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Journal of Pharmaceutical and Biomedical Analysis 37 (2005) 481–486
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Simultaneous determination of cryptotanshinone, tanshinone I and tanshinone IIA in traditional Chinese medicinal preparations containing *Radix salvia miltiorrhiza* by HPLC

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Accepted 2 November 2004
Available online 23 December 2004

Abstract

A reversed-phase high performance liquid chromatographic method was established for the simultaneous determination of tanshinones in five kinds of traditional Chinese medicinal preparations (TCMPs) containing *Radix salvia miltiorrhiza* (Chinese herbal name: Danshen). Tanshinones including cryptotanshinone, tanshinone I and tanshinone IIA were successfully separated on a Diamonsil C_{18} column (150 mm × 4.6 mm i.d., 5 μm). The mobile phase was a mixture of methanol, tetrahydrofuran, water and glacial acetic acid (20:35:44:1, v/v/v/v), employing isocratic elution at a flow rate of 1.0 mL/min. Detection was accomplished at 254 nm. The compounds were identified by comparing their retention times and UV spectra in the 200–400 nm range with authentic standards. Regression equations revealed good linear relationship between the peak areas of the constituents and their concentrations (correlation coefficients: 0.9998 for cryptotanshinone, 0.9999 for tanshinone I and 1.0000 for tanshinone IIA). The relative standard deviations ($n = 6$) of retention time and peak area were less than 0.25% and 1.00%, respectively. The recoveries were between 96.2% and 102.5%. The proposed method has been successfully applied to the simultaneous determination of the tanshinones in five kinds of Chinese herbal preparations containing Danshen within 20 min.

Keywords: Cryptotanshinone; Tanshinone I; Tanshinone IIA; RP-HPLC; Traditional Chinese medicinal preparation

1. Introduction

Traditional Chinese medicinal herbs have been widely used for over 2000 years. Most traditional Chinese medicinal preparations (TCMPs) are composed of complex constituents, and proper methods are required for quality control of them. Many high-performance liquid chromatographic (HPLC) [1–4] and capillary electrophoresis [5,6] methods have been developed for the determination of marker constituents in TCMPs.

Radix salvia miltiorrhiza, a commonly used herbal medicine in China, has the Chinese herbal name of Danshen. Due to its better performance and fewer side effects as confirmed in the long-time clinical use, Danshen is widely adopted in traditional Chinese medicinal preparations to treat coronary heart diseases, particularly angina pectoris and myocardial infarction [7–9].

Tanshinones are the hydrophobic active components which have been isolated from Danshen. It is reported that tanshinones can dilate coronary arteries, increase coronary flow, modulate mutagenic activity and protect the myocardium against ischaemia. They also have some activity as a broad-spectrum bactericide [10]. Among tanshinones in Danshen, cryptotanshinone, tanshinone I, and tanshinone IIA are present in the greatest amount. Fig. 1 shows the chemical structure of the above tanshinones.

In the Chinese Pharmacopoiea [11], tanshinone IIA is selected as the marker component for the quality control of Danshen and Fufang Danshen tablet employing the mixture

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doi:10.1016/j.jpba.2004.11.015

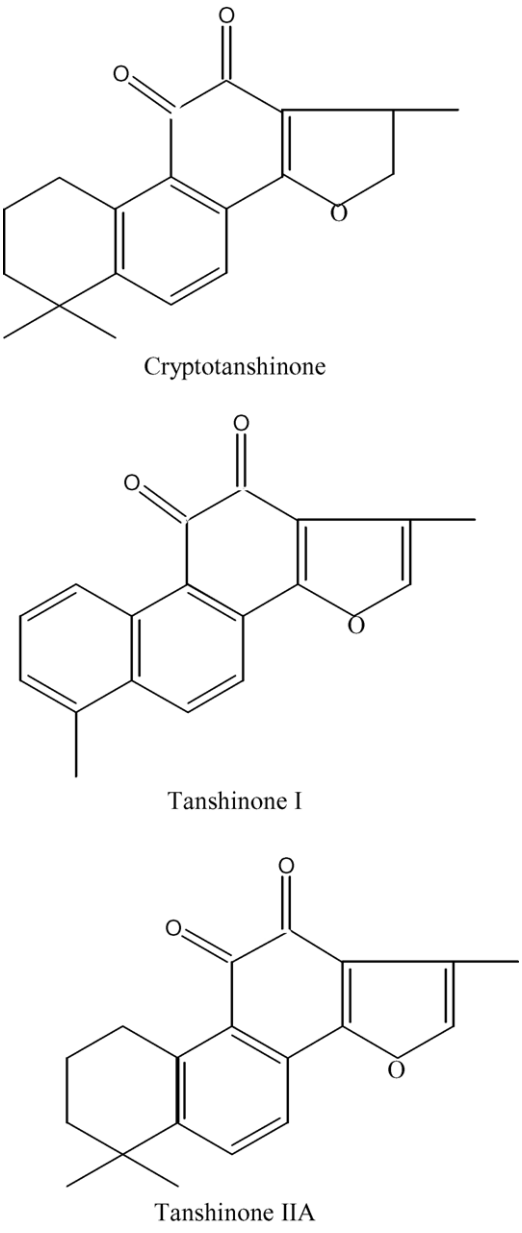

Fig. 1. Chemical structures of the tanshinones.

of methanol and water as mobile phase. Whereas, when using the mobile phase recommended by the Chinese Pharmacopoiea, cryptotanshinone and tanshinone I can not be baseline resolved and the retention time is rather long. In this case, it is not practical for the simultaneous determination of the three tanshinones. So, for the quality control of the manufacturing process of TCMPs containing Danshen and for the therapeutic monitoring of tanshinones, it is necessary to develop an efficient method for the simultaneous determination of the three tanshinones.

The present paper describes the development of an effective HPLC method for the simultaneous determination of cryptotanshinone, tanshinone I and tanshinone IIA in five TCMPs containing Danshen, using isocratic mobile phase that offers certain advantages in its high resolution and time saving. With the use of DAD detector, the identification of the peaks is more accurate.

2. Experimental

2.1. Materials and reagents

Authentic standards of cryptotanshinone, tanshinone I and tanshinone IIA were purchased from National Institute for Control of Pharmaceuticals and Biological Products (Beijing, China). All other reagents were of analytical grade. The water used was doubly-distilled. The five kinds of TCMPs containing Danshen were purchased from Jia-Shi-Tang and Ji-An-Tang pharmaceutical stores (Beijing, China). The TCMPs and their compositions, manufacturer, batch number and sample weight for analysis are listed in Table 1.

2.2. Apparatus and chromatographic conditions

All analyses were performed on an HP1100 liquid chromatograph or an Agilent 1100 liquid chromatograph (Hewlett Packard, Palo Alto, CA, USA) which consisted of a quaternary pump, an on-line degasser, a column thermostat, a model 0497 injection valve (sample loop 20 μL) and a photodiode-array detector or a multi-wavelength detector. The chromatographic data were recorded and processed with an HP chemstation software. The analytical column was a Diamonsil C_{18} (150 mm × 4.6 mm i.d., 5 μm) column. The column temperature was not controlled.

The mobile phase was a mixture of methanol, tetrahydrofuran, water and glacial acetic acid (20:35:44:1, v/v/v/v) employing isocratic elution at the flow rate of 1.0 mL/min. The column effluents were monitored at 254 nm. Injection volume was 10 μL.

An SB 3200 ultrasonic generator (50 KHz, 120 W) from the Shanghai Branson Ultrasonics Co. Ltd (Shanghai, China) was used to extract tanshinones from the samples. An Avanti J-25 high performance refrigerated centrifuge (Beckman, Fullerton, CA, USA) was employed to centrifuge the sample solutions. A versatile plant pulverizer (Tianjin, China) was used to make the medicines into powder.

2.3. Preparation of standard solutions

Standard stock solutions of cryptotanshinone and tanshinone IIA were directly prepared in methanol. Tanshinone I was firstly dissolved in a small volume of chloroform and then diluted to the mark with methanol. The standard stock solutions were stored at 4 °C. Working standard solutions containing each of the three compounds were prepared by diluting the stock solutions with methanol to proper volumes. The standard stock solutions and working solutions were all prepared in dark brown calibrated flasks.

Table 1
Traditional Chinese medicinal preparations (TCMPs) containing *Radix Salvia miltiorrhiza* (Danshen) analyzed in this paper

Sample no.	Name of medicine	Composition	Manufacturer	Batch no.	Sample weight (g)
I	Fufang Danshen tablet	Salvia Miltiorrhiza (450 g), Radix Notoginseng (141 g), Borneol (8 g).	Daheng beisheng Pharmaceutical Co., Ltd., Beijing, China	010508 030110	0.25
II	Huoxue Tong mai tablet	Salvia Miltiorrhiza, Flos Carthami, Radix Curcumae, Radix Notoginseng, Radix Ginseng, Fructus Lycii	Beijing Tongrentang Pharmaceutical Manufacturer, Beijing, China	1120009	0.50
III	Danqi tablet	Salvia Miltiorrhiza, Radix Notoginseng, Borneol	Guoyitang Pharmaceutical Co., Ltd, Guangdong, China	20021101	2.00
IV	Zhengxintai capsule	Herba Visci, Salvia Miltiorrhiza, Radix Puerariae, Radix Astragali, Rhizoma Chuanxiong, Fructus Crataegi	Qikai Pharmaceutical Co., Ltd., Guizhou, China	20020101	1.00
V	Compound Dan shen drippling pills	Salvia Miltiorrhiza, Radix Notoginseng, Borneol	Tasly Pharmaceutical Co., Ltd, Tianjin, China	20020806 20021114	2.00

2.4. Preparation of sample solutions

The sugar coats of 20 tablets of Fufang Danshen tablet or Danqi tablet were washed off with water, then the tablets were dried and the average tablet weight determined. Then Fufang Danshen tablet, Huoxue Tongmai tablet and Danqi tablet were all pulverized into fine powder in plant-pulverizer. Compound Danshen drippling pills were ground into pieces. Pulverized medicinal preparations of suitable amounts (as shown in Table 1) were quantitatively transferred into 50 mL centrifuge tubes and extracted with 10 mL methanol in ultrasonic bath at room temperature for 30 min, then centrifuged at a speed of $2856 \times g$ for 10 min. The extraction procedure was repeated for one time. The extracts were combined and diluted to 25 mL with methanol, an aliquot of the solution was filtered through a 0.45 μm nylon syringe filter. 10 μL of the filtrate was injected into the HPLC system for analysis.

3. Results and discussion

3.1. Selection of the mobile phase

In Chinese Pharmacopeia, the quality of Danshen and Fufang Danshen tablet was controlled by the content of tanshinone IIA and the mobile-phase was methanol–water = 73:27 or 75:25 (v/v). In this paper, the pharmacopoeial mobile phase was firstly used for the separation of the three tanshinones, whereas, it was found that the retention time was rather long (the retention time of tanshinone IIA was 31 min) and cryptotanshinone and tanshinone I could not be baseline resolved. Pan et al. [12], employed methanol, tetrahydrofuran, water and glacial acetic acid as mobile phase for the separation of the tanshinones in *Radix salvia miltiorrhiza*. But when using Pan's mobile phase ratio [12] for the separation of the TCMPs, cryptotanshinone couldn't be separated with the complex matrices eluted before it. As a consequence, in the preliminary studies, the optimum chromatographic conditions were investigated by varying the content of methanol and tetrahydrofuran in the mobile phase while keeping the concentration of water and acetic acid constant, it was observed that the relative ratio of methanol to tetrahydrofuran in the mobile phase exerted a striking influence on the k' values of the tanshinones (as shown in Fig. 2.). As a result, the optimum mobile phase was considered to be methanol:tetrahydrofuran:water:HAc = 20:35:44:1(v/v/v/v) with respect to better resolution and shorter retention time. Under these experimental conditions investigated, the retention times for cryptotanshinone, tanshinone I and tanshinone IIA were 7.61, 9.50 and 16.54 min, respectively.

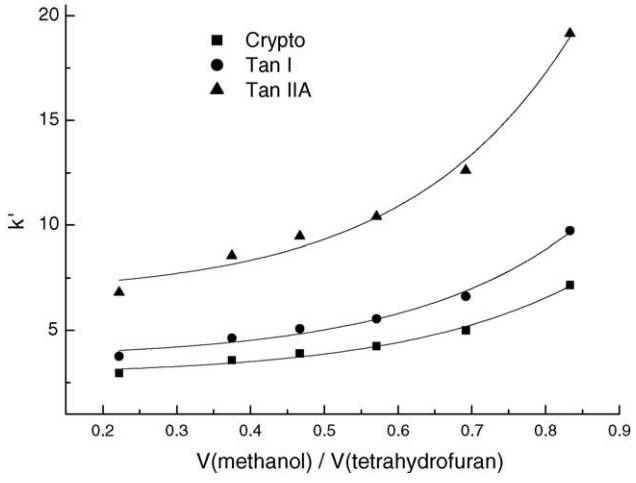

Fig. 2. Effect of volume ratio of methanol to tetrahydrofuran in the mobile phase on the retention of the tanshinones.

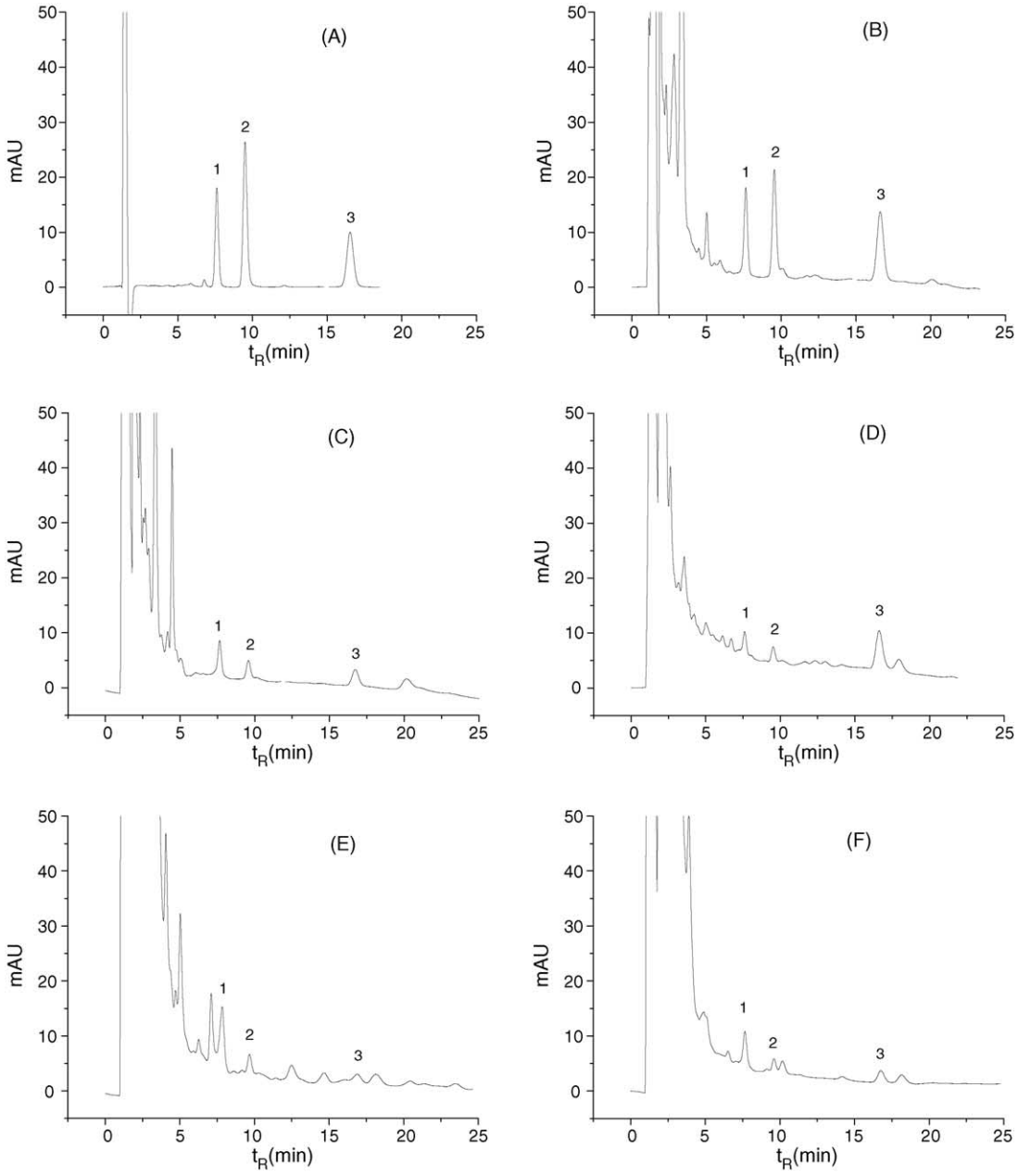

Fig. 3. HPLC chromatograms of the authentic standards and the five TCMPs containing *Radix salvia miltiorrhiza* (Danshen). (A) Authentic standards, (B) Fufang Danshen tablet, (C) Huoxue Tongmai tablet, (D) Danqi tablet, (E) Zhengxintai capsule, (F) compound Danshen drippling pill HPLC conditions, column: Diamonsil C_{18} column (150 mm × 4.6 mm i.d., 5 μm). Mobile phase: a mixture of methanol, tetrahydrofuran, water and glacial acetic acid (20:35:44:1, v/v/v/v), employing isocratic elution at a flow rate of 1.0 mL/min. Chromatograms were recorded at 254 nm.

3.2. Selection of the detection wavelength and identification of the compounds

The on-line UV spectra of the tanshinones were obtained on DAD detector. The maximum absorbance of cryptotanshinone, tanshinone I and tanshinone IIA were 262, 245 and 272 nm, respectively. As each of the three tanshinones has moderate absorbance at 254, 254 nm was selected as the detection wavelength for the simultaneous determination of the three compounds. Identification of the tanshinones in complex TCMP samples was performed by characterizing the sample peak in terms of retention time and UV spectrum. The excellent agreement between standard and sample spectra found in all analyzed samples indicates that, under the proposed analytical conditions, the determination of the tanshinones is not subjected to interferences from other components in the matrix. Typical chromatograms of the standards and the five TCMP samples are depicted in Fig. 3.

Table 2
HPLC data for the calibration graphs and limit of detection

Compounds	Linear Regression	Linear Range (μg/mL)	r	LOD (μg/mL)
Cryptotanshinone	$Y = 0.10 + 36.38X$	0.39–31.55	0.9998	0.03
Tanshinone I	$Y = 8.15 + 60.09X$	0.38–30.72	0.9999	0.02
Tanshinone IIA	$Y = 3.02 + 43.70X$	0.39–31.55	1.0000	0.03

X denotes concentration (μg/mL) of the tanshinones, Y denotes peak area $n = 7$.

Table 3
Repeatabilities of retention time and peak area of the tanshinones ($n = 6$)

Compounds	Retention time		Peak area	
	S.D. (min)	R.S.D. (%)	S.D.	R.S.D. (%)
Cryptotanshinone	0.013	0.17	2.16	0.70
Tanshinone I	0.019	0.20	5.10	1.00
Tanshinone IIA	0.042	0.25	0.74	0.23

3.3. Calibration graphs and the limit of detection

All calibration graphs were plotted based on linear regression analysis of the integrated peak areas (Y) versus concentrations (μg/mL, X) of the tanshinones in the standard solution at seven different concentrations (each concentration injected three times). The regression equations, correlation coefficients, and linear ranges for the analysis of the tanshinones are shown in Table 2.

The limit of detection value (LOD) was calculated as the amount of the injected sample which gave a signal-to-noise ratio of 3. The LOD values of the method for the three components are also listed in Table 2.

3.4. Repeatability test

The results of repeatability of the proposed method, on the basis of retention time and integrated peak area of the standard solutions are listed in Table 3. The results indicated that there is little variability in the instrumental response and thus showed very good repeatability.

3.5. Reproducibility

The within-day precision of the method for each tanshinone was evaluated using the extract of Huoxue Tongmai tablet by 11 same-day replicate assays. The relative standard deviations (R.S.D.) were 0.94% for cryptotanshinone, 1.26% for tanshinone I, and 1.76% for tanshinone IIA, respectively.

3.6. Recovery test

In order to check the accuracy of the developed method, recovery experiments were carried out by adding authentic standards to the samples and then extracted as the above procedure. The results are reported in Table 4. It can be seen that the recoveries lied between 96.2% and 102.5%, indicating that the proposed method has an adequate degree of accu-

Table 4
Analytical results of recoveries

Medicine	Sample weight (g)	Compound	Added (μg)	Found (μg)	Recovery (%)
Fufangdanshen tablet	0.25	Crypto	98.60	101.08	102.5
		Tan I	96.00	96.24	100.2
		Tan IIA	98.60	100.48	101.9
Huoxuetongmai tablet	0.50	Crypto	98.60	95.97	97.3
		Tan I	96.00	97.13	101.2
		Tan IIA	98.60	97.33	98.7
Danqi tablet	1.00	Crypto	39.44	40.07	101.6
		Tan I	38.40	37.83	98.5
		Tan IIA	39.44	40.20	101.9
Zhengxintai capsule	1.00	Crypto	39.44	38.03	96.4
		Tan I	38.40	37.35	97.3
		Tan IIA	39.44	38.59	97.8
Compound Danshen drippling pill	1.00	Crypto	39.44	38.69	98.1
		Tan I	38.40	36.94	96.2
		Tan IIA	39.44	37.96	96.2

Crypto denotes Cryptotanshinone, Tan I denotes Tanshinone I and Tan IIA denotes Tanshinone IIA.

Table 5
The measurement results of tanshinones in the TCMPs containing *Radix salvia miltiorrhiza* (Danshen) ($n = 3$)

Medicine	Batch no.	Content (μg/g)			Content (μg/tablet, pill, capsule)		
		Crypto	Tan I	Tan IIA	Crypto	Tan I	Tan IIA
I	010508	744.08 ± 17.32	583.01 ± 9.86	886.98 ± 19.86	169.28 ± 3.94	132.63 ± 2.24	201.79 ± 4.52
	030110	1170.93 ± 10.25	626.97 ± 8.69	964.71 ± 22.17	303.45 ± 2.66	162.48 ± 2.25	250.00 ± 5.74
II	1120009	168.27 ± 1.84	50.62 ± 0.64	129.11 ± 1.41	103.22 ± 1.13	31.05 ± 0.39	79.20 ± 0.86
III	20021101	25.18 ± 0.86	9.31 ± 0.39	66.27 ± 1.65	8.27 ± 0.28	3.06 ± 0.13	21.77 ± 0.54
IV	20020101	160.10 ± 1.41	29.32 ± 0.35	53.61 ± 0.69	73.65 ± 0.65	13.49 ± 0.16	24.66 ± 0.32
V	20020806	38.00 ± 1.31	11.32 ± 0.66	22.73 ± 0.64	0.95 ± 0.03	0.28 ± 0.02	0.57 ± 0.02
	20021114	41.09 ± 1.96	14.78 ± 0.28	18.99 ± 0.93	1.03 ± 0.05	0.37 ± 0.01	0.47 ± 0.02

racy for the determination of the three tanshinones in the five TCMPs containing Danshen.

3.7. Robustness

Robustness of the method was studied and showed that chromatographic patterns did not significantly change when different solvent sources were used in conjunction with a different HPLC system. Stability studies of the sample solutions found them to be stable for at least 24 h when stored at 4 °C.

3.8. Sample analysis

The established method has been applied to the determination of the three tanshinones in five TCMPs containing Danshen. The contents of tanshinones in Huoxue Tongmai tablets, Danqi tablets, compound Danshen drippling pills and Zhengxintai capsules were reported for the first time. The contents ($n = 3$) of the tanshinones are listed in Table 5. It can be seen that the contents of tanshinone I and tanshinone IIA in both batches of Fufang Danshen tablet are almost the same, and the contents of tanshinone IIA can meet the demand of Chinese Pharmacopoeia (>0.2 mg/tablet). While the content of cryptotanshinone has large difference in the two batches, one of the possible reasons is that the crude drug Danshen used in the two batches are not from the same source, the other reason is that cryptotanshinone in the tablets manufactured in 2001 has decomposed gradually during the long-term storage. The determination results further reveal the importance of quality control of the TCMPs.

4. Conclusions

Compared with the method recommended by Chinese Pharmacopoiea and other methods appeared in journals, the proposed method has some distinguished progresses especially in its giving a good resolution between cryptotanshinone and tanshinone I, and offering a relatively short retention time which is a prerequisite in routine analysis of pharmaceutical preparations. The validated HPLC method has the advantages of simplicity, precision, rapidity and reliability, allowing its quality control of manufacturing process of TCMPs containing Danshen.

Acknowledgement

The authors thank the Modern Research Center for Traditional Chinese Medicine of Peking University for financial support.

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