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ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 45 (2007) 775–784
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

Improved quality control method for Fufang Danshen preparations through simultaneous determination of phenolic acids, saponins and diterpenoid quinones by HPLC coupled with diode array and evaporative light scattering detectors

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Received 8 March 2007; received in revised form 7 July 2007; accepted 11 July 2007
Available online 20 July 2007

Abstract

A herb-combined prescription, mainly derived from roots of *Salvia miltiorrhiza* and *Panax notoginseng*, has been widely used for improving coronary or cerebral circulation in China as well as in Western countries. Multiple commercially available preparations, known as Fufang Danshen preparations (FDPs), produced by various manufacturers with the raw materials from different sources, pose a serious challenge to the quality control of this herb medicine. Previous pharmacological studies identify three types of bioactive components correlated with the clinical effect of those herb preparations. Those mainly include four phenolic acids, four saponins and four diterpenoid quinones. In this report, by using high performance liquid chromatography (HPLC) coupled with diode array and evaporative light scattering detectors (DAD–ELSD), we developed an improved quality control method for those herb medicines. A simultaneous separation and quantification of the 12 components was performed on a C_{18} column, in which the mobile phase consisted of (A) 0.1% aqueous formic acid and (B) acetonitrile using a gradient elution. The optimum detection wavelength was set at 281 nm, the drift tube temperature of ELSD was set at 113 °C, the nitrogen flow rate at 3.1 L/min, and the gain = 4. All calibration curves showed good linear regression ($r^2 > 0.9927$) within test ranges. The method developed showed good precision and accuracy with overall intra- and inter-day variations of 0.64–4.79% and 0.69–4.96%, respectively, and the overall recoveries of 93.50–107.69% for the 12 compounds analyzed. This method was successfully applied to quantify the twelve components in ten commercial samples from three formulas by seven independent manufacturers. This readily available, low-cost and reliable HPLC–DAD–ELSD method improved the quality control of traditional Chinese medicinal preparations consisting of complex compounds with different structures such as FDPs.

Keywords: Fufang Danshen preparations; HPLC–DAD–ELSD; *Salvia miltiorrhiza*; *Panax notoginseng*; Quality control

1. Introduction

The herb-combined prescription, mainly derived from the roots of *Salvia miltiorrhiza* (Danshen in Chinese) and *Panax notoginseng* (Sanqi in Chinese), have been widely used in China for at least 30 years, to a lesser extent, in Japan, the United States, and many European countries for improving coronary and cerebral circulation. Various preparations of this combined prescription, known as Fufang Danshen preparations (FDPs), are commercially available, and among them, Fufang Danshen tablet (FDT), Compound Danshen dripping pill (CDDP), Danqi tablet (DT) are the three most commonly used formulas. Pharmacological research revealed that these preparations carry many biological activities, such as activating blood circulation, dilating coronary artery and antagonizing myocardial ischemia, and their therapeutic effects have been exhibited for treating coronary heart disease, cardiac angina and atherosclerosis in clinic [1–3]. To ensure the clinical efficacy and safety of these products, quality control of FDPs is of significant importance.

Generally, therapeutic effects of Chinese herb-combined prescription are integrative results of multiple bioactive components. Chemical analysis and pharmacological studies on

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doi:10.1016/j.jpba.2007.07.013

Danshen, Sanqi and FDPs have further linked phenolic acids, diterpenoid quinones and saponins to their pharmacological activities and therapeutic efficacy [4–10]. Phenolic acids and diterpenoid quinones from Danshen have been shown to inhibit platelet aggregation, antagonize thrombosis, scavenge oxygen free radicals, protect cardiac microvascular endothelial cells, dilate coronary arteries and increase coronary flow, modulate mutagenic activity and protect the myocardium against ischemia [4–8]. Saponins from Sanqi, meanwhile, can antagonize thrombosis, dilate blood vessel and protect cardiac microvessels [9,10]. Currently, multiple products of FDPs are made by independent manufacturers using raw herbs from different areas. An accurate measurement of bioactive components, e.g. phenolic acids, diterpenoid quinones and saponins in these products becomes essential for quality control of their therapeutic efficacy.

Up to now, many analytical methods have been established for the quality control of the crude drug of Danshen or Sanqi, or their combined prescriptions. For instance, the prevailing HPLC–UV or DAD [11–24], coulometric electrode array (CEA) [25], nonaqueous capillary electrophoresis [26], capillary electrochromatography [27], and LC–MS [28] methods have been used to determine phenolic acids, diterpenoid quinones or both of them in Danshen and its herbal preparations. While HPLC–ELSD [29–32], HPLC–UV or DAD [24,33–40], and LC–MS [40,41] methods have been used for quantification of saponins in Sanqi and its herbal preparations. However, except those used in HPLC–UV and HPLC–ELSD methods, the equipments used in other methods are relatively expensive and may be unavailable in every laboratory. In addition, most of the reported methods dealt with the qualitation or quantification of one or two types of components from only one comprising herb (Danshen or Sanqi) except our previously reported method [24]. In Chinese Pharmacopoiea [42], even no bioactive saponins of Sanqi were considered as marker compounds for the quality control of all the FDPs. Though our previous study established the HPLC–DAD method [24] to simultaneously determine seven major components of both Danshen and Sanqi from FDTs for the first time, some improvement was needed to include other major components such as danshensu, rosmarinic acid, ginsenoside Rd, dihydrotanshinone I and tanshinone I for a better quality control of FDT and other FDPs. Specifically, the baseline at 203 nm was unstable due to gradient elution, and the detection of notoginsenoside R_1 at 203 nm was easily interfered by adjacent peaks of two phenolic acids. Considering all these factors discussed above, it is urgent to improve the quality control of FDPs by developing a simple, low-cost and reliable method through simultaneous quantification of these three types of bioactive components.

In recent years, an on-line coupled HPLC–DAD–ELSD method has attracted ever-increasing attention and has been successfully applied to simultaneous quantification of multi-components in traditional Chinese herbal medicines and their preparations [43–45]. This method is potentially ideal for routine analysis and quality evaluation of FDPs. UV and ELSD signals contain complementary information for each other. The sensitive UV detection reveals strongly UV absorbing compounds such as phenolic acids and diterpenoid quinones in FDPs, while the versatile ELSD reveals none or poorly UV absorbing compounds such as saponins in providing a stable and flat baseline even with gradient elution. The major advantage is the simultaneous measurement of active compounds with important savings on cost and time.

In this paper, using on-line coupled HPLC–DAD–ELSD, an improved quality control method for three formulas of FDPs, i.e. FDT, CDDP and DT, was developed through simultaneous determination of 12 bioactive components, including four major phenolic acids, namely danshensu (**1**), protocatechuic aldehyde (**2**), rosmarinic acid (**3**), and salvianolic acid B (**4**), respectively; four major saponins, namely notoginsenoside R_1 (**5**), ginsenoside Rg_1 (**6**), ginsenoside Rb_1 (**7**), and ginsenoside Rd (**8**), respectively; and four major diterpenoid quinones, namely dihydrotanshinone I (**9**), cryptotanshinone (**10**), tanshinone I (**11**), and tanshinone IIA (**12**). The structures of these 12 compounds were shown in Fig. 1.

2. Experimental

2.1. Samples, chemicals and reagents

DT (AB: batch no. 040803) was purchased from Ji-An-Tang Pharmaceutical store (Beijing, PR China), FDT (BYS: batch no. 03121024; TRT: batch no. 040602; HQYT: batch no. 050308; HS: batch no. 060203; LYS: batch no. 060459) and CDDP (batch nos. 20030604; 20040303; 20051205; 20060216) were purchased from Baixin, Jianjun, Xiansheng and Yi-Shan-Tang Pharmaceutical stores (Nanjing, Jiangshu, PR China). Reference compounds danshensu, protocatechuic aldehyde, notoginsenoside R_1, ginsenoside Rg_1, salvianolic acid B, ginsenoside Rb_1, cryptotanshinone and tanshinone IIA were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, PR China); tanshinone I, dihydrotanshinone I and ginsenoside Rd were purchased from Sikehua biotechnology Co. Ltd. (Chengdu, PR China); rosmarinic acid was purchased from Kangjiu chemical engineering Co. Ltd. (Shanghai, PR China). The purity of each reference compound was determined to be above 98% by HPLC analysis. HPLC grade acetonitrile was purchased from Merck (Darmstadt, Germany); deionized water was purified by Milli-Q system (Millipore, Bedford, MA, USA); formic acid was purchased from the first chemical company of Nanjing (Nanjing, PR China); analytical grade methanol was purchased from Hanbang Science & Technology (Nanjing, PR China).

2.2. Apparatus

An Agilent 1100 liquid chromatograph system (Agilent Technologies, Palo Alto, CA, USA) was used, consisting of a dual pump, an auto-sampler, a DAD coupled with an ELSD (Alltech Associates, Deerfield, USA), and an HP ChemStation software (Agilent Technologies, USA). The column configuration consisted of an Agilent Zorbax Extend reversed-phase C_{18} column (5 μm, 250 mm × 4.6 mm) and an Agilent Zorbax C_{18} guard column (5 μm, 4.6 mm × 12.5 mm).

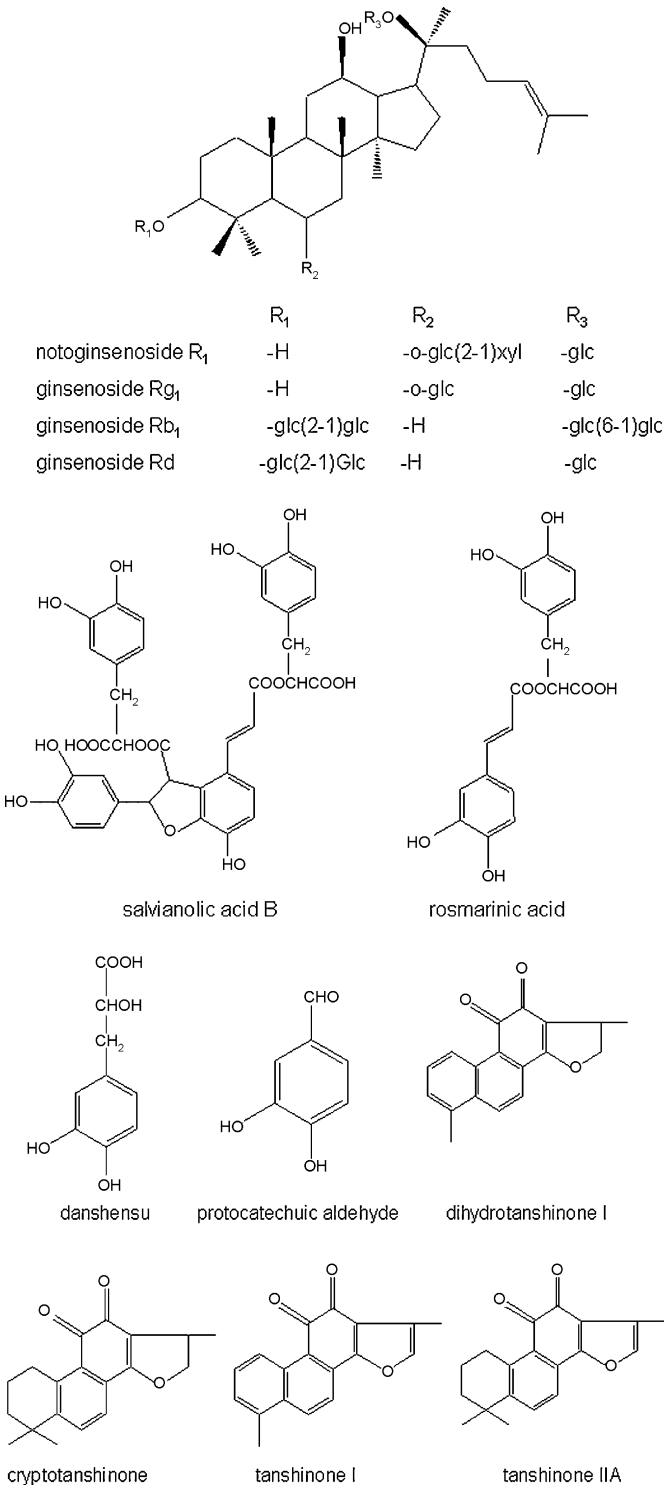

Fig. 1. Chemical structures of the twelve active components determined.

2.3. Chromatographic conditions

The mobile phase was composed of (A) aqueous formic acid (0.1%, v/v) and (B) acetonitrile using a gradient elution of 5–14% B at 0–10 min, 14–17% B at 10–12 min, 17–19% B at 12–16 min, 19–21% B at 16–30 min, 21–23% B at 30–40 min, 23–30% B at 40–50 min, 30–55% B at 50–65 min, 55–75% B at 65–80 min, and 75–90% B at 80–90 min, and the re-equilibration time of gradient elution was 15 min. The flow rate was 1.0 mL/min, the column temperature was maintained at 30 °C, the optimum wavelength was set at 281 nm, the drift tube temperature for ELSD was set at 113 °C, and the nitrogen flow rate was 3.1 L/min.

2.4. Preparation of sample solutions

Twenty tablets of FDTs or DTs were scraped off the sugar coats, and then were pulverized into fine powder in mortar after the average tablet weight was determined; CDDPs were ground into pieces. The powder of FDTs or DTs (0.5 g) and CDDPs (80 pills) were respectively transferred into dark brown calibrated flasks and extracted with 25 mL, 70% methanol in ultrasonic bath for 30 min, 70% methanol was added to compensate the lost volume. The solution was filtered through a 0.45 μm membrane filter before injecting 10 μL to HPLC analysis.

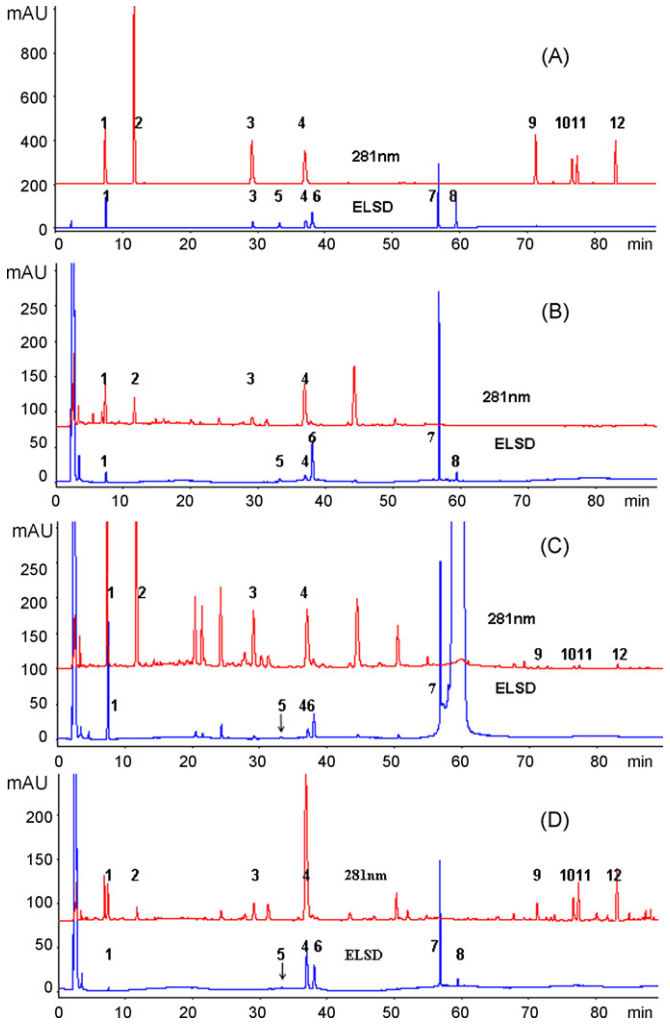

Fig. 2. Typical HPLC–DAD–ELSD chromatograms of the standard solution and various commercial FDPs: (A) Standard solution; (B) Danqi tablet; (C) Compound Danshen dripping pill;(D) Fufang Danshen tablet. Peak (**1**) refers to danshensu; (**2**) protocatechuic aldehyde; (**3**) rosmarinic acid; (**4**) salvianolic acid B; (**5**) notoginsenoside R_1; (**6**) ginsenoside Rg_1; (**7**) ginsenoside Rb_1; (**8**) ginsenoside Rd; (**9**) dihydrotanshinone I; (**10**) cryptotanshinone; (**11**) tanshinone I; (**12**) tanshinone IIA.

Table 1
Regression data, LODs and LOQs for the twelve analytes of the assay

Analyte	Detection styles	Regression equation[a]	r^2	Linear range (μg/mL)	LOD[b] (μg/mL)	LOQ[c] (μg/mL)
1	281 nm	$Y = 6.0986X - 11.4889$	1.0000	3.55–1704.00	0.07	0.22
2	281 nm	$Y = 42.8527X - 25.3908$	1.0000	1.78–852.00	0.04	0.13
3	281 nm	$Y = 16.3999X - 36.3970$	1.0000	1.92–920.00	0.05	0.14
4	281 nm	$Y = 8.6665X - 123.1673$	0.9997	3.43–1644.00	1.14	2.29
5	ELSD	$y = 1.4136x - 0.6724$	0.9927	12.85–1028.00	1.29	1.93
6	ELSD	$y = 1.5791x - 0.9923$	0.9957	20.15–1612.00	1.01	2.02
7	ELSD	$y = 2.0050x - 1.6001$	0.9947	10.93–1312.00	1.09	2.73
8	ELSD	$y = 1.7135x - 1.0510$	0.9993	7.60–912.00	0.76	1.90
9	281 nm	$Y = 42.0474X - 14.7740$	1.0000	0.53–254.00	0.10	0.20
10	281 nm	$Y = 19.5678X - 8.7836$	0.9999	0.62–298.00	0.06	0.15
11	281 nm	$Y = 20.5451X - 42.4421$	0.9997	0.67–320.00	0.07	0.14
12	281 nm	$Y = 31.3505X - 9.2955$	0.9998	0.64–308.00	0.06	0.15

[a] Y is the peak area in UV chromatograms monitored at 281 nm, X the compound concentration injected, and y, x are the logarithmic values of area and concentration in ELSD chromatograms.
[b] Limits of detection.
[c] Limits of quantification.

2.5. Validation procedure

2.5.1. Calibration curves, limits of detection and quantification

Standard stock solutions of four phenolic acids, four saponins and four diterpenoid quinones were respectively prepared in 30, 50 and 100% methanol, and 0.5, 0.5 and 1 mL of each kind of standard stock solution was transferred to a 2 mL volumetric flask to make 70% methanol solution of the 12 reference compounds, the concentration of each compound being 1.704 mg/mL (**1**), 0.852 mg/mL (**2**), 0.920 mg/mL (**3**), 1.644 mg/mL (**4**), 1.028 mg/mL (**5**), 1.612 mg/mL (**6**), 1.312 mg/mL (**7**), 0.912 mg/mL (**8**), 0.254 mg/mL (**9**), 0.298 mg/mL (**10**), 0.320 mg/mL (**11**) and 0.308 mg/mL (**12**), respectively. The stock solution was further diluted with 70% methanol to make 12 different concentration ranges including 1, 1/2, 1/4, 1/8, 1/12, 1/16, 1/20, 1/40, 1/80, 1/120, 1/240 and 1/480 of the original concentration. The solutions were filtered through a 0.45 μm membrane filter and an aliquot of 10 μL was injected into HPLC for analysis. The calibration curve was performed with at least nine appropriate concentrations in triplicate. For compounds **1**–**4** and **9**–**12** all the 12 concentration ranges of the original concentration were appropriate for the calibration curves; for compounds **5** and **6**: 1, 1/2, 1/4, 1/8, 1/12, 1/16, 1/20, 1/40 and 1/80 of the original concentration; and for compounds **7** and **8**: 1, 1/2, 1/4, 1/8, 1/12, 1/16, 1/20, 1/40, 1/80 and 1/120 of the original concentration. For the four phenolic acids and four diterpenoid quinones by DAD, their regression equations were calculated in the form of $Y = AX + B$, where Y and X was peak area and compound concentration, while for the four saponins by ELSD detection, their regression equations could be described as $Y = aX^b$, so the calibration curves should be obtained in double logarithmic coordinates.

The dilute solution of the 12 reference compounds was further diluted to a series of concentrations with 70% methanol for the gain of the limits of detection (LOD) and quantification (LOQ). The LOD and LOQ under the present chromatographic conditions were determined at a signal-to-noise (S/N) ratio of 3 and 10, respectively.

2.5.2. Precision, repeatability and stability

Intra- and inter-day variations were utilized to determine precision of the developed assay by analyzing standard solutions with three concentrations. The intra-day variation was determined by analyzing the six replicates on the same day and inter-day variation was determined in 3 consecutive days. The R.S.D. was taken as a measure of precision.

To confirm the repeatability, five different working solutions prepared from each kind of FDP sample (DT: AB batch no. 040803; FDT: HQYT, batch no. 050308; CDDPs: batch no. 20051205) were analysed. The R.S.D. and relative error (RE) were taken as the measures of precision and accuracy of repeatability test. Stability was tested with a working solution prepared from each sample above at room temperature and analyzed at 0, 2, 4, 8, 12, 24, 48, and 72 h within 3 days. And the RE of the mean determined concentration compared to the nominal concentration at each time point was taken as the measure of stability.

2.5.3. Accuracy

Accuracy was determined by the recovery test. However, it is very difficult to get a good estimation of the true recovery due to lack of blank matrix, the recovery of the spiked solution added to the sample was determined here. Appropriate amounts of powder samples of DT (AB: batch no. 040803, 0.25 g), FDT (HQYT: batch no. 050308, 0.25 g) and CDDPs (batch no. 20051205; 40 pills) were respectively weighted and spiked with known amount of each reference compound by adding standard stock solutions of four phenolic acids, four saponins and four diterpenoid quinones, and then analyzed as described in Section 2.6. Each sample was analyzed in triplicate. The total amount of each analyte was calculated from the corresponding calibration curve, and the recovery of each analyte was calculated by the

following equation:

$$\text{recovery}\,(\%) = \frac{\text{amount}_{\text{determined}} - \text{amount}_{\text{original}}}{\text{amount}_{\text{spiked}}} \times 100$$

where $\text{amount}_{\text{determined}}$ is the determined total amount of each analyte, $\text{amount}_{\text{original}}$ the original amount of each analyte in DT, FDT and CDDPs measured in Section 2.6, and $\text{amount}_{\text{spiked}}$ is the spiked amount of each analyte.

Table 2
Intra- and inter-day variations of the assay

Analyte/concentration spiked (μg/mL)	Intra-day ($n = 6$)		Inter-day ($n = 3$)	
	Detected (μg/mL)	R.S.D. (%)	Detected (μg/mL)	R.S.D. (%)
1				
852.00	868.31 ± 13.13	1.51	861.29 ± 14.05	1.63
85.20	84.50 ± 1.55	1.83	85.40 ± 1.44	1.69
14.20	14.42 ± 0.18	1.28	14.30 ± 0.19	1.32
2				
426.00	433.71 ± 7.76	1.79	429.38 ± 8.21	1.91
42.60	41.11 ± 0.45	1.10	41.04 ± 0.56	1.35
7.10	7.15 ± 0.08	1.12	7.13 ± 0.08	1.09
3				
460.00	466.58 ± 10.71	2.30	463.99 ± 9.16	1.97
46.00	43.89 ± 0.40	0.91	43.62 ± 0.56	1.28
7.67	7.62 ± 0.20	2.69	7.72 ± 0.11	1.37
4				
822.00	831.35 ± 11.79	1.42	827.70 ± 16.97	2.05
82.20	81.48 ± 1.15	1.41	81.65 ± 0.95	1.16
13.70	13.36 ± 0.42	3.11	13.49 ± 0.42	3.12
5				
514.00	498.35 ± 16.83	3.38	506.28 ± 46.74	4.10
128.50	118.06 ± 4.52	3.83	123.82 ± 9.71	4.12
51.40	48.60 ± 2.33	4.79	44.85 ± 4.17	4.84
6				
806.00	802.06 ± 19.12	2.38	805.52 ± 19.76	2.45
201.50	208.16 ± 9.41	4.52	195.73 ± 9.34	4.77
80.60	81.80 ± 3.85	4.71	76.25 ± 3.92	4.94
7				
656.00	664.3 ± 10.65	1.60	658.88 ± 9.52	1.44
164.00	159.37 ± 5.50	3.45	159.02 ± 7.20	4.53
65.60	65.60 ± 3.33	4.39	62.97 ± 2.97	4.72
8				
456.00	458.09 ± 13.37	2.92	455.59 ± 14.53	3.19
114.00	114.16 ± 3.71	3.25	112.50 ± 4.53	4.03
45.60	45.60 ± 1.85	4.06	41.61 ± 2.03	4.88
9				
127.00	126.70 ± 0.81	0.64	126.51 ± 0.87	0.69
12.70	12.04 ± 0.36	3.02	12.30 ± 0.18	1.46
2.12	2.13 ± 0.02	1.01	2.12 ± 0.04	1.89
10				
149.00	153.03 ± 3.09	2.02	152.28 ± 2.77	1.82
14.90	14.89 ± 0.12	0.78	14.93 ± 0.22	1.42
2.48	2.42 ± 0.07	2.99	2.44 ± 0.08	3.46
11				
160.00	150.64 ± 6.52	4.33	151.97 ± 7.53	4.96
16.00	16.54 ± 0.54	3.24	16.38 ± 0.65	3.96
2.67	2.63 ± 0.03	1.33	2.65 ± 0.04	1.57
12				
154.00	161.78 ± 2.77	1.71	160.10 ± 2.97	1.86
15.40	15.27 ± 0.13	0.86	15.20 ± 0.15	1.01
2.57	2.61 ± 0.06	2.42	2.59 ± 0.05	1.76

2.6. Quantification of 12 compounds in FDPs

Ten commercial samples of the three formulas of FDPs (FDT, DT and CDDP) were prepared as described in Section 2.4. An aliquot (10 μL) of the filtrate was directly subject to HPLC–DAD–ELSD analysis. Each sample was determined in triplicate. The content of each analyte was calculated from the corresponding calibration curve.

3. Results and discussion

3.1. Extraction method development

In order to achieve quantitative extraction, variables involved in the procedure such as solvent, extraction method and extraction time were optimized. Pure and aqueous methanol or

Table 3
Repeatability of the assay ($n = 5$)

Analyte	R.S.D. (%)			RE (%)		
	DT	CDDPs	FDT	DT	CDDPs	FDT
1	4.18	3.38	1.44	−0.84	−0.56	1.32
2	4.56	2.68	3.60	1.85	0.28	1.79
3	3.35	1.86	1.53	2.45	1.48	−1.50
4	0.12	4.12	1.06	0.61	−0.57	0.30
5	4.08	3.58	2.07	1.17	0.30	1.22
6	3.17	3.16	0.56	0.20	−0.97	−0.67
7	0.49	1.16	2.11	0.16	−1.02	0.05
8	3.24	nd[a]	3.47	3.77	nd	0.85
9	nd	4.21	1.52	nd	2.43	1.20
10	nd	3.98	1.02	nd	0.59	−1.08
11	nd	2.94	1.13	nd	−0.91	−1.43
12	nd	2.38	1.23	nd	−2.55	−0.86

[a] Not detected.

Table 4
Stability of the assay

Analyte	Nominal (mg/g)	RE (%)						
		2 h	4 h	8 h	12 h	24 h	48 h	72 h
DT								
1	5.98	1.83	0.49	−0.39	4.85	−1.28	−1.85	2.27
2	0.54	0.24	−1.91	−0.69	−0.86	−4.12	−2.62	−3.26
3	0.30	1.33	0.11	−2.02	−0.68	0.91	1.10	−0.72
4	8.26	0.06	−0.20	0.86	2.59	1.85	0.35	0.17
5	2.56	−1.92	4.25	−2.81	−0.90	0.74	−1.54	−1.03
6	15.15	−1.33	0.68	−1.23	−0.43	−0.40	1.72	−0.55
7	12.63	−0.11	0.60	2.61	−0.22	0.45	1.20	0.36
8	2.65	−2.41	−1.02	−0.73	−1.89	3.06	−2.19	−1.31
9	nd[a]	nd	nd	nd	nd	nd	nd	nd
10	nd	nd	nd	nd	nd	nd	nd	nd
11	nd	nd	nd	nd	nd	nd	nd	nd
12	nd	nd	nd	nd	nd	nd	nd	nd
CDDPs								
1	8.90	−2.15	−3.65	4.41	−1.96	2.68	−2.92	3.44
2	2.61	3.02	−2.03	−1.67	3.29	2.59	−2.43	−1.93
3	1.35	1.34	−2.16	0.35	1.81	0.57	−1.56	−2.99
4	3.50	4.29	0.31	−3.72	3.61	4.71	−4.68	−3.59
5	0.69	4.51	−4.32	0.38	−2.82	4.63	1.24	−2.70
6	3.09	3.74	1.92	−4.76	2.72	1.99	−0.07	−3.11
7	3.94	0.98	0.03	−0.25	0.60	−0.08	−0.44	−2.63
8	nd	nd	nd	nd	nd	nd	nd	nd
9	0.02	2.79	−4.84	−4.06	−2.97	3.73	4.04	−4.84
10	0.04	−2.60	4.43	−3.60	−4.27	−2.26	4.77	−3.94
11	0.07	−0.02	−4.21	−3.48	2.08	3.35	−0.38	2.53
12	0.04	−2.73	0.62	−3.67	−4.71	−2.21	−3.56	1.56
FDT								
1	3.78	−0.67	0.25	0.78	−1.36	−2.92	−0.18	−1.14
2	0.24	−1.53	1.25	3.03	3.36	−2.48	0.77	0.15
3	1.33	0.60	−1.57	1.36	0.01	−0.76	−0.11	1.36
4	23.63	−0.20	1.57	2.41	−1.68	0.04	−1.08	−0.24
5	2.45	−0.10	−0.76	3.34	3.21	−3.72	1.89	−0.50
6	10.51	0.65	−1.57	−0.88	0.43	−0.14	0.01	0.82
7	9.59	1.42	−1.37	3.56	1.97	0.82	1.69	0.63
8	2.35	−0.19	2.93	−1.86	−0.01	−1.06	1.21	−3.46
9	0.33	1.90	−0.38	0.94	−0.84	0.12	−0.16	−1.05
10	0.93	2.04	0.20	0.23	−0.56	0.15	0.09	−0.39
11	1.40	−0.68	−0.32	0.45	0.02	1.98	−0.32	−0.15
12	1.16	−0.27	0.25	−0.84	2.17	0.68	1.34	−0.84

[a] Not detected.

ethanol solutions were tried as the extraction solvent, the best solvent was found to be 70% methanol, which gave rise to optimum extraction of all the 12 components with broad range of polarity in high yield. Compared to Soxhlet extraction and refluxing extraction, the ultrasonic treatment procedure was found to be the best extraction method for all the 12 components. In order to investigate extraction time, powdered FDT (HQYT: batch no. 050308, 0.50 g) samples were extracted with 25 mL 70% methanol for 10, 20, 30, 45, and 60 min, respectively. The results suggested that all

Table 5
Accuracy of the assay

Analyte/samples	Original mean (mg)	Spiked mean (mg)	Detected mean (mg)	Recovery[a] mean (%)	R.S.D. (%) ($n = 3$)
1					
DT	1.51	1.07	2.51	93.93	3.82
CDDPs	8.49	5.33	14.17	106.55	2.20
FDT	0.96	1.78	2.69	97.71	2.06
2					
DT	0.14	0.53	0.64	95.51	2.52
CDDPs	2.49	2.66	5.31	106.17	1.71
FDT	0.06	0.89	0.94	99.26	2.63
3					
DT	0.07	0.58	0.69	106.50	4.21
CDDPs	1.29	2.88	4.16	99.96	2.16
FDT	0.34	0.96	1.27	97.56	2.47
4					
DT	2.09	1.03	3.18	106.37	3.45
CDDPs	3.34	5.14	8.15	93.73	1.08
FDT	5.99	1.71	7.83	107.69	2.36
5					
DT	0.65	1.61	2.17	94.69	4.33
CDDPs	0.66	1.61	2.19	95.57	4.84
FDT	0.63	0.86	1.44	94.81	4.64
6					
DT	3.83	2.52	6.19	93.50	3.57
CDDPs	2.94	2.52	5.35	95.74	4.53
FDT	2.69	1.34	4.11	105.27	4.62
7					
DT	3.19	2.05	5.29	102.28	4.43
CDDPs	3.75	2.05	5.85	102.12	4.18
FDT	2.46	0.76	3.19	96.88	4.78
8					
DT	0.67	1.43	2.06	97.21	3.05
CDDPs	nd[b]	1.43	nd	nd	nd
FDT	0.60	0.76	1.40	104.50	3.25
9					
DT	nd	0.01	0.01	105.71	4.11
CDDPs	0.02	0.01	0.03	107.19	4.98
FDT	0.08	0.21	0.29	99.21	3.70
10					
DT	nd	0.02	0.02	106.52	4.47
CDDPs	0.04	0.02	0.05	103.61	4.90
FDT	0.24	0.25	0.48	96.34	4.94
11					
DT	nd	0.02	0.02	106.41	4.21
CDDPs	0.06	0.02	0.08	100.39	4.09
FDT	0.35	0.27	0.54	106.70	4.92
12					
DT	nd	0.02	0.02	103.64	4.35
CDDPs	0.04	0.02	0.05	96.26	4.71
FDT	0.29	0.26	0.55	98.62	3.42

[a] Recovery (%) = ((amount$_{determined}$ − amount$_{original}$)/amount$_{spiked}$) × 100.
[b] Not detected.

Table 6
Each content of twelve active components in various commercial FDPs (mg/g) ($n = 3$)

Samples	Four major phenolic acids from Danshen					Four major saponins from Sanqi					Four major diterpenoid quinones from Danshen				
	1	**2**	**3**	**4**	Total	**5**	**6**	**7**	**8**	Total	**9**	**10**	**11**	**12**	Total
DT															
040803															
Mean	5.98	0.54	0.30	8.26	15.07	2.56	15.15	12.63	2.65	32.98	nd[a]	nd	nd	nd	nd
S.D.	0.310	0.030	0.010	0.010		0.130	0.480	0.060	0.090						
CDDPs															
20030604															
Mean	8.91	1.76	1.25	1.39	13.31	0.71	2.25	3.57	nd	6.53	0.01	0.02	0.04	0.02	0.08
S.D.	0.235	0.079	0.055	0.060		0.031	0.112	0.082			0.001	0.001	0.001	0.001	
20040303															
Mean	10.24	2.27	1.46	2.07	16.03	0.67	3.77	5.00	nd	9.45	0.01	0.03	0.05	0.03	0.12
S.D.	0.503	0.103	0.054	0.040		0.028	0.068	0.037			0.001	0.001	0.001	0.001	
20051205															
Mean	8.90	2.61	1.35	3.50	16.36	0.69	3.09	3.94	nd	7.71	0.02	0.04	0.07	0.04	0.16
S.D.	0.372	0.078	0.024	0.176		0.029	0.102	0.038			0.001	0.002	0.002	0.001	
20060216															
Mean	9.26	2.90	1.29	3.55	17.00	0.71	3.61	4.93	nd	9.26	0.02	0.04	0.07	0.04	0.17
S.D.	0.424	0.114	0.047	0.138		0.031	0.153	0.178			0.001	0.002	0.003	0.002	
FDTs															
BYS															
Mean	3.78	0.22	1.21	16.68	21.90	1.24	5.37	5.30	1.34	13.25	0.38	1.00	0.98	0.85	3.20
S.D.	0.135	0.001	0.059	0.818		0.050	0.229	0.222	0.050		0.013	0.041	0.043	0.038	
TRT															
Mean	8.60	0.45	1.84	26.95	37.85	2.43	13.96	28.20	3.38	47.97	0.50	1.28	2.03	2.11	5.91
S.D.	0.414	0.012	0.088	1.034		0.111	0.191	0.578	0.127		0.022	0.055	0.065	0.065	
HQYT															
Mean	3.78	0.24	1.33	23.63	28.98	2.45	10.51	9.59	2.35	24.89	0.33	0.93	1.40	1.16	3.82
S.D.	0.057	0.008	0.023	0.256		0.053	0.064	0.203	0.091		0.006	0.011	0.017	0.014	
HS															
Mean	2.29	0.11	2.34	51.28	56.02	1.15	3.72	3.81	1.12	9.80	0.57	1.66	1.97	2.15	6.36
S.D.	0.070	0.005	0.106	2.049		0.049	0.183	0.081	0.039		0.027	0.070	0.078	0.105	
LYS															
Mean	2.51	0.18	1.93	34.74	39.36	1.29	6.26	7.43	2.37	17.35	0.44	2.13	1.63	2.54	6.73
S.D.	0.083	0.008	0.064	1.695		0.051	0.312	0.208	0.087		0.021	0.053	0.077	0.120	

[a] Not detected.

the 12 components were almost completely extracted within 30 min.

3.2. Optimization of chromatographic conditions

In the present study, 12 major components belonging to three types from Danshen (phenolic acids and diterpenoid quinones) and Sanqi (saponins) were chosen as chemical markers to simultaneously evaluate the quality of both Danshen and Sanqi in FDPs. As shown in Fig. 1, the compounds have very broad range of polarity, so gradient elution was carried out to separate these components in FDPs. By optimizing the compositions of mobile phase, 0.1% (v/v) formic acid–acetonitrile was finally utilized as mobile phase, and all 12 compounds could be eluted with baseline separation in 90 min (Fig. 2).

On basis of UV maximal absorption of phenolic acids and diterpenoid quinones, the detection at 281 nm in DAD was chosen, providing an optimum S/N for simultaneously quantitative analysis of both phenolic acids **1**–**4** and diterpenoid quinones **9**–**12**. ELSD connected in series with DAD was utilized for monitoring saponins **5**–**8**, and the operating conditions were optimized according to the data computed with the ELSD software: the nitrogen flow rate was set at 3.1 L/min, the drift tube temperature was determined to be 113 °C, and the gain = 4. At the selected detection mode, all these 12 compounds had acceptable LOD and LOQ (Table 1). The identity of each peak in Fufang Danshen preparation samples was confirmed by comparison of retention time and UV spectrum of each peak with that of reference compound. In addition, spiking samples with the reference compounds further confirmed the identities of the peaks. Representative HPLC–DAD–ELSD chromatograms of the 12 reference compounds and three formulas of FDPs were shown in Fig. 2.

3.3. Method validation

Table 1 listed linear calibration curve with r^2, linear range, LOD and LOQ of each compound determined. As a result, all the marker substances showed good linearity ($r^2 > 0.9927$) in a relatively wide concentration range. The LOD and LOQ for four phenolic acids and four diterpenoid quinones in UV detection ranged from 0.04 to 1.14 μg/mL and from 0.13 to 2.29 μg/mL, and for four saponins in ELSD detection ranged from 0.76 to 1.29 μg/mL and 1.90 to 2.73 μg/mL, respectively. Table 2 showed the results of precision test of the 12 analytes. It indicated that the overall R.S.D. of the intra- and inter-day were 0.64–4.79% and 0.69–4.96%, respectively. Table 3 showed that the overall precision and accuracy of repeatability test were 0.12–4.56% and −2.55–3.77%, respectively. Table 4 showed that the overall stability variations were −4.84 to 4.85%. And then in Table 5, it was found that the overall recoveries of DT, FDT and CDDP were 93.50–106.52%, 94.81–107.69% and 93.73–107.19% for the analytes, respectively. The results of recovery test indicated that the method developed was accurate enough for the determination of the 12 bioactive components in FDPs. Therefore, the HPLC–DAD–ELSD method is precise, accurate and sensitive enough for simultaneous quantitative evaluation of four major phenolic acids, four major diterpenoid quinones and four major saponins in FDPs.

3.4. Sample analysis

The HPLC–DAD–ELSD method developed was successfully applied to simultaneous determination of the twelve compounds in ten commercial samples from three formulas of FDPs by seven independent manufacturers, i.e. DT, FDT and CDDP. The contents ($n = 3$) of the 12 marker compounds analyzed were listed in Table 6. The results showed that the content of each compound or the total content of certain type of compounds in different FDPs varied markedly: All diterpenoid quinones were hardly detected in DT, with low content in CDDP, but high in FDT samples; among the four phenolic acids, the content of salvianolic acid B in CDDP was much lower than that in DT and FDT samples, while the contents of danshensu and protocatechuic aldehyde in CDDP were much higher than that of DT and FDT samples; as for saponins, ginsenoside Rd in CDDP samples could not be detected owing to the disturbance of adjuvant, and the total content of saponins in DT was higher than that in CDDP and nearly all FDT samples. The variation might result from different proportion of Danshen to Sanqi and different processing procedures for preparation of different FDPs. It could also be seen that the total contents of the three types of compounds varied insignificantly in CDDP samples of 4 consecutive years, but varied obviously in FDT samples of five different manufacturers. The reason might be mainly due to the quality consistency of the crude materials in CDDP samples is better than that in FDT samples in the present study. In addition, compared to phenolic acids **1**–**4** and diterpenoid quinones **9**–**12**, saponins **5**–**8**, another major type of bioactive components in FDPs, were also determined as major ingredients, and the total content of four saponins accounted for 30–70% of that of all 12 compounds analyzed. As a result, it is necessary and rational to improve the quality control of FDPs by simultaneous quantification of the multiple active components from both Danshen and Sanqi.

4. Conclusions

It is evident from a number of reports that hydrophilic phenolic acids, lipophilic diterpenoid quinones and saponins are responsible for the overall biological activities of FDPs, techniques for their simultaneously qualitative and quantitative analysis in commercial products are therefore of great importance. By using on-line coupled HPLC–DAD–ELSD, we have developed an improved quality control method for FDPs through simultaneous quantification of hydrophilic phenolic acids, lipophilic diterpenoid quinones, and saponins. The method was successfully applied to simultaneous determination of twelve major components of four major phenolic acids, four major diterpenoid quinones, and four major saponins in ten commercial samples of three formulas of widely used FDPs. Additionally, this method developed was fully validated with respect to precision, repeatability and accuracy. It is helpful to scientifically control the quality of FDPs.

Acknowledgement

This research was financially supported by the key program (30530870) of National Science Foundation of China.

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