High-performance liquid chromatographic determination of tanshinones in the roots of *Salvia miltiorrhiza* and related traditional Chinese medicinal preparations

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ABSTRACT **Purpose**: This paper describes a validated high-performance liquid chromatographic method to quantitate four tanshinones as markers; dihydrotanshinone I, cryptotanshinone, tanshinone I and tanshinone IIA for use in the quality control of the roots of *Salvia miltiorrhiza* and its related traditional Chinese medicinal preparations. **Methods:** Separation was achieved using a Zorbax Extend C18 reserved-phase column (5μm, 250×4.6mm) at 20 °C with a gradient mixture of deionized water and acetonitrile at a flow rate of 1.2ml/min. **Result:** The limits of quantitation were 0.13, 0.08, 0.06 and 0.05μg/ml for dihydrotanshinone I, cryptotanshinone, tanshinone I and tanshinone IIA, respectively. This method provided good reproducibility and sensitivity for the quantification of four tanshinones with overall RSD values for intra-day and inter-day precision and accuracy better than 3.8% and higher than 94.9%, respectively. The recovery of the method was 95.4-104.4% for all the tanshinones and showed good linearity (r>0.9998) over a relatively wide concentration range. **Conclusions:** This assay was successfully applied to the determination of four tanshinones in the roots of *Salvia miltiorrhiza* and its related traditional Chinese medicinal preparations. The results indicated that the HPLC assay could be readily utilized as a quality control method for the roots of *Salvia miltiorrhiza* and its related traditional Chinese medicinal preparations.

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INTRODUCTION

The roots of *Salvia miltiorrhiza* (Danshen), a commonly used herbal medicine in China, are widely used to treat coronary heart disease, cerebrovascular disease, hepatitis, hepatocirrhosis and chronic renal failure (1-4). Due to its purported biological activity and fewer side effects as confirmed by pharmacological investigations and clinical use, many traditional Chinese medicinal preparations (TCMPs) containing Danshen, such as Fufang Danshen tablets (FDT), Compound Danshen Dripping pills (CDDP), Danshen injection (DSI) and Xiangdan injection (XDI), have been developed. These TCMPs are mainly used to treat coronary heart disease, heart-stroke, and associated cerebrovascular and cardiovascular diseases (5-9).

To ensure clinical efficacy, quality control of *Salvia miltiorrhiza* and its related TCMPs is critical. Since Danshen is the major component of FDT, CDDP, DSI and XDI, quality control of Danshen content is thus essential to guarantee the quality of these TCMPs. Tanshinones are hydrophobic components isolated from Danshen. It has been reported that tanshinones possess anti-toxin properties, modulate immunological diseases, dilate coronary arteries, increase coronary flow and protect the myocardium against ischaemia (10-13). In addition, tanshinones have attracted particular attention because they have exhibited significant

antibacterial, anti-dermatophytic, anti-neoplastic and anti-platelet aggregation activities (14-16).

In the Pharmacopoeia of the People's Republic of China (2005 Edition) (1), quality control of Danshen and FDT has relied mainly on the determination of tanshinone IIA. A number of studies on the quantitation of tanshinones in Danshen and its related TCMPs by thin layer chromatography (TLC), spectrophotometry or HPLC have been reported. However, only a few tanshinones were determined in these papers (17-21). According to the literature (22) and our study, dihydrotanshinone I, cryptotanshinone, tanshinone I and tanshinone IIA (Fig. 1) are the main tanshinones contained in Danshen and its related TCMPs.

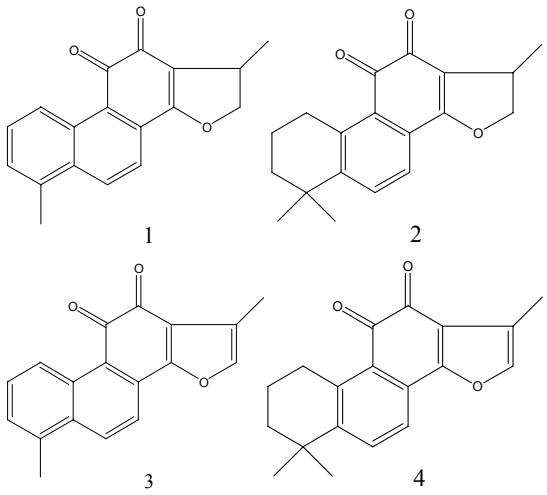

Figure 1: The structures of selected tanshinones: 1, dihydrotanshinon I; 2, cryptotanshinone; 3, tanshinone I; 4, tanshinone IIA

Therefore simultaneous determination of these four tanshinones could, to some extent, reflect the overall quantity of Danshen and its related TCMPs.

In this report, we describe a simple, rapid and accurate method of analysis using reversed-phase HPLC (RP-HPLC) to simultaneously determine dihydrotanshinone I, cryptotanshinone, tanshinone I and tanshinone IIA in Danshen and in four TCMP samples.

MATERIALS AND METHODS

Chemicals and Materials

HPLC grade acetonitrile and methanol (E. Merck, Darmstadt, Germany) were used for the HPLC analysis. Deionized water was purified by a Milli-Q system (Millipore, Bedford, MA, USA). Phosphoric acid and chloroform were of analytical grade from Beijing Beihua Fine Chemicals Co., Ltd. (Beijing, China). The roots of *Salvia miltiorrhiza* and TCMPs samples were purchased from drugstores around China (Table 4).

Dihydrotanshinone I [**1**], cryptotanshinone [**2**], tanshinone I [**3**] and tanshinone IIA [**4**] were purchased from the National Institute for Control of Biological and Pharmaceutical Products of China, and their purity was over 98% by HPLC analysis.

Apparatus and chromatographic conditions

An Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) comprising a quaternary solvent delivery system, an on-line degasser, an autosampler, a column temperature controller and photodiode array detector coupled with an analytical workstation was used. The column configuration was an Agilent Zorbax Extend C_{18} reserved-phase column (5μm, 250×4.6 mm) with an Agilent Zorbax Extend C_{18} guard column (5μm, 10×4.6mm). The sample injection volume was 10 μl.

The detection wavelength was set at 270nm, the flow rate was 1.2ml/min and the column temperature was maintained at 20 °C. The mobile phase consisted of A (deionized water) and B (acetonitrile). Gradient elution was as follows: Initially 45% B at 0 min, linearly increasing to 60% at 3 min, maintaining 60% B from 3 min to 14 min, linearly increasing B to 80% at 15 min and then linearly increasing B to 82% at 20 min until the end of the run. After each analysis, 45% mobile phase B was pumped and maintained for 10 min to re-equilibrate the system for baseline stability. An SB 3500 ultrasonic generator (50 KHz, 350 W) from Shanghai Branson Ultrasonics Co. Ltd (Shanghai, China) was used to aid the extraction of tanshinones from the samples.

Table 1: Calibration curves of tanshinones.

Analyte	Retention Time (min)	Standard curve	r 2	Test range (μg ml-1)	LOD (μg ml-1)	LOQ (μg ml-1)
Dihydrotanshinone I	8.02	Y=24.321x+6.525	0.9998	1.4-64.1	0.05	0.13
Cryptotanshinone	11.95	Y=26.835x+11.065	0.9998	6.9-171.9	0.03	0.08
Tanshinone I	13.23	Y=30.049x+0.850	0.9998	4.1-82.70	0.03	0.06
Tanshinone II A	18.13	Y=41.220x+23.234	0.9999	30.86-185.14	0.02	0.05

Y: peak area; x: concentration of analyte (μg ml^{-1})

An FW100 pulverizer (24000 rpm/min, 460 W) from Tianjin City Taisite Instrument Co. Ltd (Tianjin, China) was used to comminute the Danshen. Quantitative filter paper (No.201, 9 cm) supplied by Hangzhou Fuyang Special Industry Co., Ltd (Hangzhou, China) and the membrane filters (13 mm, 0.45 μm) were obtained from Tianjin Tengda Filtration Instrument Co., Ltd (Tianjin, China).

Preparation of calibration standard solutions

A methanol stock solution containing compounds **1**, **2**, **3** and **4** was prepared and diluted to the appropriate concentration range (Table 1) for the establishment of calibration curves. Each calibration curve was analyzed three times with six different concentrations using the same HPLC conditions as described above.

Sample preparation

The dried roots of *Salvia miltiorrhiza* were sheared to be about 1cm in length and then comminuted by pulverizer. After the coating was removed by abrasive cloth, FDT and CDDP were ground in a mortar. Each solid sample (100 mesh, 0.300g) was suspended in methanol: chloroform (7:3, v/v, 10 ml) and sonicated for 0.5h. After filtration, the filtrate was transferred to a 25 ml volumetric flask and evaporated to dryness at 28 °C. The evaporated residue was dissolved in methanol and made up to volume in a 10 ml volumetric flask. One milliliter of either DSI or XDI liquid sample was diluted to 6 ml with deionized water. The solutions were filtered through a membrane filter (0.45 μm) and then injected into the HPLC.

Quality control samples

On different days, QC samples containing the relevant reference compounds (low, medium and high concentrations) were used to verify the calibration curve. The calibration curve could be used on subsequent days when the coefficient of variability (CV) of the QC samples was less than 2%, failing which a new calibration curve would be established.

Precision and accuracy

The measurements of intra-day and inter-day variability were utilized to determine the precision of the method. Three different concentrations (low, middle and high) of the four standards were prepared. The relative standard deviation (RSD) was used as a measure of precision. The intra-day variability was examined within one day (n=5) and inter-day variability was determined in triplicate on 3 separate days. Recovery experiments were carried out by spiking FDT (0.25g, brown powder) with different amounts (low, medium and high) of authentic standards with known contents of marker compounds and the samples were treated according to the sample preparation procedure.

Limits of detection (LOD) & limits of quantitation (LOQ)

LOD is defined as the lowest amount of analyte which can be detected. It is formally defined as $X\text{-}X_B=3S_B$, where X is the signal from the sample, X_B is the signal from the analytical blank and S_B is the SD of the reading for the analytical blank (26). LOQ is the lowest amount of an analyte which can be quantitated and similar to LOD, LOQ is formally defined as $X\text{-}X_B=10S_B$.

RESULTS AND DISCUSSION

Optimization of Extraction Procedure and Chromatographic Conditions

In order to achieve quantitative extraction, variables involved in the procedure such as solvent, extraction method and extraction time were optimized. A series of solvents such as methanol (MeOH), methanol:chloroform ($CHCl_3$) (7:3, 5:5, 3:7, v/v) and $CHCl_3$ were tested as the extraction solvents of tanshinones (23-25). The best solvent was found to be a mixture of MeOH: $CHCl_3$ (7: 3, v/v) (Table 2), which allowed extraction of all the tanshinones in high yield. MeOH or $CHCl_3$ alone were not efficient for the complete extraction of the four compounds of interest.

Table 2: Comparison of different extraction solvents.

Extraction Solvent	The peak area of tanshinones			
	1	**2**	**3**	**4**
MeOH	194.8	392.6	614.2	2016.1
MeOH :$CHCl_3$=7:3	287.7	539.0	863.1	2747.3
MeOH :$CHCl_3$=5:5	165.9	324.3	504.7	1655.2
MeOH :$CHCl_3$=3:7	140.7	276.8	433.6	1426.8
$CHCl_3$	177.3	127.0	418.2	826.7

The ultrasonic treatment procedure was found to be the best extraction method for the tanshinones. In order to investigate extraction time, powdered FDT (0.300g) samples were extracted with 10 ml MeOH: $CHCl_3$ (7:3, v/v) for 10, 20, 30, 45, 60 min, respectively. The results suggested that all the tanshinones were almost completely extracted within 30 min (Table 3).

Table 3: Comparison of extraction time.

Extraction Time	The peak area of tanshinones			
	1	**2**	**3**	**4**
10 min	347.5	1100.1	498.5	1130.5
20 min	464.4	1752.0	1012.9	2459.7
30 min	452.9	1811.2	1071.3	2522.4
45 min	464.8	1797.8	1063.0	2527.8
60 min	436.9	1778.3	1052.5	2457.9

Therefore, the optimal extraction method of tanshinones was ultrasonic extraction with 10ml of the mixture of MeOH: $CHCl_3$ (7:3, v/v) for 30 min.

A good separation is assumed when the analyzed peaks are baseline separated within a short analysis time. To obtain chromatograms with good separation, various stationary phases, mobile phases, column temperatures, detection wavelengths and flow rates were investigated. For the assay of tanshinones in Danshen and its related TCMPs, a Zorbax Extend C18 was found to be better than BDS-Hypersil C18, YMC-Pack ODS-A C18 or Luna C18. Various mixtures of water/acetonitrile or water/methanol were used as mobile phase and the results indicated that water/acetonitrile system was better than that of water/methanol. Due to the similar retention behaviors of compounds **2** and **3**, the mobile phase B (acetonitrile) was maintained at 60% from 3 min to 14 min to achieve a baseline separation of these two compounds. It was also found that the best separation was achieved when the column temperature was kept at 20 °C using a flow rate of 1.2ml/min and detection wavelength of 270 nm.

Linearity and range

Figure 2 shows a typical chromatogram of four tanshinones, **1**, **2**, **3** and **4** with retention times 8.02, 11.95, 13.23 and 18.13 min, respectively.

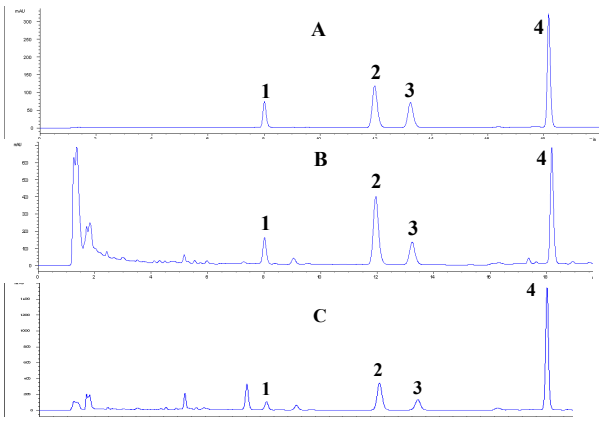

Fig. 2: Representative HPLC chromatograms of (A) standard solution at medium concentration, (B) FDT (Xianzhi,Liaoning, China), (C) Danshen (Shanxi, China) 1, dihydrotanshinon I; 2, cryptotanshinone; 3, tanshinone I; 4, tanshinone II A.

Table 4: Intra-and inter-day variability.

	Inter-day (n=9)			Intra-day (n=5)		
Concentration (μg ml^{-1})	Found	RSD[a] (%)	Accuracy[b] (%)	Found	RSD (%)	Accuracy (%)
Dihydrotanshinone I						
5.83	5.69±0.19	3.3	97.6	5.53±0.05	0.85	94.9
34.95	13.00±0.17	1.3	104.0	35.10±0.06	0.18	100.4
58.25	60.49±0.73	1.2	103.8	60.20±0.63	1.04	102.9
Cryptotanshinone						
13.75	14.04±0.53	3.8	102.1	13.24±0.02	0.12	96.3
82.50	85.3±1.71	2.0	96.7	83.22±0.39	0.47	100.9
158.13	165.75±1.85	1.1	104.8	164.81±0.35	0.21	104.2
Tanshinone I						
8.27	8.15±0.16	2.0	98.5	8.03±0.02	0.26	97.1
49.60	50.05±1.4	2.7	100.9	49.53±0.31	0.63	99.9
74.40	75.49±1.5	2.0	101.5	75.94±0.23	0.30	102.1
Tanshinone II A						
38.6	37.04±1.04	1.0	95.9	37.23±0.07	0.18	96.4
92.6	89.44±2.01	2.3	96.6	90.71±0.22	0.24	98.0
177.4	172.31±4.70	2.7	97.1	178.90±0.45	0.25	100.8

[a]*RSD(%) = (SD/mean)×100*

[b]*Recovery(%) = (mean of measured concentration/spiked concentration)×100*

Table 5: Recoveries of the four tanshinones (n=4).

tanshinone spiked (μg ml^{-1})	Found (μg ml^{-1})	RSD[a] (%)	Recovery (%)
Dihydrotanshinone I			
3.0	2.94±0.10	3.3	98.1
36.2	35.41±0.57	1.6	97.8
48.6	48.37±1.35	2.8	98.8
Cryptotanshinone			
8.8	9.07±0.13	1.5	103.1
96.8	95.07±1.38	1.5	98.2
142.1	138.28±2.35	1.7	97.3
Tanshinone I			
8.9	9.24±0.31	3.4	103.9
48.9	47.53±1.5	3.2	97.2
63.4	60.45±3.5	5.8	95.4
Tanshinone IIA			
38.5	40.20±2.39	5.9	104.4
84.7	81.13±2.81	3.5	95.8
126.6	117.26±4.0	3.4	95.6

[a]*RSD(%) = (SD/mean)×100,* [b]*Recovery(%) = (mean of measured concentration/spiked concentration)×100*

The four peaks in the samples were monitored in the UV range from 200-400nm with a DAD-detector and assessment of peak purity showed peak homogeneity thereby indicating the specificity of the method. Linear ranges and correlation co-efficients are depicted in Table 1. All the standard compounds showed good linearity (r>0.9998) in a relatively wide concentration range.

System suitability

The intra-day precision is shown in Table 4 where the RSDs ranged from 0.12% to 1.04%. The inter-day precision was determined from nine determinations over 3 separate days for each concentration and the results were in the range of 1.00%-3.80%. The recovery of the four standards ranged from 95.4% to 104.4% (Table 5).

Stability testing was performed on a sample solution left on the bench and was analyzed every 12 h over 3 days at room temperature. The analytes were found to be relatively stable over 72 h (RSD<5%).

LOD and LOQ

The LODs of the four tanshinones were 0.05, 0.03, 0.03 and 0.02 μg ml^{-1} and the LOQs were 0.13, 0.08, 0.06 and 0.05 μg ml^{-1}, for **1**, **2**, **3** and **4,** respectively (Table 1).

Sample analysis

Forty samples were prepared as previously described. A volume of 10 μl of filtered solution of each sample was injected into the instrument and each sample was determined in triplicate. Peaks in the chromatograms were identified by comparing their retention times and on-line UV spectra with those of the reference standards.

The method was successfully applied to determinate four tanshinones in Danshen and in four Danshen-containing TCMPs, FDT, CDDP, XDI and DSI, respectively. The contents of the four major tanshinones in 40 samples are shown in Table 6. From Table 6, it is obvious that the quality of fourteen Danshen samples from different locations varied quite drastically and could account for uncertain and unstable curative effects of the TCMPs. Furthermore, it can be seen that the contents in four of the TCMPs also varied markedly and are hardly detectable in DSI and XDI, low in content in CDDP and high in Danshen and FDT samples. The reason for the variation might be due to different processing procedures of Danshen during the manufacturing process. It is also obvious from Table 6 that the contents of the tanshinones in FDT varied greatly amongst the different manufacturers. The variation of contents may be due to different quality of the raw material, the difference of production procedure, storage and transportation, etc., amongst others.

In the Pharmacopoeia of People's Republic of China (2005 Edition), tanshinone IIA is claimed to be present in not less than 0.2% of Danshen crude drug and 200μg/tablet for FDT. In our 14 samples of Danshen crude drugs analyzed, there are 11 samples (N0s.21, 22, 23 and 27-34) that meet the specified standard by Chinese Pharmacopoeia, while all FDT samples determined meet this standard. Considering the purported medicinal properties of tanshinone I, crytotanshinone and dihydrotanshinone I, it is recommended that not only tanshinone IIA but also tanshinone I, crytotanshinone and dihydrotanshinoneI should be determined for use as quality control markers for Danshen crude drug and TCMPs containing Danshen.

CONCLUSIONS

This paper describes a simple, accurate and precise method for the simultaneous determination of the four major tanshinones in Danshen, FDT, CDDP, DSI and XDI. The data obtained from 40 samples reveal that the contents of tanshinones in Danshen and FDT varied considerably. The contents are very low in CDDP and almost undetectable in DSI and XDI. The current HPLC method has thus been shown to be suitable for use as a method for the quality control of *Salvia miltiorrhiza* and its related TCMPs to assure their clinical efficacy.

Table 6: Contents of tanshinones in samples of Danshen and its related TCMPs.

NO	Batch Number or harvest time[h]	Collection Province	Content[f](n=3) Tanshinone IIA	Tanshinone I	Cryptotanshinone	Dihydrotanshinone I
1 [a]	30902	Guangdong	573.2±9.3	219.5±9.0	444.2±8.1	135.9±5.9
2 [a]	31207	Beijing	531.3±9.0	201.5±9.3	528.5±21.4	123.0±6.1
3 [a]	40703	Shenzhen	534.6±6.3	144.3±6.7	291.6±5.2	93.9±3.2
4 [a]	30113	Guangxi	534.5±21.2	87.2±3.3	192.6±3.6	42.5±3.4
5 [a]	40210	Henan	714.4±18.6	477.4±3.4	768.7±6.2	270.3±21.6
6 [a]	9919	Hebei	411.6±6.7	210.5±12.7	273.5±3.1	84.2±3.2
7 [a]	20040342	Anhui	696.6±9.5	297.2±3.5	834.6±2.5	159.5±3.5
8 [a]	40237	Shanghai	621.5±39.2	249.6±6.6	777.4±24.6	183.6±9.7
9 [a]	4120369	Beijing	483.3±9.4	285.3±9.5	222.2±3.3	141.3±6.4
10 [a]	20040561	Yunnan	666.1±9.3	309.7±6.2	744.4±3.5	207.5±6.7
11 [a]	40614	Sichuang	609.4±3.5	354.3±9.3	507.3±9.7	135.7±3.2
12 [a]	20040307	Hebei	447.2±12.5	240.7±3.4	453.5±3.2	111.2±3.7
13 [a]	40110	Fujiang	429.3±33.5	141.8±6.5	279.3±6.3	75.4±3.3
14 [a]	40505	Guangxi	642.6±15.3	129.3±6.3	354.4±6.1	78.6±2.8
15 [a]	20301	Jiangxi	507.2±6.4	237.5±3.2	369.6±3,5	81.8±3.1
16 [a]	31101	Guangdong	675.5±2.6	144.3±6.3	300.3±6.3	87.3±6.2
17 [a]	40343	Guangdong	576.2±15.3	141.6±6.6	72.1±3.0	ND
18 [a]	40208	Guangdong	777.4±24.5	264.8±6.2	426.3±6.3	135.5±3.6
19 [a]	301900	Guangdong	615.2±3.3	408.3±9.7	1863.5±27.6	399.7±6.4
20 [a]	40427	Beijing	528.4±27.5	252.2±9.4	510.3±9.7	114.5±6.2
21 [b]	200406	Shandong	2.01±0.09	1.82±0.03	2.63±0.03	1.37±0.01
22 [b]	200407	Sichuang	5.03±0.27	1.22±0.02	4.08±0.05	3.72±0.04
23 [b]	200308	Shanxi	7.45±0.32	2.01±0.03	5.49±0.05	3.51±0.03
24 [b]	200309	Liaoning	0.44±0.02	0.17±0.01	0.23±0.00	0.07±0.00
25 [b]	200409	Henan	1.79±0.08	1.16±0.02	0.98±0.01	0.48±0.01
26 [b]	200408	Zhejiang	0.40±0.02	0.36±0.01	0.17±0.00	0.10±0.00
27 [b]	200107	Shanxi	2.23±0.11	2.03±0.04	1.81±0.02	3.95±0.04
28 [b]	200309	Liaoning	2.37±0.10	1.27±0.03	2.59±0.03	2.13±0.02
29 [b]	200402	Henan	2.45±0.12	1.09±0.02	1.53±0.01	1.34±0.01
30 [b]	200408	Shanxi	2.03±0.09	1.41±0.03	0.75±0.01	0.98±0.01
31 [b]	200407	Hebei	2.48±0.09	0.98±0.01	1.29±0.01	0.82±0.01
32 [b]	200403	Shanxi	2.27±0.08	1.20±0.01	1.39±0.01	0.94±0.01
33 [b]	200301	Shanxi	2.60±0.09	1.95±0.03	1.81±0.02	2.05±0.02
34 [b]	200303	Shandong	5.11±0.14	3.29±0.05	3.09±0.04	2.41±0.02
35 [c]	040710	Shanghai	ND	ND	ND	ND
36[d]	040828	Guangdong	ND	ND	ND	ND
37 [e]	20031001	Tianjing	ND	2.8±0.3	5.4±0.4	ND
38 [e]	20030618	Tianjing	ND	2.9±0.2	5.5±0.5	ND
39 [e]	20040218	Tianjing	ND	2.7±0.2	5.4±0.4	5.3±0.4
40 [e]	20040508	Ttianjing	2.7±0.2	2.7±0.3	5.3±0.3	5.2±0.3

[a]: Fufang Danshen tablet, [b]: Danshen crude drug, [c]: Danshen injection, [d]: Xiangdan injection, [e]: Compound Danshen dripping pill, [f] Content = mean±SD(n=3), units of Danshen crude drug is mg/g, units of FDT is μg(tanshinone)/300mg tablet, units of CDDP is μg(tanshinone)/27mg dripping pill, units of DSI and XDI is mg/10ml injection, [g]ND: not detected, [h]: Batch Number used in the TCMPs and Harvest time used in Danshen crude drug.

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