A Uniform HPLC Method Developed for the Analysis of *Salvia miltiorrhiza, Panax notoginseng,* and Fufang Danshen

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Abstract

Fufang Danshen (FFDS) is a famous typical Chinese complex prescription, which is mainly composed of Radix *Salvia miltiorrhiza* Bunge (SM) and Radix *Panax notoginseng* (PN). An HPLC method is developed to analyze SM, PN, and FFDS effectively; the effective analysis is achieved by using a gradient elution procedure with a mobile phase consisting of acetonitrile and 0.025% aqueous phosphoric acid (v/v). Through this method, 33 peaks in FFDS are clearly exhibited, and the components that make up the 33 peaks in FFDS are evaluated. Also, the chemical ingredients are compared between the single herbs (SM and PN) and the complex prescription (FFDS). The result indicate that the chemical ingredients in FFDS are not simply a combination of SM and PN. In addition, the HPLC method is suitable for the routine quality control of SM, PN, and FFDS, which could present a uniform quality control method for single medicines and one of the most commonly used Traditional Chinese Medicine-complex prescriptions.

Introduction

Traditional Chinese medicine (TCM) is becoming increasingly popular in many countries. With this increased usage, the assessment of the safety, quality, and efficacy of these medicines has become an important concern for health professionals and health authorities (1). TCM is a complex mixture, containing usually hundreds of chemically different constituents. A prescription in TCM frequently consists of several Chinese herbs; for example, Danggui-Shaoyao-San is a combination of six Chinese herbs (2,3). A chromatographic fingerprint of a TCM can be a comprehensive quantitatable identification method to confirm authenticity, as well as to evaluate the quality, consistency, and stability of TCM herbs and products (4,5). For complex prescription, a uniform high-performance liquid chromatography (HPLC) method is important to compare the chemical ingredients between single herbs and complex prescriptions (6). Therefore, to develop a stable, highly efficient, and uniform HPLC method would be helpful to control the quality and compare the components of single herbs and complex prescriptions.

Salvia miltiorrhiza (SM), a popular Chinese herb, has been widely and successfully used mainly for angina pectoris, myocardial infarction, and stroke (7). Panax notoginseng (PN) is used for the treatment of haemoptysis, haematemesis, metrorrhagia, haematoma, and sharp pains in the chest and abdomen (8). Fufang Danshen (FFDS) is a famous typical Chinese complex prescription, which is mainly composed of SM and PN. It is widely used as an effective medicine in the treatment of coronary heart disease, angina pectoris, and myocardial infarction (5). Studies on the chemical compositions of both SM and PN have been reported extensively in the literature, revealing a number of active constituents for each herb (9–16). These constituents are phenolic acids, tanshinones, saponins, etc. In the last 20 years, the analytical technique that has dominated the separation and characterization of phenolic acids is HPLC with reversed-phase column technology (17). An acid inhibitor often needs to be added into the mobile phase in HPLC analysis (18). HPLC is also the most powerful and the most frequently used technique for the determination of saponins, and the main problem in their HPLC analysis is detection. The majority of saponins have to be traced at lower UV wavelengths (ranging from 200 to 210 nm), which creates a problem regarding additives in saponins analysis (19). Therefore, one approach to solving this problem is to adopt other detectors, such as evaporative light-scattering detection (ELSD) or mass spectrometry (MS) coupled to HPLC (20–22).

There is much information on analyzing the chemical components of SM in FFDS or the chemical ingredients of PN in FFDS (22,23). But, to the best of our knowledge, there is little information on simultaneously analyzing the components of SM and PN in FFDS during the course of a single chromatographic run because the co-existence of phenolic acids in SM and saponines in PN makes it difficult. The primary aim of the present study is to develop an effective and uniform gradient HPLC method for

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analyzing SM, PN, and FFDS and to compare the change of components between single herbs and complex prescription.

Experiment

Chemicals and materials

HPLC-grade acetonitrile (Tedia, Jacksonville, FL) and methanol (YuWang Industry, SanDong, China) were used for HPLC analysis. The water was purified in a Milli-Q water purification system (Millipore, Bedford, MA). Formic acid (Acros, Trenton, NJ), acetic acid (Acros), and phosphoric acid (KeMiOu Chemical Co., TianJin, China) were of HPLC grade for HPLC separations. Analytical-grade EtOH was used for extraction and sample preparation. Authentic reference standards of protocatechuic acid, danshensu, rosmarinic acid, lithospermic acid, salvianolic acid B, notoginsenoside-R_1, ginsenoside-Re, Rg_1, and Rb_1 were purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). SM and PN were purchased from Tianshili (Tianjin, China).

Preparation of samples for HPLC

SM and PN were powdered, and 1.0 g powdery material of SM, PN, and FFDS [$m_{(SM)} : m_{(PN)} = 5:3$] was refluxed with 65% EtOH (v/v, 13 mL/g) for 3 h, respectively. The extracts were evaporated under reduced pressure, and the residues were redissolved to 10 mL by methanol. All solutions were filtered through a 0.45-µm filter membrane before HPLC analysis.

Instrumentation and conditions

HPLC analysis was performed using a Waters 2690 system (Waters, Milford, MA) equipped with an automatic sample injector and 996PDA Detector and Millennium32 chromatogram workstation. Separation was performed on a Hypersil ODS2 column (5 µm, 4.6 × 250-mm i.d., Dalian Elite Analytical Instruments, Dalian, China) at 35°C. Solvent A (acetonitrile) and solvent B (0.025% aqueous phosphoric acid, v/v) were used as the mobile phase components (1.0 mL/min). The gradient condition was as follows: 0–3 min, 5% A; 3–20 min, linear change to 21% A; 20–32 min, linear change to 26% A; 32–65 min, linear change to 85% A; 65–70 min, 85% A. The injecting volume of SM and PN was adjusted according to FFDS to ensure the corresponding amount was the same. In this paper, 5 µL SM, 3 µL PN, and 8 µL FFDS were respectively injected into the column. Considering saponins have a feeble UV absorption at a lower wavelength, the UV detection wavelength range was set as 200–400 nm and was monitored at 203 nm.

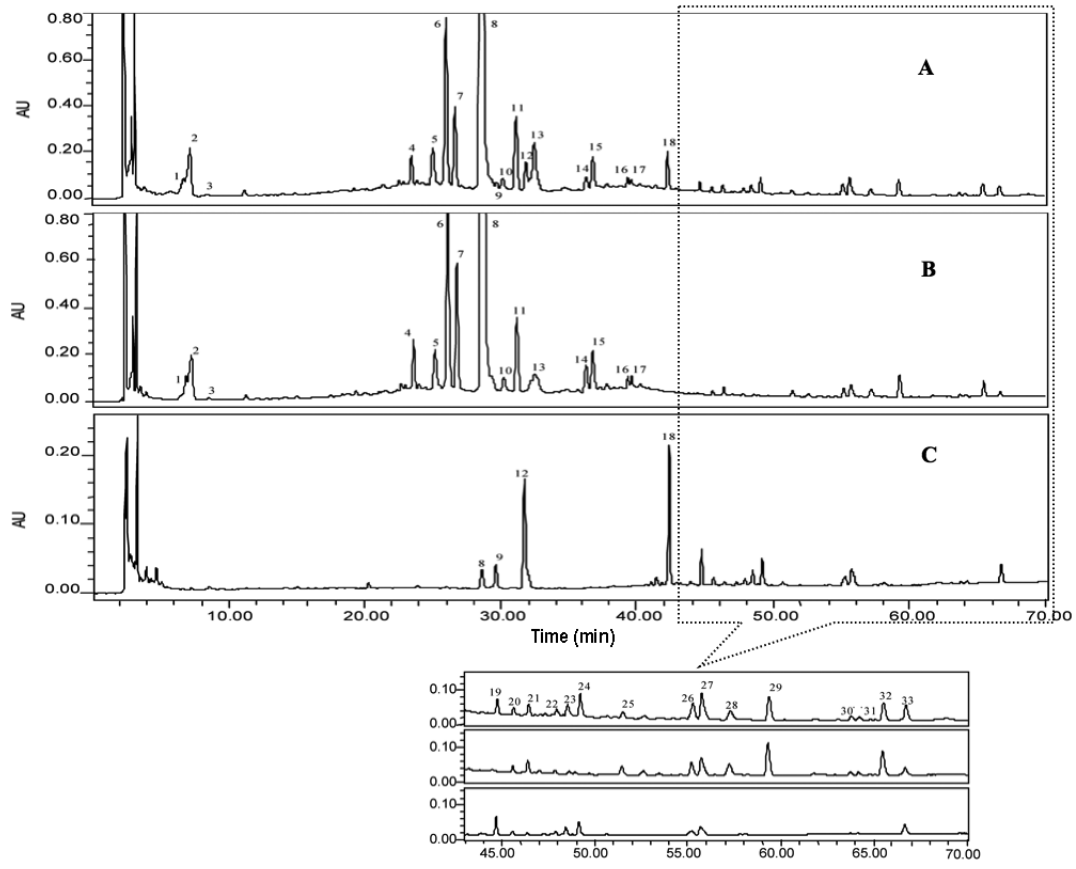

Figure 1. Chromatograms of FFDS (A), SM (B), and PN with acetonitrile–0.025% aqueous phosphoric acid (v/v) at 203 nm (C). $m_{(SM)} : m_{(PN)} = 5:3$.

Repeatability of HPLC method

Five portions of FFDS samples were extracted, respectively, according to the method described in the plant material and sample preparation section. The repeatability experiment was carried out by calculating the RSDs of the peak areas of these five samples.

Results and Discussion

Optimization of HPLC condition

TCMs are complex mixtures usually containing hundreds of different chemical constituents (2). Some of them have strong UV absorption, such as flavones, and others have only feeble UV absorption at lower UV wavelengths, ranging from 200 to 210 nm, such as saponins. Thus, it is important to select a suitable mobile phase and suitable additive to analyze herbs effectively.

Considering the existence of saponines in FFDS, aqueous methanol was inferior to aqueous acetonitrile because the end absorption of methanol would disturb the detection of saponines at lower UV wavelengths. On the other hand, eluent acidification was necessary to inhibit the ionization of phenolic acids. Acetic acid, formic acid, and ammonium acetate were too weak an additive to be useful for the phenolic acids present, and they also would disturb the detection of saponines. Phosphoric acid, as a middle-to-strong acid, had no UV absorption and was very suitable to be added to depress the tailing of the peaks of phenolic compounds in the analysis of FFDS. The concentration of 0.025% phosphoric acid was selected to ensure the reproducibility of the fingerprints of FFDS. The five-slope gradient of the mobile phase (see the HPLC–MS method section) could achieve maximum throughput and optimal resolution.

The repeatability of HPLC method

The HPLC fingerprints of SM, PN, and FFDS were respectively obtained according to the developed uniform HPLC method previously described. The results, detected at 203 nm, are presented in Figure 1. All samples of SM, PN, and FFDS were separated effectively, and 33 peaks were separated distinctly in the plot of FFDS (Figure 1A).

The results of the repeatability experiment of the acetonitrile–0.025% aqueous phosphoric acid system used to analyze FFDS are presented in Table I. The relative standard deviation of 33 peak areas were all less than 7.54%, which indicated that the HPLC method was reliable.

Identification of some peaks in FFDS

The HPLC fingerprint of the reference standards was also obtained according to the developed HPLC method previously described, and it is shown in Figure 2. Identification of the peaks in the fingerprint profiles of FFDS, SM, and PN were carried out by comparing the retention time of the fingerprint profile of reference standards with those of FFDS, SM, and PN. Through comparison, peaks 2, 3, 6, 7, 8, 9, 12, and 18 in HPLC fingerprints of FFDS, SM, and PN were identified, respectively, as danshensu, protocatechuic acid, rosmarinic acid, lithospermic acid, salvianolic acid B, notoginsenoside-R_1, ginsenoside-Rg_1, and ginsenoside-Rb_1.

Components origination of FFDS

Because all samples of SM, PN, and FFDS were analyzed by a uniform method, the origin of the components in FFDS was determined and whether or not new components were produced could be judged clearly by comparing the plot of the complex prescription (FFDS, Figure 1A) with those of single herbs (SM and PN, Figures 1B and 1C). For example, there was no presentations of peaks 4, 5, and 6 in the plot of PN (Figure 1C), but they clearly appear in the plots of SM (Figure 1B) and FFDS (Figure 1A). Thus, it can be concluded that peaks 4, 5, and 6 in FFDS originated from SM, but not from PN. And by the same method of comparison, peaks 9, 12, and 18 originated from PN but not from SM. Meanwhile, the co-existing components in PN and SM were also found. For example, peaks 26 and 27 existed in SM, PN, and FFDS; therefore peaks 26 and 27 were from SM and PN. To sum up, by the comparison of FFDS with SM and PN it was demonstrated that peaks 1–7, 10, 11, 13–17, 25, and 28–32 in FFDS originated from SM, peaks 9, 12, 18, 19, and 24 originated from PN, and peaks 8, 20–23, 26, 27, and 33 originated simultaneously from SM and PN. Thus, the components of FFDS originated from SM and PN or were co-contributed by SM and PN, and no new components appeared and no components disappeared when co-decocting SM and PN into FFDS.

Comparison of the components of FFDS with those of SM and PN

In the HPLC experiments, the injecting volume of SM and PN was adjusted according to FFDS to ensure that the corresponding amount was the same. That is to say, the same amount of SM was present in 8 µL of FFDS as in 5 µL of SM, and the the same amount of PN was present in 8 µL FFDS as in 3 µL of PN.

Table I. Reproducibility of Analysis for Sample FFDS (*n* = 5)

Peak No.*	RSD (Area) %	Peak No.	RSD (Area) %
1	7.54	18	5.31
2	1.33	19	1.80
3	3.35	20	0.43
4	1.25	21	5.88
5	1.75	22	3.87
6	3.51	23	3.88
7	4.08	24	3.67
8	2.38	25	3.59
9	6.73	26	0.86
10	4.31	27	1.65
11	1.55	28	3.03
12	0.51	29	1.17
13	1.96	30	5.59
14	3.02	31	3.51
15	2.80	32	1.47
16	1.64	33	5.39
17	4.87		

*Peak numbers are the same as in Figure 1.

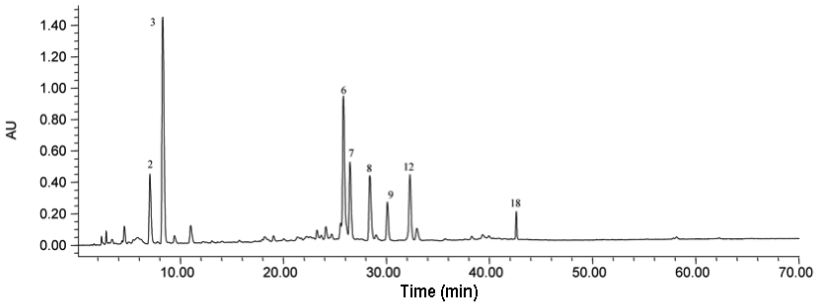

Figure 2. HPLC fingerprint of the reference standards. The peaks in chromatogram are as follows: 2, danshensu; 3, protocatechuic acid; 6, rosmarinic acid; 7, lithospermic acid; 8, salvianolic acid B; 9, notoginsenoside R1; 12, ginsenoside Rg1; 18, ginsenoside Rb1.

Table II. The Ratios of Peak Areas of Samples SM and PN to those of FFDS Samples

Peak No.*	Peak ratio			Peak No.*	Peak ratio		
	SM	FFDS‡	PN		SM	FFDS	PN
1	0.96	1	–	18	–	1	1.26
2	0.88	1	–	19	–	1	1.21
3	0.76	1	–	20	0.74	1	0.48
4	1.02	1	–	21	1.09	1	0.14
5	0.75	1	–	22	0.41	1	0.46
6	1.05	1	–	23	0.24	1	0.64
7	1.28	1	–	24	–	1	0.61
8	1.06	1	0.07	25	1.23	1	–
9	–	1	0.64	26	0.70	1	0.33
10	0.83	1	–	27	0.73	1	0.38
11	0.93	1	–	28	1.10	1	–
12	–	1	1.25	29	1.33	1	–
13	0.47	1	–	30	0.91	1	–
14	1.93	1	–	31	0.91	1	–
15	1.10	1	–	32	1.32	1	–
16	1.18	1	–	33	0.50	1	0.72
17	1.49	1					

* Peak numbers are the same as in Figure 1.
‡ FFDS ratio [$m_{(SM)}$: $m_{(PN)}$ (5:3)].

Therefore, this uniform method not only could be used to judge the origin of the components, but also could be used to compare the quantitative change of the ingredients.

A method of peak area ratios, which is the relative peak areas of SM or PN to the corresponding peak areas of FFDS, was used to compare the quantitative change of components in SM, PN, and FFDS (shown in Table II). From the results in Table II, it was determined that the areas of peaks 9, 13, and 24 obviously increased in FFDS, and the areas of peaks 14 and 17 clearly decreased. The quantities of most components changed between the single herbs and the complex prescription. The results indicated that the chemical ingredients in FFDS were not simply the combination of SM and PN. The reason for this phenomenon may be that the co-decoction of SM and PN could change the dissolving amount of the chemical ingredients of the single herbs.

Though there are no new ingredients produced in FFDS, the content of the ingredients changed. Corresponding with these changes, the therapeutic activity of FFDS was a little different from that of SM or PN. That is to say, the therapeutic activity not only had a relationship with the ingredients, but it also had a relationship with the content of the ingredients. This result could constitute a solid rationale for the compatibility rule in TCM with both scientific merits and intrinsic reasoning.

Conclusion

The acetonitrile–0.025% aqueous phosphoric acid system was used as a uniform HPLC method to analyze SM, PN, and FFDS effectively. By this means, the origin of the chemical ingredients in FFDS was defined. The components of FFDS are originated from SM and PN or are co-contributed by SM and PN. No new ingredients appeared and no components disappeared in FFDS by the co-decocting of SM and PN. The amount of most components in the complex prescription differed from single herbs. In addition, the present method has been validated to be reproducible and reliable. It is suitable for the routine quality control of SM, PN, and FFDS, which could present a means for quality control of single herbs and the most commonly used TCM-complex prescriptions by a uniform method.

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