Quantitative Determination of Four Diterpenoids in Radix Salviae Miltiorrhizae Using LC-MS-MS

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An HPLC-ESI-MS-MS method has been developed for the quantitative determination of four diterpenoids, dihydrotanshinone I, cryptotanshinone, tanshinone I, and tanshinone IIA in Radix Salviae Miltiorrhizae (RSM, the root of *Salvia miltiorrhiza* BGE.). The diterpenoids were chromatographically separated on a C18 HPLC column, and the quantification of these diterpenoids was based on the fragments of $[M+H]^+$ under collision-activated conditions and in Selected Reaction Monitioring (SRM) mode. The quantitative method was validated, and the mean recovery rates from fortified samples ($n=5$) of dihydrotanshinone I, cryptotanshinone, tanshinone I, and tanshinone IIA were 95.0%, 97.2%, 93.1%, and 95.9% with variation coefficient of 6.0%, 4.3%, 3.7%, and 4.2%, respectively. The established method was successfully applied to the quality assessment of seven batches of RSM samples collected from different regions of China.

Key words HPLC-ESI-MS-MS; quantitative analysis; *Salvia miltiorrhiza*; diterpenoid; tanshinone

Radix Salviae Miltiorrhizae (RSM, the root of *Salvia miltiorrhiza* BGE., Lamiaceae), is a well-known traditional Chinese herbal medicine called "Danshen" (丹参) widely used in China to treat various cardiovascular diseases. More than 30 diterpenoids have been isolated from the root of *Salvia miltiorrhiza* BGE.[1,2] Among these constituents, tanshinones, such as tanshinone IIA and tanshinone I, have been confirmed to have antiinflammatory, antiischemic, antioxidant, and antitumor activities.[3—6] Therefore quantitative evaluation of the contents of tanshinones in RSM is significant for the quality control of this medicinal herb and products containing it. Several analytical techniques including HPLC, capillary electrophoresis (CE), and high-speed countercurrent chromatography-mass spectrometry (HSCCC-MS) have been used for the determination of diterpenoids in RSM.[7—10]

Liquid chromatography combined with tandem mass spectrometry (LC-MS-MS), a relatively new technique with rapidly growing popularity, has been successfully applied to elucidate the structures of the active components in medicinal plants. Recently, it has also been employed to determine the contents of bioactive components, such as saponins and flavonoids, in herbal medicines.[11—14] Although mass spectrometry is a more expensive and complex option than other HPLC detectors, LC-MS-MS can greatly simplify the sample pretreatment procedures and shorten separation times of HPLC due to the high selectivity and sensitivity of MS-MS detection, thus dramatically reducing the total analysis time.

In this study, an HPLC-MS-MS method has been developed and validated for the quantitative determination of four major diterpenoids, dihydrotanshinone I, cryptotanshinone, tanshinone I, and tanshinone IIA (Fig. 1) in crude and processed RSM samples.

Results and Discussion

HPLC-ESI-MS-MS Analysis of Four Diterpenoids For the acquisition of the mass spectra and the respective daughter-ion mass spectra, dihydrotanshinone I, cryptotanshinone, tanshinone I, and tanshinone IIA standard solutions were introduced by direct infusion. The spectra of these four diterpenoids showed similar behaviors in their MS/MS fragmentation. Water and CO were easily eliminated from the precursor ion $[M+H]^+$. In the MS-MS spectra obtained from $[M+H]^+$ of dihydrotanshinone I (Fig. 2), the ions of the *m/z* 261, 251, 233 and 205 were deduced to represent the $[M+H-H_2O]^+$, $[M+H-CO]^+$, $[M+H-H_2O-CO]^+$, and $[M+H-H_2O-2CO]^+$, respectively. The most abundant fragment ion for each tanshinone was chosen for selected reaction monitoring (SRM) quantification. The ion pairs for SRM experiments are listed in Table 1.

Since the mobile phase plays an important role in the ionization process, the composition and ratio of the mobile phase for the LC-MS-MS determination of four diterpenoids were investigated. Figure 3 shows the MS spectra of tanshinone IIA in HPLC-MS-MS analysis using methanol/water and acetonitrile/water systems as the mobile phase. It was found that the latter mobile phase can facilitate the production of molecular ion $[M+H]^+$ (*m/z* 295) ion. The addition of *ca.* 0.5% of formic acid into the mobile phase was found to insure the stability of production of molecular ion $[M+H]^+$. The retention time of dihydrotanshinone I, cryptotanshinone, tanshinone I, and tanshinone IIA was 5.82, 8.12, 9.13, 13.29 min, respectively (Fig. 4).

Validation Results of Quantitative Methods Linearity

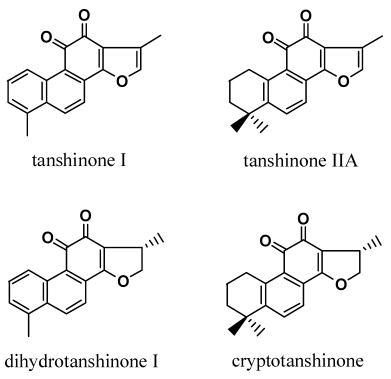

Fig. 1. Structures of Four Diterpenoids in RSM

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was tested for each of the reference standards at concentrations of 0.050, 0.10, 0.20, 0.50, 1.0, 2.0, 5.0, 10, and 20 μg/ml with the correlation coefficients larger than 0.999. The intraday precisions of injection were evaluated using the results of five replicate injections of the standard solutions containing the four tanshinones at a concentration of 1.0 μg/ml. The repeatability of the quantitative procedure was based on the results of five analyses of one batch of RSM sample. The relative standard deviations (RSD) of precision and repeatability of four diterpenoids were both less than 5%.

The recovery studies were conducted by spiking the same batch of RSM sample five times with dihydrotanshinone I, cryptotanshinone, tanshinone I, and tanshinone IIA standards at the same concentration of *ca.* 1.2 mg/g, respectively. The mean recovery rates of dihydrotanshinone I, cryptotanshinone, tanshinone I, and tanshinone IIA were 95.0%, 97.2%, 93.1%, and 95.9% with RSD of 6.0%, 4.3%, 3.7%, and 4.2%, respectively.

Limits of detection (LOD) and limit of quantification (LOQ) of the four diterpenoids were the analyte concentrations producing a signal of at least 3.14 times and 10 times, respectively, of the standard deviation of seven determinations of a crude drug sample. In our experiment, it was deter-

Table 1. The Ion Pairs for Selected Reaction Monitoring (SRM) Experiments

Diterpenoids	Precursor ion $[M+H]^+$ (m/z)	Fragment ion (m/z)	Dwell time (ms)	Range of monitoring time (min)
Dihydrotanshinone I	279	261	1200	0—7.2
Cryptotanshinone	297	251	800	7.2—10.5
Tanshinone I	277	249	800	7.5—10.5
Tanshinone IIA	295	277	1200	10.5—16

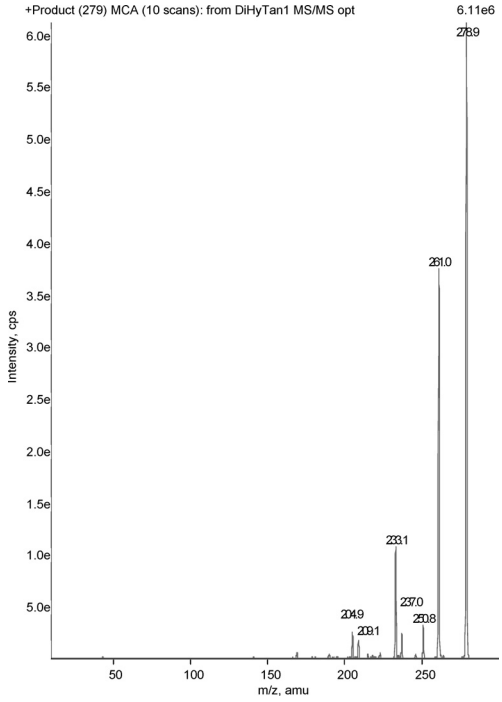
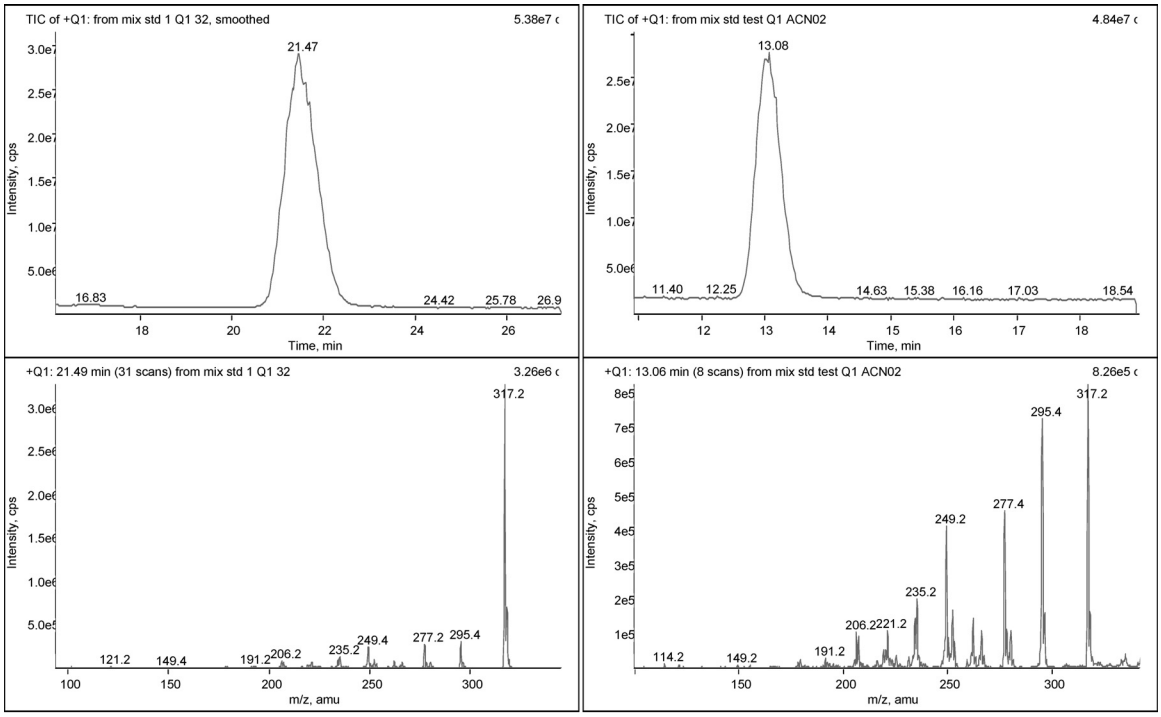


Fig. 2. ESI-MS-MS Spectra of Dihydrotanshinone I Obtained from $[M+H]^+$



(A) (B)

Fig. 3. Effects of the Mobile Phase on the Production of $[M+H]^+$ in ESI-MS Spectra

(A) MS spectrum of tanshinone IIA in positive mode using methanol/water (70 : 30, v/v) as the mobile phase. (B) MS spectrum of tanshinone IIA in positive mode using acetonitrile/water (70 : 30, v/v) as the mobile phase. Column, Alltima C18 (2.1 mm×150 mm, 5 μm); flow rate, 0.21 ml/min; injection volume, 5 μl.

mined by fortifying the extracted Radix Ginseng (acting as the blank matrix) with *ca.* 5 μg (*ca.* 0.2 mg/l in solution) of each standard. The LOQ and LOQ values of the four diterpenoids were on the same order of magnitude. All of the validation data for the four diterpenoids are summarized in Table 2.

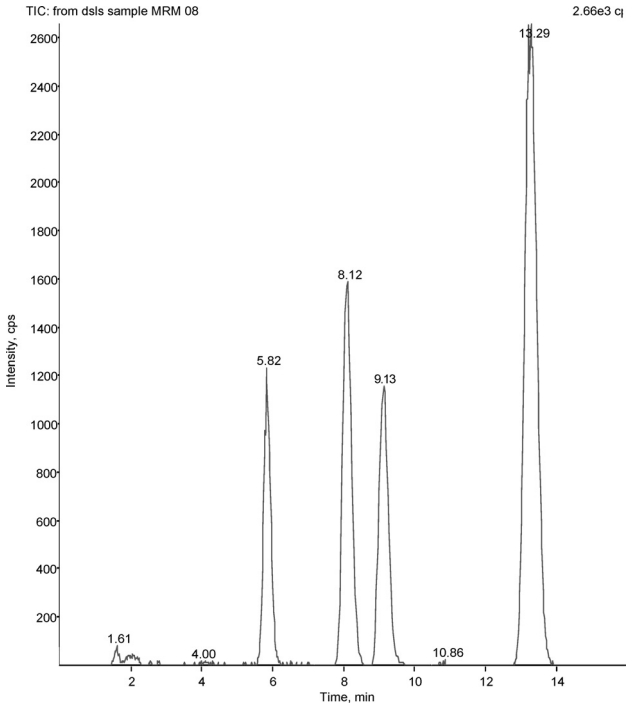


Fig. 4. Total Ion Chromatogram (TIC) Spectrum of Four Diterpenoids in RSM Samples

Column, Alltima C18 (2.1 mm×150 mm, 5 μm); mobile phase, CH_3CN–1.6% HCOOH in H_2O (7 : 3); flow rate, 0.21 ml/min; injection volume, 5 μl.

Quantitative Analysis of RSM Samples Seven batches of crude RSM samples collected from representative regions of China were quantitatively determined using the developed LC-MS-MS method (Table 3). The LC-MS-MS SRM chromatogram of a typical RSM sample indicating the retention time of each tanshinone is shown in Fig. 4. It was found that the amounts of the four diterpenoids varied based on the harvest area of the medicinal plants. Particularly for samples collected in Zhongjiang, Sichuan Province, the contents of dihydrotanshinone I and tanshinone I were markedly lower than those in samples from other provinces. Furthermore, the method was also applied to the determination of four diterpenoids in three batches of processed RSM samples purchased from a Hong Kong market. The results indicated that the contents of the four pharmacologically active compounds in the processed RSM samples were much lower than those in the crude drugs (Table 4).

The contents of the individual diterpenoids in crude and processed RSM samples determined using the LC-MS-MS method were somehow lower or higher than those determined using HPLC-UV in our laboratory (unpublished data). Compared with HPLC-UV techniques, determination of the tanshinones in RSM samples using LC-MS-MS has the advantages of much higher selectivity (Fig. 5) utilizing the characteristic reaction monitoring (Table 1) for each tanshinone. Therefore, this technique can provide satisfactory resolution of the peaks and eliminate the interference even with a short elution time of these diterpenoids on the HPLC col-

Table 2. Validation Data of LC-MS-MS Quantitative Method for Four Diterpenoids in RSM Samples

Diterpenoids	Linearity (mg/l)	Precision RSD (%)	Repeatability RSD (%)	Recovery (%)	MDL (μg/g)	LOQ (μg/g)
Dihydrotanshinone I	0.05—10	3.6	4.4	95.0	17	53
Cryptotanshinone	0.05—20	1.4	2.3	97.2	21	66
Tanshinone I	0.05—5.0	2.7	2.0	93.1	22	70
Tanshinone IIA	0.05—20	2.8	2.2	95.9	19	61

Table 3. Contents of Four Diterpenoids in Crude RSM Samples from Different Regions of China

Source	Dihydrotanshinone I (mg/g)	Cryptotanshinone (mg/g)	Tanshinone I (mg/g)	Tanshinone IIA (mg/g)
Zhong Jiang-1, Sichuan, China	Lower than LOQ	0.24	Lower than LOQ	0.64
Zhong Jiang-2, Sichuan, China	0.086	0.32	0.064	1.3
Yi Nan, Shandong, China	0.48	1.8	1.1	2.4
Lu Shi, Henan, China	0.86	1.5	0.94	1.7
Ping Ding Shan, Henan, China	0.69	1.2	1.4	2.1
Luo Yang, Henan, China	0.39	0.86	0.73	1.6
Shang Luo, Shaanxi, China	0.46	0.83	0.99	1.7

Table 4. Contents of Four Deterpenoids in Processed RSM Samples Purchased from Three Companies in Hong Kong

Source	Dihydrotanshinone I (mg/g)	Cryptotanshinone (mg/g)	Tanshinone I (mg/g)	Tanshinone IIA (mg/g)
Company A	0.062	0.18	Lower than LOQ	0.37
Company B	Lower than LOQ	0.19	Lower than LOQ	1.0
Company C	0.11	0.24	0.24	0.69

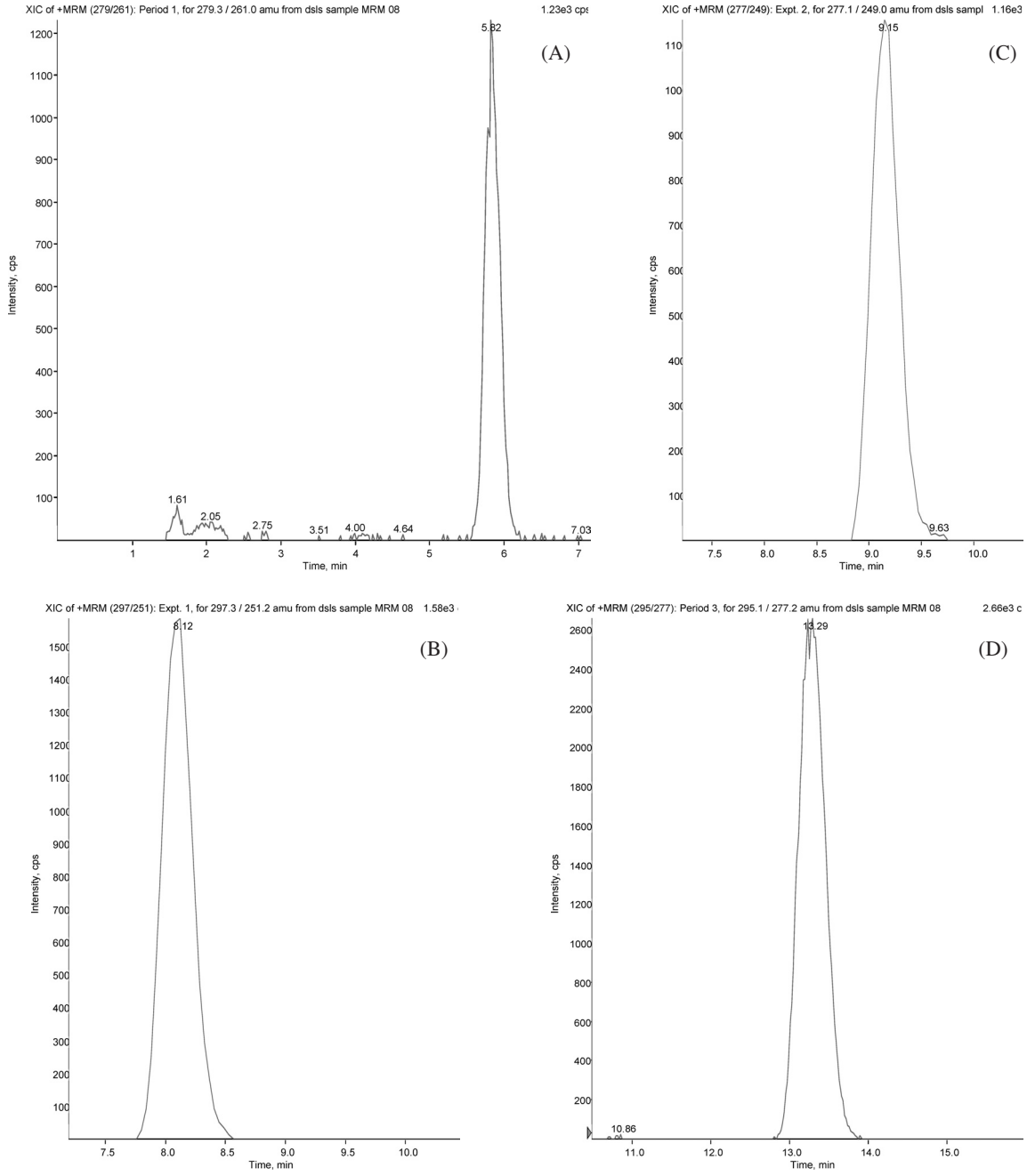


Fig. 5. Four LC-MS-MS SRM Chromatograms Used for Selective Quantification of the Diterpenoids in RSM Samples

(A) Dihydrotanshinone I; (B) cryptotanshinone; (C) tanshinone I; (D) tanshinone IIA. Column, Alltima C18 (2.1 mm×150 mm, 5 μm); mobile phase, CH_3CN–1.6% HCOOH in H_2O (7 : 3); flow rate: 0.21 ml/min; injection volume: 5 μl.

umn. Thus, accurate quantitative analysis does not solely rely on the HPLC baseline separation which requires sophisticated optimization of chromatographic conditions. The simultaneous quantification of four tanshinones in RSM samples using the LC-MS-MS method can be achieved in 15 min, about the half time required when using the HPLC-UV method. However, the RSDs of precision, repeatability, and recovery of the LC-MS-MS method are higher than those with the HPLC-UV method. In addition, the LOQ values of dihydrotanshinone I and tanshinone I in the LC-MS-MS method are several times greater than those of the HPLC-UV method due to the poorer reproducibility of the ESI-MS-MS instrument. As a result, the contents of dihydrotanshinone I and tanshinone I in several batches of RSM samples (Tables 3, 4) which were less than the value of their LOQ were not calculated, although they were obviously detected in the SRM chromatograms.

Experimental

Standards and Chemicals Dihydrotanshinone I, cryptotanshinone, tanshinone I, and tanshinone IIA were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) (purity>98%). Acetonitrile of HPLC grade and methanol and dichloromethane of analytical reagent grade were obtained from Labscan (Stillorgan, Ireland). Formic acid of guarantee reagent grade for HPLC analysis was from Merck (Darmstadt, Germany). Water for HPLC analysis was purified with a Milli-Q water system (Millipore Corp., Bedford, MA, U.S.A.).

Crude Drugs Seven samples of crude drugs were collected from the

cultivation base of *Salvia miltiorrhiza* in different regions of China and were air-dried according to the procedure described in the Chinese Pharmacopoeia (2000 edition). All were identified by one of the authors (Z. Zhao) and deposited in the Center of Chinese Materia Medica, Hong Kong Baptist University. Three samples of processed RSM (after washing with water, the crude drugs were softened and sliced into pieces) were purchased from three Chinese herbal medicine companies in Hong Kong.

Sample Preparation About 25 mg of powdered crude drug was accurately weighed into a 25-ml amber volumetric flask. The volumetric flask was made up with methanol–dichloromethane mixture (4 : 1, v/v) and sonicated for 60 min. Then the mixture was filtered through a 0.45-μm PTFE filter (Iwaki Glass, Tokyo, Japan) to obtain the test solution.

HPLC-ESI-MS-MS Analytical Conditions HPLC analysis was carried out on a C18 column (Alltech, Alltima C18, 2.1 mm×150 mm, 5 μm) with isocratic elution with 70% acetonitrile and 30% formic acid aqueous solution (1.6%). The flow rate was 0.21 ml/min, and the injection volume was 5 μl. Perkin-Elmer Sciex API 365 triple-quadrupole mass spectrometers (Sciex, Toronto, Canada), equipped with an ion-spray (pneumatically assisted electrospray) interface were employed. The standards of tanshinones were analyzed by direct-flow injection to optimize the ESI-MS-MS conditions. The quantification of tanshinones in RSM samples was based on the fragments of $[M+H]^+$ of tanshinones under collision-activated condition. SRM mode (Fig. 5) was chosen for the detection. The monitoring conditions of the four tanshinones are listed in Table 1.

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References

1) Editorial Board of China Herbal, State Administration of Chinese Traditional Medicine, "China Herbal," Vol. 7, ed. by Ying X. X., Yu H. A., Shanghai Medicine and Technology Press, Shanghai, China, 1999, pp. 169—186.
2) Kong D. Y., *Chin. J. Pharm.*, **20**, 279—285 (1989).
3) Kang B. Y., Ching S. W., Kim S. H., Ryu S. T., Kim T. S., *Immunopharmacology*, **49**, 355—361 (2003).
4) Cui L., Wu T., Liu Y. Y., *Acta Pharm. Sin.*, **25**, 678—684 (2004).
5) Liang Y., Yang Y. M., Yuan S. L., *Chin. Trad. Herb. Drugs*, **31**, 304—306 (2000).
6) Niu X. L., Lehimori K., Yang X., Hirota Y., Hoshiai K., Li M., Nakzawa H., *Free Radical Res.*, **33**, 305—312 (2002).
7) Hu P., Liang Q.-L., Luo G. A., Zhao Z. Z., Jiang Z. H., *Chem. Pharm. Bull.*, **53**, 677—683 (2005).
8) Pan X. J., Niu G. G., Liu H. Z., *J. Chromatogr. A*, **922**, 371—375 (2001).
9) Chen A. J., Zhang J. Y., Li C. H., Hu Z. D., Chen X. G., *J. Sep. Sci.*, **27**, 569—575, (2004).
10) Wu F. Y., Dai D. S., Wang Y. M., Luo G. A., *Chem. J. Chin. Univ.*, **23**, 1968—1700 (2002).
11) Wang X. M., Sakuma T., Adjaye E. A., Shui G. K., *Anal. Chem.*, **71**, 1579—1584 (1999).
12) Li W. K., Gu C. G., Zhang H. J., Awang D. V. C., Fitzloff J. F., Fong H. H. S., Breemen R. B. V., *Anal. Chem.*, **72**, 5417—5422 (2000).
13) Liu Z. Y., Wang G. Q., Zhang H., Yu C., Ji S., Zhu D. Y., *Chin. Trad. Pat. Med.*, **25**, 144—146 (2003).
14) Liang S. P., Li C., *J. Chin. Mass Spectrom. Soc.*, **24**, 85—86 (2003).