quantified by using external standard method.

3.4.1. Linearity and limit of detection

The standard curves were prepared under six different concentrations and five injections were made at each level. Good linear relationship can be gained, and the correlation coefficients of all the calibration curves were found to be higher than 0.992. For different components, the limit of detection (S/N = 3) ranged from 0.5 to 2 ng/ml, and the limit of quantification (S/N = 10) ranged from 2.5 to 10 ng/ml.

3.4.2. Repeatability and recovery

The reliability of the analytical method was estimated by the small coefficients of variation and good recovery. The inter-day repeatability of the method was assessed by repeated analysis of the standard solutions at three concentration levels. R.S.D. was below 4.8%.

The recovery of each target compounds was studied by adding the standard solutions of known concentrations to the samples. Mean recoveries were 96–101% (five parallel trials of additions of standard to sample).

3.5. Application

The method was applied to analyzing Gantaikang capsule and Yiganxintai tablet. Sample solutions and standard solutions were injected into HPLC. The results are listed in Table 3.

4. Conclusion

In this paper, a method has been developed for simultaneous determination of the active constituents in compound preparations of *S. miltiorrhiza* and *S. marianum*. The HPLC-DAD-ESI MS method may be conveniently and sensitively used for quality assurance of compound preparations containing *S. miltiorrhiza* and *S. marianum*, and also for quality control in pharmaceutical factories.

Acknowledgments

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