Journal of Medicinal Plants Research Vol. 5(10), pp. 1850-1858, 18 May, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

Simultaneous determination of five major active depsides in the freeze-dried Dan-Shen injection by LC

Shou-Jun Jiang[1], Bin Zhu[1], Dan-Dan Zhou[2], Gang-Li Wang[3], Ying-Xin Wang[4] and Rui-Chao Lin[3]*

[1]Guangxi Institute for Food and Drug Control, Xin Min Road 1-1, 530021 Nanning, Guangxi, People's Republic of China.
[2]Shanghai University of Traditional Chinese Medicine, Cai Lun Road 1200, 201203 Shanghai, People's Republic of China.
[3]National Institute for the Control of Pharmaceutical and Biomedical Products, Tiantan Xili 2, 100050 Beijing, People's Republic of China.
[4]Harbing Pharm Group Co. LTD Second Chinese Medicine Factory, Ji Chang Road 243,150078 Haerbing, Heilongjiang, People's Republic of China.

Accepted 9 March, 2011

To establish an high-performance liquid chromatography-diode array detection (HPLC-DAD) method for the first time to simultaneously determine five major bioactive depsides, salianic acid A , protocatechuic acid, protocatechuic aldehyde, salvianolic acid G and salvianolic acid D in freeze-dried Dan-Shen injection. Chromatographic separation was carried out on a Phenomenex Hydro-C18 reserved-phase column (4.6×250 mm, 4 μm) by stepwise gradient elution with acetonitrile and water (0.1% trifluoroacetic acid, v/v) as the mobile phase. The UV detection wavelength was set at 281 nm for salianic acid A and protocatechuic aldehyde, 260 nm for protocatechuic acid, 228 nm for salvianolic acid G and 322 nm for salvianolic acid D, respectively. Good linear relationships were observed between peak areas and concentrations with r^2 values above 0.9997 for all the analytes. Average recoveries for the five major actives ranged from 98.3 to 99.4%. This method was rapid, sensitive, accurate, and could be readily applied to the quality assessment of freeze-dried Dan-Shen injection.

Key words: Column liquid chromatography, depsides, freeze-dried Dan-Shen injection, quality control.

INTRODUCTION

Traditional Chinese Medicine (TCM) has played an important role in the prevention and treatment of diseases in China for thousands of years. In recent decades, TCM has been given increasingly attention worldwide. It is widely accepted that a vast number of chemical and constituents and complex synergistic effects exist between these ingredients that are responsible for the therapeutic effects of TCM. Traditional Chinese Medicine injection (TCM injection) as a kind of TCM preparations has been used in clinical practice for the treatment of critical disease widely. Due to the special administer way and complicated chemical constituents of TCM injection, quality control of the TCM injections has always been a tremendous challenge. In the past, the strategy of quality control of TCM injection was focus mainly on tracking one or two marker compounds or the total amount of major active constituent group, which is not enough to control the quality of TCM injection. Therefore, sensitive and reliable holistic analytical approach is necessary for the quality control of TCM injection. Simultaneous determination of multiple constituents present in TCM preparation is significantly important (Cheng, 2003).

Freeze-dried Dan-Shen injection made from the aqueous extracts of *Salvia miltiorrhiza* Bunge is one of most widely used TCM preparation. It had the functions of

*Corresponding author. E-mail: linrch307@sina.com. Fax: 86-10-67023650.

promoting the circulation of blood and activating meridians, and presented excellence curative effects on the treatment of angina pectoris and coronary disease (Pharmacopoeia Committee of People's Republic of China, 2004). The chemical constituents of S. *miltiorrhiza* cover two chemical types: diterpenoid quinones and water-soluble phenolic acids. Before 1970s, the studies were mainly focused on the lipophilic diterpenoid, which have been considered to be responsible for the clinical efficacy (Gu, 1981; Lin, 2000). But with the pharmacological and clinical investigation on water-soluble hydrophilic depsides in *S. miltiorrhiza*, it was found that hydrophilic depsides were the real active principles other than the lipophilic diterpenoids reported previously (Chen, 1981; Li, 1997; Zhou, 1999; Ling, 1999; Du, 2004). Up to now, a number of analytical methods have been reported for the quantification of hydrophilic depsides including high performance liquid chromatography (LC) coupled with ultraviolet detection (UV), diode array detection(DAD), evaporative light-scattering detector (ELSD), electrochemical detection (ED) and mass spectrometry (MS) detections (Yuan, 2005; Qin, 2006; Liu, 2007; Li, 2009). However, quantitative determination of water soluble constituents in *S. miltiorrhiza* and its preparations has been focused only on one or two compounds, which could not reflect the overall quality of *S. miltiorrhiza* and its preparations.

In our recent studies for the chemical components and pharmacological activities of freeze-dried Dan-Shen injection, 16 hydrophilic depsides have been isolated and identified from freeze-dried Dan-Shen injection, which are the main active components being effective in cardiovascular disease and inhibiting inflammation. In the active quality criteria of freeze-dried Dan-Shen injection, only Salianic acid A (Dan-Shensu) was quantified by HPLC method, which could not reflect the real and comprehensive active constituents and was inadequate to control the quality of freeze-dried Dan-shen injection. In order to ensure the stability and efficacy of freeze-dried Dan-Shen injection in clinical usage, more objective and effective quality methods are needed to evaluate and assess its quality. This paper aimed to develop a new, simple and reliable analytical method for the simultaneous quantification of five major bioactive compounds (salianic acid A, protocatechuic acid, protocatechuic aldehyde, salvianolic acid G and salvianolic acid D) (Figure 1) in freeze-dried Dan-Shen injection by HPLC-DAD. This was the first report on the simultaneous determination of five major phenolic acids in the samples of freeze-dried Dan-Shen injection. The results suggested that contents of the five major phenolic acids were appropriate used to evaluate the quality of freeze-dried Dan-Shen injection.

EXPERIMENTAL

Chemicals and materials

Salianic acid A, protocatechuic acid and protocatechuic aldehyde standards (purity > 98.0%) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Salvianolic acid G and salvianolic acid D were isolated from freeze-dried Dan-Shen injection by the authors. Their identities were confirmed by ^{1}H NMR, ^{13}C NMR and MS spectral analysis, and their purity were over 98.0% by HPLC analysis. The freeze-dried Dan-Shen injection (batch numbers: 050203, 050302, 050804, 051009, 051208, 060405, 060214, 070715, 070801, 070709 and 070812) were supplied by Harbing Pharm Group Co. LTD Second Chinese Medicine Factory (Harbing, China). HPLC grade acetonitrile and trifluoroacetic acid were purchased from Merck Company (Merck, Darmstadt, Germany). Glacial acetic acid was of analytic grade and obtained from Beijing Reagent Company (Beijing, China). Deionized water was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

Sample preparation

The contents obtained from the sample of freeze-dried Dan-Shen injection are mixed, and the weight (20 mg) is accurately measured in a 10 ml volumetric flask. The volume is dissolved and diluted with 0.5% glacial acetic acid solution and mixed well. The solutions were then filtered through a 0.45 μm filter membrane before analysis.

LC and LC-ESI-MS Conditions

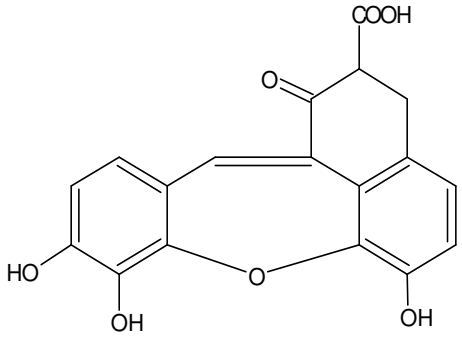
Analyses were performed on a Waters Alliance 2695 liquid chromatograph system (Milford, MA, USA), consisting of a vacuum degasser, quaternary gradient pump, autosampler and photodiode array detector (PAD) was operated with the Waters Empower soft. A phenomenex hydro C_{18} reserved-phase column (4.6 × 250 mm, 4 μm). Diode array scanning from 200 to 400 nm was used for the separation. The binary gradient elution system consisted of solvent A (acetonitrile) and solvent B (0.1% trifluoroacetic acid, v/v). Separation was achieved by using the following gradient program: 0 to 8 min, 93% B, 8 to 30 min, 93 to 70% B, 30 to 45 min, 70 to 55% B, 45 to 50 min, 55 to 55% B, and finally, reconditioning at 93% B isocratic for 10 min. The column temperature was maintained at 30°C. The flow-rate was 1.0 ml min^{-1}. Injection volume was 10 μl. UV detection wavelength was set at 281 nm for salianic acid A and protocatechualdehyde, 260 nm for protocatechuic acid, 228 nm for salvianolic acid G and 322 nm for salvianolic acid D, respectively.

An Agilent 1100 Series HPLC and 6320 Ion Trap LC/MS system (Agilent Technologies, Palo Alto, CA, USA) was employed in negative electrospray ionization mode for unambiguous and confirmatory identification of the chromatographic peaks. The column, elution program, column temperature, flow-rate and injection volume remained unchanged. The mobile phase composition required some minor adjustment in order to maximize ionization and signal intensity. Thus, solvent A (acetonitrile) and solvent B (0.4% formic acid, v/v) were used for LC-ESI-MS analysis. A post-column spilt directed around 25% of the flow towards the MS. The MS conditions were set as follows: negative ion mode, Auto MS^{n}, the turbo-gas temperature was set at 350°C, the ion-spray voltage (IS) was adjusted at 4000 v, high purity nitrogen served as nebulizer gas with a flow rate of 10 L min^{-1} and a pressure of 25 psi, scan range m/z 100–1,000.

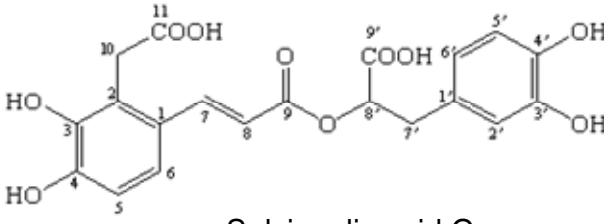
Validation of the method

Calibration curves, limits of detection and limits of quantification

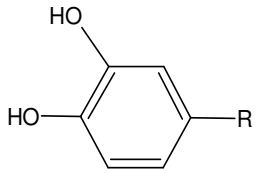
Aqueous methanolic stock solution(60%, v/v), containing salianic acid A (486.7 μg ml^{-1}), protocatechuic aci (50.40 μg ml^{-1}),



Salvianolic acid D



Salvianolic acid G



R= -$CH_2CH(OH)COOH$ (Salianic acid A)

R= -COOH (Protocatechuic acid)

R= -CHO (Protocatechuic aldehyde)

Figure 1. Chemical structures of depsides used in the study.

protocatechuic aldehyde (48.42 μg ml^{-1}), salvianolic acid G (272.0 μg ml^{-1}) and salvianolic acid D (323.0 μg ml^{-1}) was prepared and diluted to appropriate concentration ranges with 0.5% glacial acetic acid solution for the establishment of calibration curves. Each calibration curve was performed with five different concentrations in triplicate. Calibration curves were plotted after linear regression of the peak areas versus the concentrations. The limit of detection (LOD) and limit of quantification (LOQ) were determined a signal-to-noise (S/N) of 3 or 10, respectively.

Precision and accuracy

Intra- and inter-day variations were evaluated in order to determine the precision based on the relative standard deviation (RSD). For intra- and inter- variability tests, the standard solution (salianic acid A 121.7 μg ml^{-1}, protocatechuic acid 4.04 μg ml^{-1}, protocatechuic aldehyde 12.10 μg ml^{-1}, salvianolic acid G 21.76 μg ml^{-1} and salvianolic acid D 12.92 μg ml^{-1}) was analyzed for five times a day and for five separate days, respectively.

Six samples with known quantities were spiked with the accurate amounts of reference substances and then extracted as described above. All samples were filtered through a 0.45 μm Millipore filter and injected for HPLC analysis to calculate the recoveries.

Stability

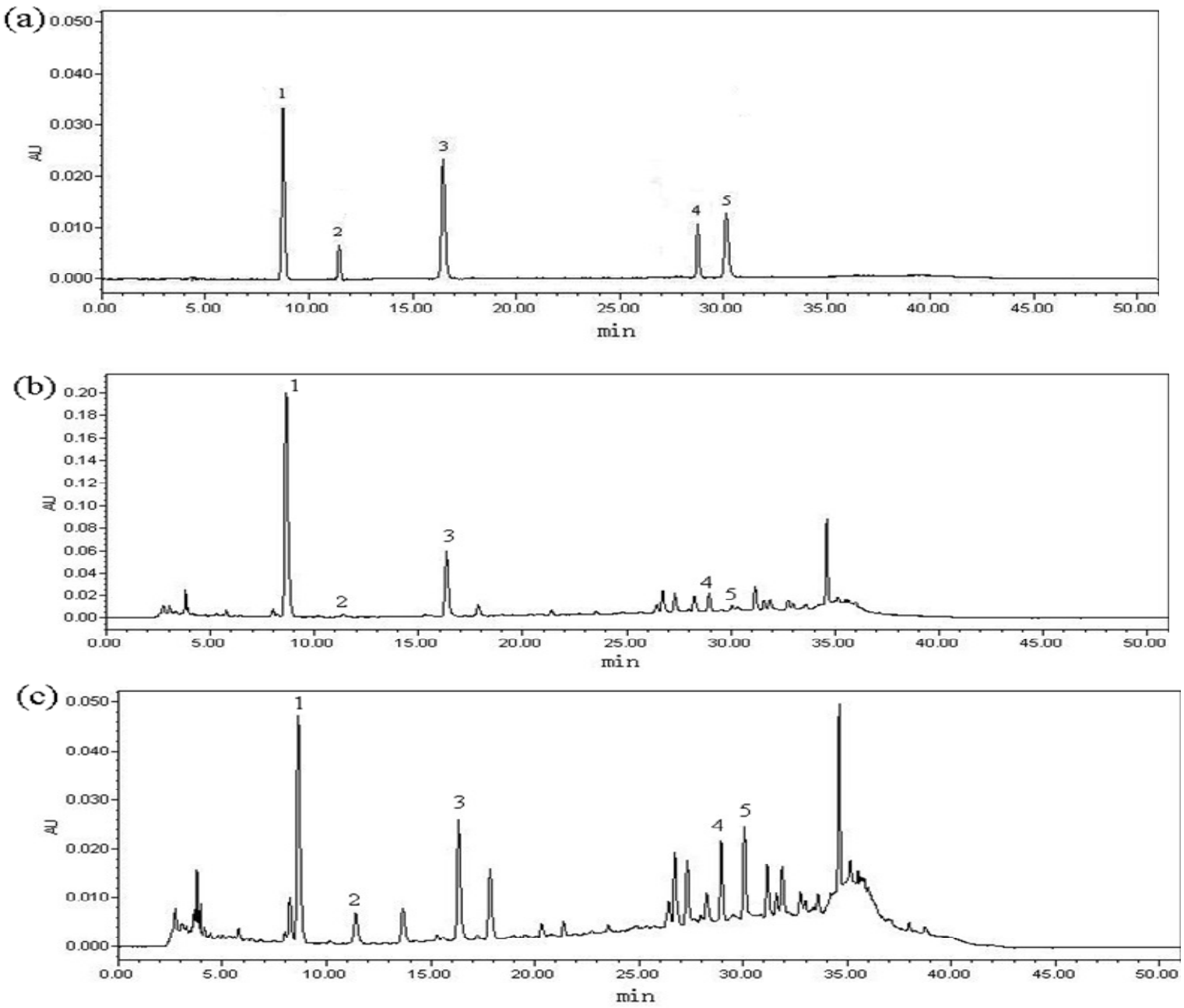
The solution stability of freeze-dried Dan-Shen injection was evaluated by exposure of test, sample and reference standards to room temperature for a period of 24 h. These were stored in tightly capped volumetric flasks and sample solutions were assayed at 0, 2, 4, 8, 12, and 24 h, respectively.

RESULTS AND DISCUSSION

Method development

The chromatographic conditions were developed using the standards, as well as the sample of freeze-dried Dan-Shen injection. Several formic acid–methanol and trifluoroacetic acid – acetonitrile gradient systems were evaluated as mobile phases. It was found that, with the trifluoroacetic acid–acetonitrile system, the sharper chromatographic peaks were not only obtained, but also more stable baseline could be obtained than with formic acid – methanol system (Figure 2). Finally, it was decided to select the gradient program described above.

Due to the prominent difference of the UV absorption properties of the five depsides, caused by the chemical structure, it is impossible to simultaneously determine them in the same UV detection wavelength. In this paper, an HPLC system equipped with DAD was employed to establish the assay method for simultaneous quantification of the five active components in freeze-dried Dan-Shen injection. According to the UV spectrograms of five depsides obtained from DAD (Figure 3), the detection wavelength was set at described above.


(a)
AU
min
1
2
3
4
5
(b)
AU
min
1
2
3
4
5
(c)
AU
min
1
2
3
4
5

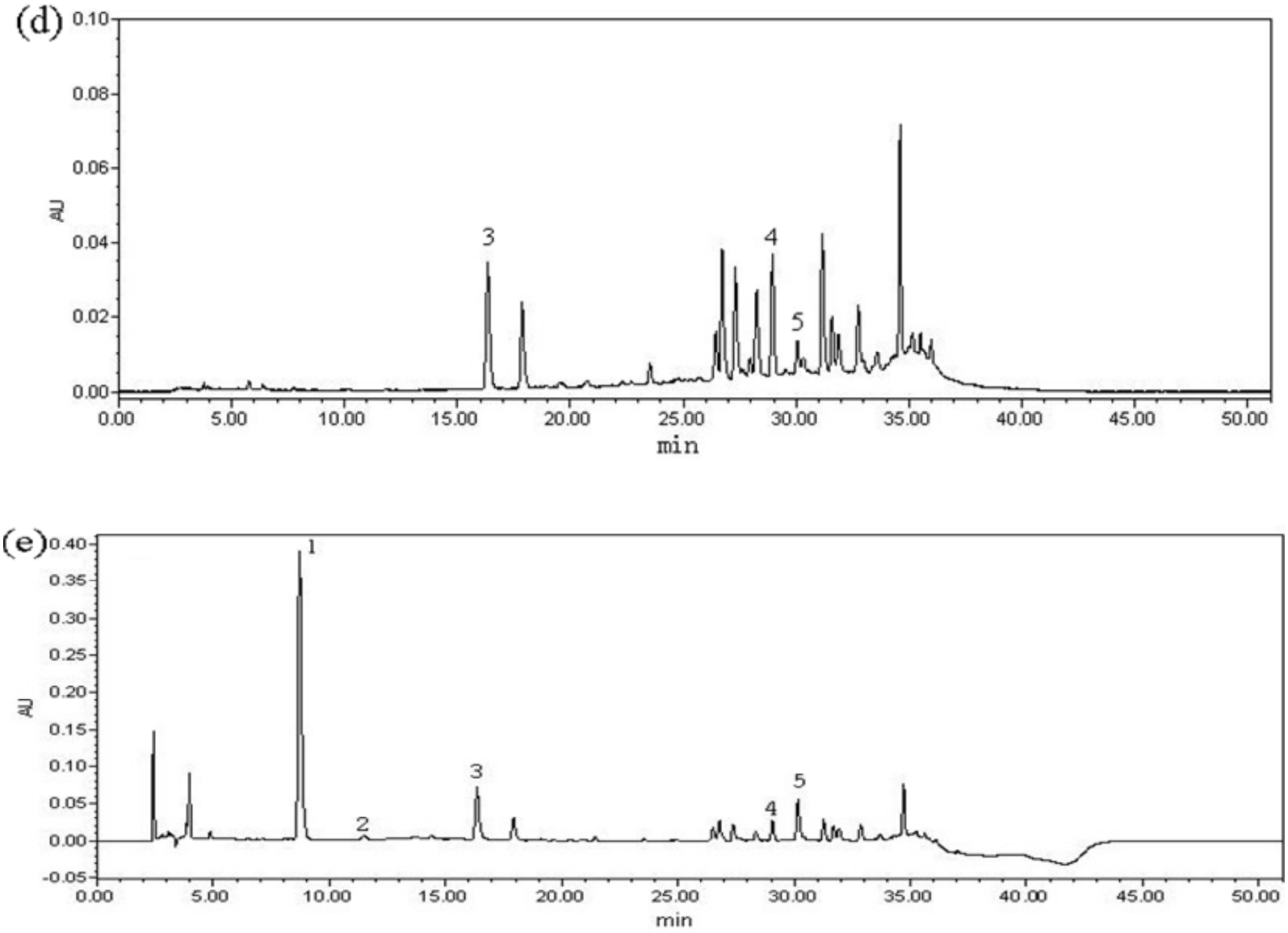
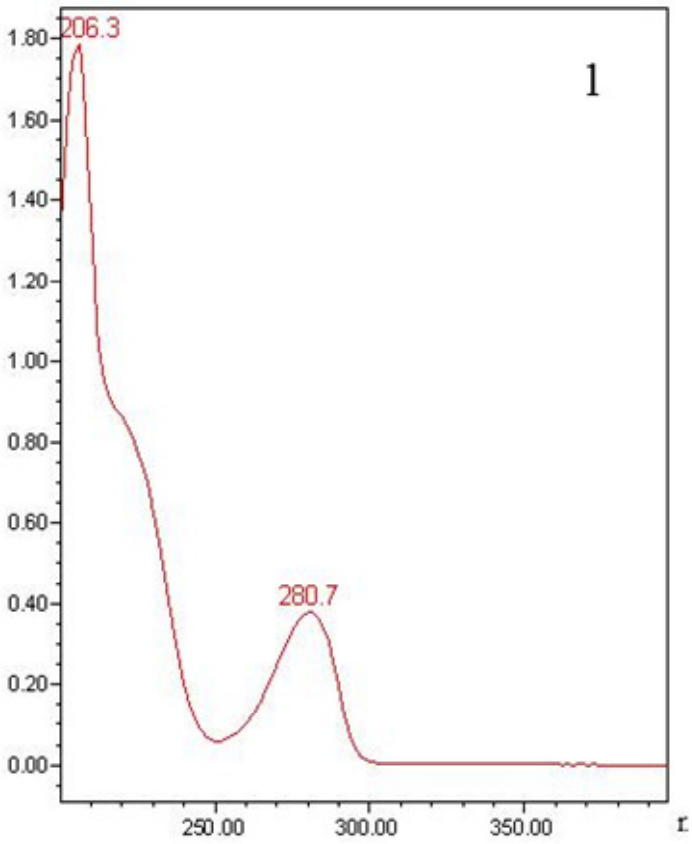
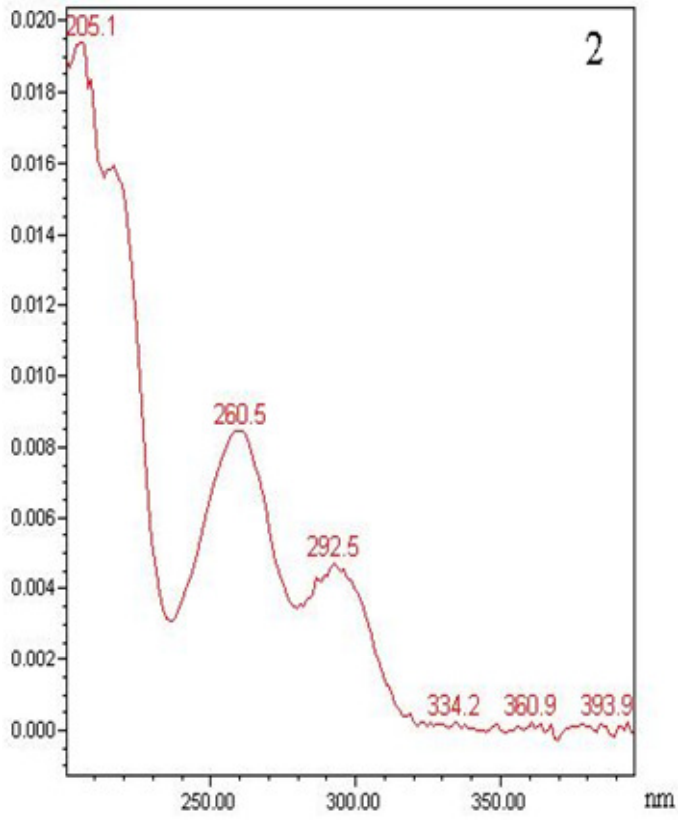
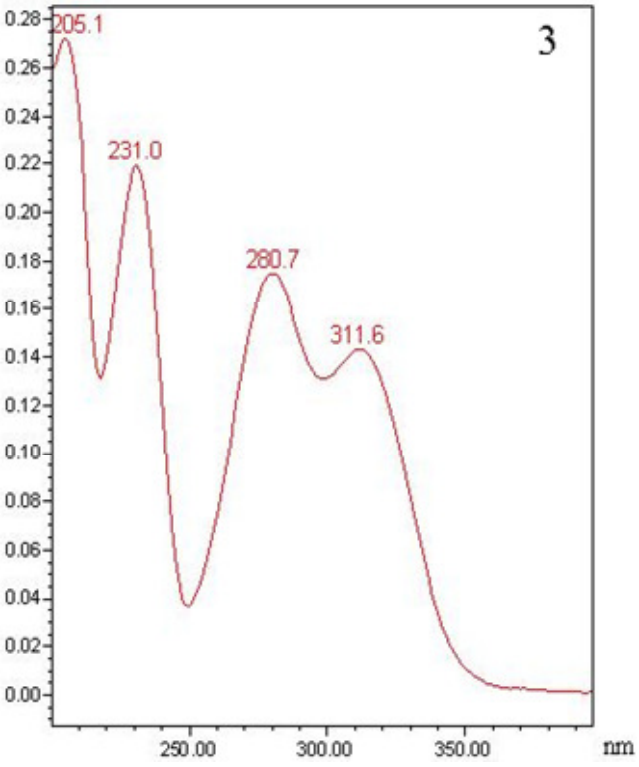
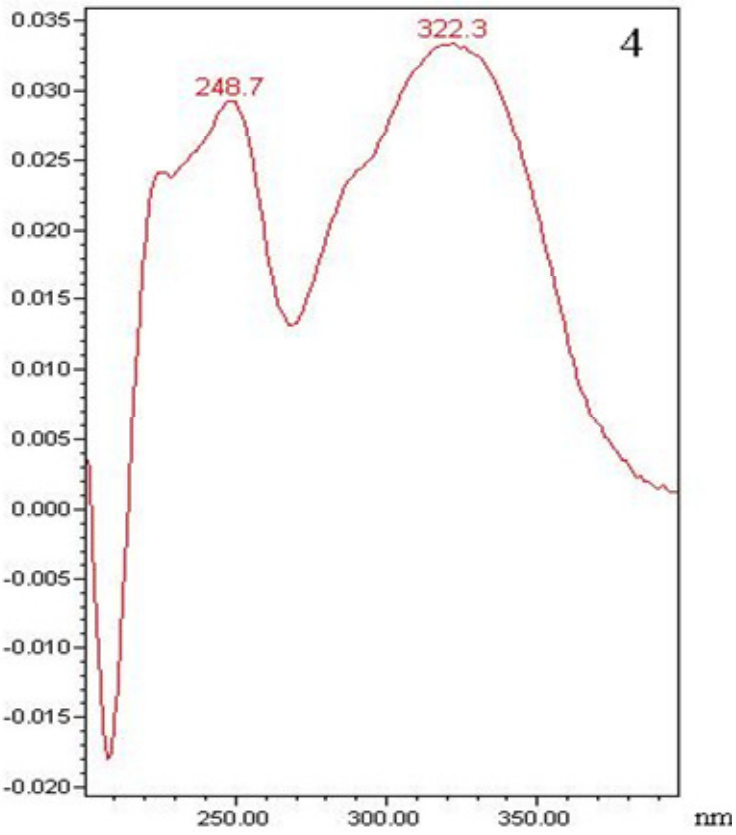


Figure 2. Representative LC-UV chromatograms obtained after analysis of freeze-dried Dan-Shen injection. (a)Standard mixture(λ=260 nm); (b) Freeze-dried Dan-Shen injection test sample(λ=281 nm); (c) Freeze-dried Dan-Shen injection test sample (λ=260 nm); (d) Freeze-dried Dan-Shen injection test sample (λ=322 nm); (e) freeze-dried Dan-Shen injection test sample (λ=228 nm). 1=Salianic acid A, 2=Protocatechuic acid, 3= Protocatechuic aldehyde, 4= Salvianolic acid D, 5= Salvianolic acid G.


1
206.3
280.7
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
250.00
300.00
350.00
r


2
205.1
260.5
292.5
334.2
360.9
393.9
0.020
0.018
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
250.00
300.00
350.00
nm


3
205.1
231.0
280.7
311.6
0.28
0.26
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
250.00
300.00
350.00
nm


4
322.3
248.7
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
-0.005
-0.010
-0.015
-0.020
250.00
300.00
350.00
nm

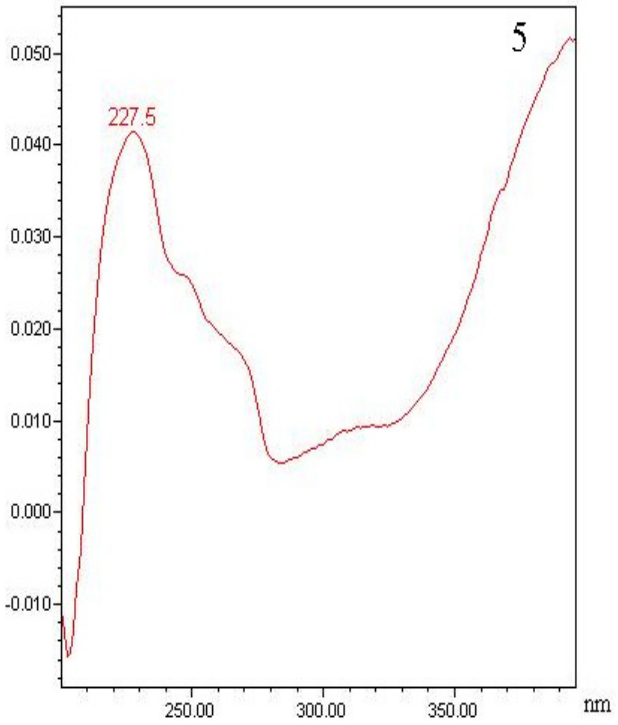


Figure 3. Representative UV spectrograms of five depsides obtained from DAD, (1) Salianic acid A; (2) Protocatechuic acid; (3) Protocatechuic aldehyde; (4) salvianolic acid D; (5) Salvianolic acid G.

Identification of five compounds

The peaks were identified by comparing the retention times and UV spectra of the standards and the sample of freeze-dried Dan-Shen injection. MS detection was also applied to identification. By carefully studying the mass spectra of these compounds and comparing with standards and reference data (Zhang, 2005), five peaks in freeze-dried Dan-Shen injection were identified (TIC) was presented in Figure 4.

Method validation

Calibration curves, limits of detection and limits of quantification

The regression equations, correlation coefficient (r^2) and concentration ranges of eight markers were given in the Table 2, where y and x were the peak area and concentration (μg ml^{-1}), respectively. The results showed good linear relationship.

Precision and accuracy

The intra- and inter-day variations (RSD) obtained for the five actives were salianic acid A 0.51 and 1.18%, protocatechuic acid 0.85 and 1.38%, protocatechuic (Table 1). A representative total ion chromatogram aldehyde 0.53 and 1.26%, salvianolic acid G 1.27 and 1.98% and salvianolic acid D 0.64 and 1.08%, respectively. The average recoveries were salianic acid A 98.6% (RSD 1.21%), protocatechuic acid 98.5% (RSD 1.73%), protocatechuic aldehyde 99.1% (RSD 1.69%), salvianolic acid G 98.3% (RSD 1.61%), salvianolic acid D 99.4% (RSD 2.27%), respectively. The values obtained indicated acceptable precision and accuracy.

Stability

Variations in RSD values for five actives were salianic acid A 0.79%, protocatechuic acid 1.36%, protocatechuic aldehyde 1.53%, salvianolic acid G 2.58%, salvianolic acid D 0.61%, respectively. These data confirmed that the sample solutions were stable up to 24 h when kept at room temperature.

Application in preparation

This method was subsequently applied to a simultaneous determination of five active compounds for 10 batches freeze-dried Dan-Shen injection sample. The average

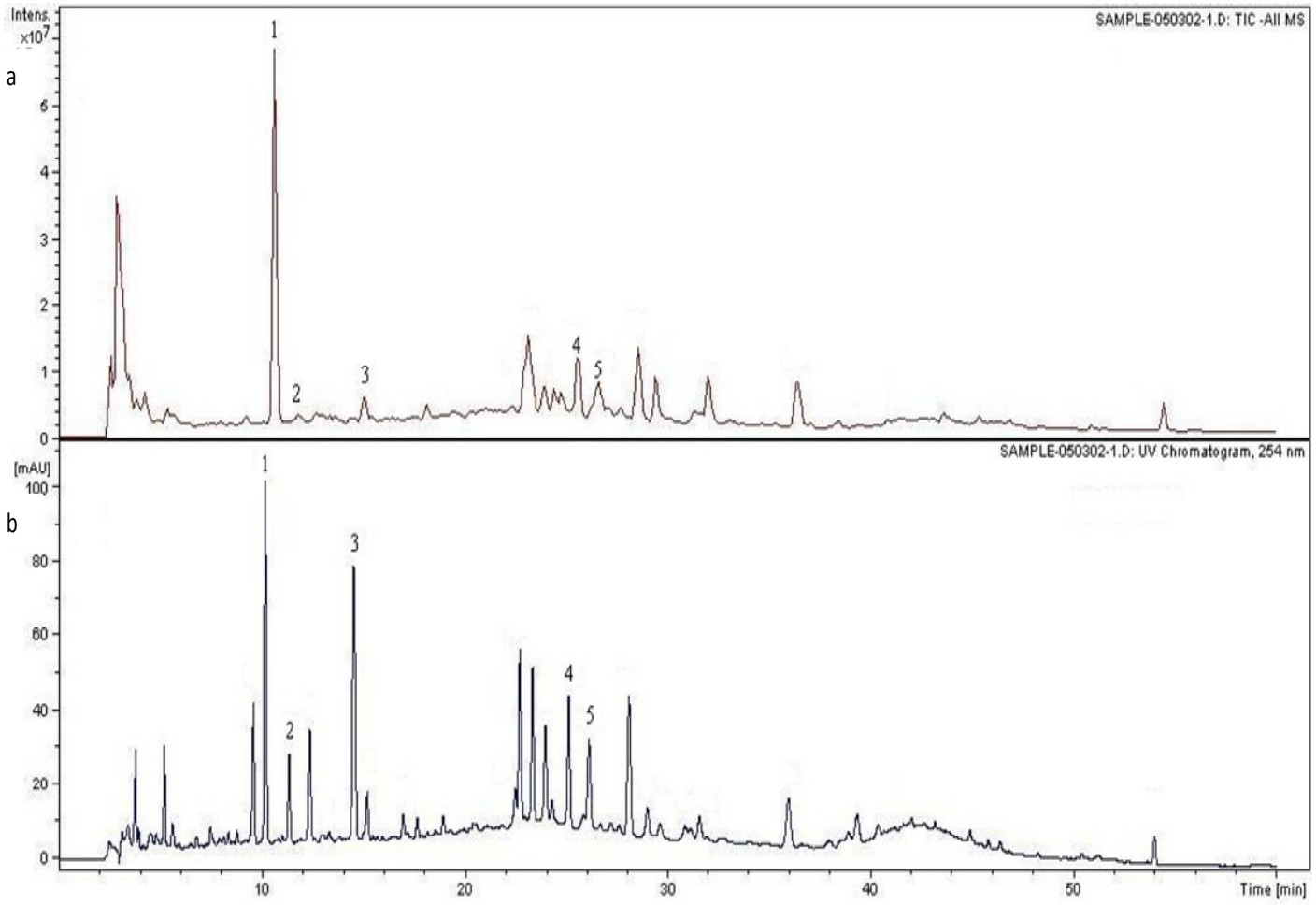


Figure 4. Representative total ion chromatogram (TIC) and UV chromatogram obtained after LC-MS analysis of freeze-dried Dan-Shen injection.(a)TIC of sample; (b) HPLC-UV chromatogram of sample. 1=Salianic acid A, 2=Protocatechuic acid, 3= Protocatechuic aldehyde, 4= Salvianolic acid D, 5= Salvianolic acid G.

Table 1. MS and MS2 data obtained from LC-MS analysis of freeze-dried Dan-Shen injection.

Peak	Identification	RT(min)	$[M - H]^-$ (m/z)	MW
1	Salianic acid A	10.5	197	198
2	protocatechuic acid	11.2	153	154
3	Protocatechuic aldehyde	14.8	137	138
4	Salvianolic acid D	25.5	417	418
5	salvianolic acid G	26.6	339	340

Table 2. Linear calibration curve, concentration range, LOD and LOQ of five compounds.

Compound	Regression equation	r	Linear range(μg ml^{-1})	LOD (μg ml^{-1})	LOQ (μg ml^{-1})
Salianic acid A	$Y=7.471\times10^3X+6.386\times10^3$	0.9998	24.34–486.72	4.05	10.5
protocatechuic acid	$Y=3.737\times10^4X-1.577\times10^3$	0.9997	1.01–50.40	0.32	0.84
Protocatechuic aldehyde	$Y=5.006\times10^4X-1.534\times10^4$	0.9999	2.42–48.42	0.41	1.07
Salvianolic acid D	$Y=1.049\times10^4X+7.745\times10^3$	0.9999	2.58-323.00	0.52	1.35
salvianolic acid G	$Y=5.176\times10^3X-8.635\times10^2$	0.9999	5.44-272.00	0.87	2.04

contents ($\bar{x}$ ±SD) were salianic acid A 114.28±10.30 mg g^{-1}, protocatechuic acid 0.76 ±0.09 mg g^{-1}, protocatechuic aldehyde 7.59 ±1.99 mg g^{-1}, salvianolic acid G 7.17±1.32 mg g^{-1} and salvianolic acid D 8.85 ± 1.32 mg g^{-1}, respectively. The results demonstrated that the technology of production was stable enough and the method was suitable to ensure the quality of products.

Conclusion

In this study, an accurate and reliable analytical method was first developed for the simultaneous determination of five active components present in freeze-dried Dan-Shen injection. The results suggested that this HPLC method could be considered as good quality criteria to control the quality of freeze-dried Dan-Shen injection.

REFERENCES

Chen ZX, Gu WH, Huang HZ, Yang XM, Sun CJ, Chen WZ, Dong YL, Ma HL (1981). Studies on the water soluble phenolic acid of *Salvia miltiorrhiza*. Chin. J. Chin. Pharm. Bul., 16(9): 536-537.

Cheng YY, Chen MJ, Tong WD (2003). An approach to comparative analysis of chromatographic fingerprints for assuring the quality of botanical drugs. Chin. J. Chem. Inf. Comput. Sci., 43(3): 1068-1070.

Du GH, Zhang JT (2004). The general situation and progress of the modern research of red sage root (Radix *Salvia militiorrhiza*). Chin. J. Her Med., 23(6): 435-440.

Gu WH (1981). Chemical constituents of the plants in *Genus Salivia*. Chin. J. Chin. Trad. Herb Drugs, 12(2): 41-48.

Li LN (1997). Water solute active components of *Salvia militiorrhiza* and related plants. Chin. J. Chin. Pharm. Sci., 6(2): 57-64.

Li YG, Song L, Wang ZT, Hu ZB (2008). Advancement in Analysis of *Salviae miltiorrhizae* Radix et *Rhizoma* (Danshen). USA. J. Chroma A., 1216(11): 1941-1953.

Lin HC, Chang WL (2000). Diterpenoids from *Salvia miltiorrhiza*. U.K. J. Phytochemistry, 53(8): 951-953.

Ling HY, Lu XZ, Zhao YL (1999). The survey on the research of water-soluble components of Chinese medicine “DANSHEN”. Chin. J. Nat. Prod. Res., 11(1): 75-81.

Liu XM, Zhang JS, Chen XG (2007). Separation and determination of three water-soluble compounds in *Salvia miltiorrhiza* Bunge and two related traditional medicinal preparations by flow injection-capillary electrophoresis. USA. J. Chromatogr B., 852: 325-332.

National Drug Standards, the New Drug to Positive Standard. Chin. B., 44: 10-14.

Pharmacopoeia Committee of People’s Republic of C hina (2004).

Qin HY, Liu WZ, Suo ZR, Zheng JB (2006). Determination of 3 water-soluble ingredients in *Salvia miltiorrhiza* Bge by HPLC with electrochemical detection. Chin. J. Pharm. Anal., 26(8): 1035-1038.

Yuan D, Pan Yn, Fu Ww, Makino T, Kano Y (2005). Quantitative analysis of the marker compounds in *Salvia miltiorrihiza* root and its phytomedicinal preparations. Jap. J. Chem. Pharm. Bull., 53: 508-514.

Zhang JL, Cui M, He Y, Yu HL (2005). Chemical fingerprint and metabolic fingerprint analysis of DanShen injection by HPLE-UV and HPLC-MS methods. USA. J. Pharm. Biomed. Anal., 36: 1029-1035.

Zhou CX, Luo HW, Niwa M (1999). Studies on isolation and identification of water-soluble components of *Salvia militiorrhiza*. Chin. J. China Pharm. Univ., 30(6): 411-416.