Simultaneous Determination of Seven Alkaloids in Fufang Zhenzhu Tiaozhi Capsule by HPLC Coupled with DAD

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Abstract: **Objective** To establish a reverse-phase liquid chromatography method for the determination of seven alkaloids (magnoflorine, columbamine, jatrorrhizine, epiberberine, coptisine, palmatine, and berberine) in Fufang Zhenzhu Tiaozhi Capsule. **Methods** Chromatography was performed on a Dionex Acclaim C_{18} column (250 mm × 4.6 mm, 5.0 μm) at 30 ℃. The mobile phase was composed of acetonitrile-potassium dihydrogen phosphate solution (0.015 mol/L, 40:60, including 1.7 g/L sodium dodecyl sulfate and phosphoric acid used to regulate pH value to 3.0), with a flow rate of 1.0 mL/min. The detection wavelength was 270 nm. **Results** The calibration curves of magnoflorine, columbamine, jatrorrhizine, epiberberine, coptisine, palmatine, and berberine were linear in the range of 1.07－10.65, 0.78－7.55, 0.75－7.50, 1.60－15.95, 2.69－26.85, 2.31－23.10, and 6.04－60.40 mg/mL. The average recoveries of magnoflorine, columbamine, jatrorrhizine, epiberberine, coptisine, palmatine, and berberine were 101.0%, 101.2%, 100.1%, 100.0%, 100.1%, 101.1%, and 99.7%, respectively. **Conclusion** The method could be used for the quantitative determination of the preparation.

Key words: alkaloids; diode array detection; Fufang Zhenzhu Tiaozhi Capsule; HPLC; traditional Chinese medicines
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Introduction

Traditional Chinese medicines (TCMs), which are widely used for health needs over thousands of years in China and some Asian countries (Howes and Houghton, 2003; Liu *et al*, 2010), have the characteristics of multiple pharmacological effects and relatively low toxicity in disease treatment by composing of many Chinese herbal medicine (CHM) with multiple bioactive components. However, the quality control of CHM is facing a great challenge at present. Therefore, the development of methods to evaluate and control the quality of CHM is of significant value.

Fufang Zhenzhu Tiaozhi Capsule (FZTC), a compound preparation composed of *Coptidis Rhizoma*, *Glycyrrhizae Radix* et *Rhizoma*, *Notoginseng Radix* et *Rhizoma*, *Ligustri Lucidi Fructus*, *Cirsii Jeponici Herba*, *Eucommiae Cortex*, *Citri Sarcodactylis Fructus*, and *Atractylodis Rhizoma*, is one of the patently and clinically approved CHM prescriptions in the treatment of dyslipidemia, which was invented by Prof. GUO Jiao (Guo *et al*, 2009; Guo, 2005). Our previous study revealed that FZTC could lower the levels of total cholesterol (TC), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-C) in serum, while increase the level of high-density lipoprotein cholesterol (HDL-C) in serum of dyslipidemic patients (Guo *et al*, 2011). Alkaloid components in *Coptidis Rhizoma* are revealed to be the main bioactive ingredients of FZTC, which may play important roles in the biological and pharmacological effects of FZTC.

Many analytical methods have been employed for the quantitative analysis of CHM or its prescriptions (Liang *et al*, 2009; Liang, Xie, and Chau, 2010; Jiang *et al*, 2010). But most of them were relied on the

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quantification of a single active component while the other bioactivities were ignored (Kang, Fan, and Zhang, 2010). According to the theory of CHM, the therapeutic actions of CHM are based on integral interaction of multiple ingredients (Tang *et al*, 2010). Thus, in order to utilize FZTC effectively and enhance the clinical safety, it is urgently needed to develop an accurate and reliable method based on the multiple components for the quality control of FZTC. However, to our knowledge, the method for the simultaneous separation and evaluation of multiple active constituents in FZTC by HPLC has not been found.

Alkaloids belonging to protoberberine alkaloids (Hao, 2007) are reported to be the main bioactive ingredients in *Coptidis Rhizoma* (Tang *et al*, 2009), and exhibit a great variety of biological and pharmacological benefits (Yu *et al*, 2006). For this reason, the alkaloids were used as indicator compounds for the quality control of many CHM prescriptions contained *Coptidis Rhizoma* (Fan *et al*, 2010; Kong *et al*, 2010; Tan *et al*, 2007). Nevertheless, available methods typically focus on a single component or a few limited components. And quantitative information about other alkaloids of *Coptidis Rhizoma* is lacking (Liu *et al*, 2010; Lay *et al*, 2010; Geng *et al*, 2010). So far, there are no reports on the simultaneous analyses as many alkaloids as possible in compound preparation. An analytical method for CHM prescriptions contained *Coptidis Rhizoma*, capable of simultaneous detection of multi components, especially alkaloids for quality control, therefore, is needed.

In our study, a simple, sensitive, and reliable analytical method for simultaneous determination of seven alkaloids, berberine, palmatine, coptisine, epiberberine, jatrorrhizine, columbamine, and mangoflorine (Fig. 1) in FZTC, was developed by HPLC method coupled with diode array detection (DAD). The validated method was successfully applied in the quantitative analysis of these seven alkaloids in FZTC samples taken from different production batches, allowing evaluation of batch quality. From these results, the proposed method in this paper is particularly suitable for the routine analysis of FZTC and its quality control.

Materials, methods, and results

Chemicals and reagents

HPLC-grade acetonitrile and methanol were purchased from Honeywell International Inc. (USA). HPLC-grade phosphoric acid was purchased from Tianjin Kermel Chemical Reagent Co., Ltd. (China). Deionized water was purified using a Purelab Ultra GE MK2 Water System (UK). All other reagents used were of analytical or better grade. Reference substances of berberine, palmatine, coptisine, jatrorrhizine, and magnoflorine were purchased from Chengdu Herb purify Co., Ltd. (China), the purity (≥ 98.0%) of these

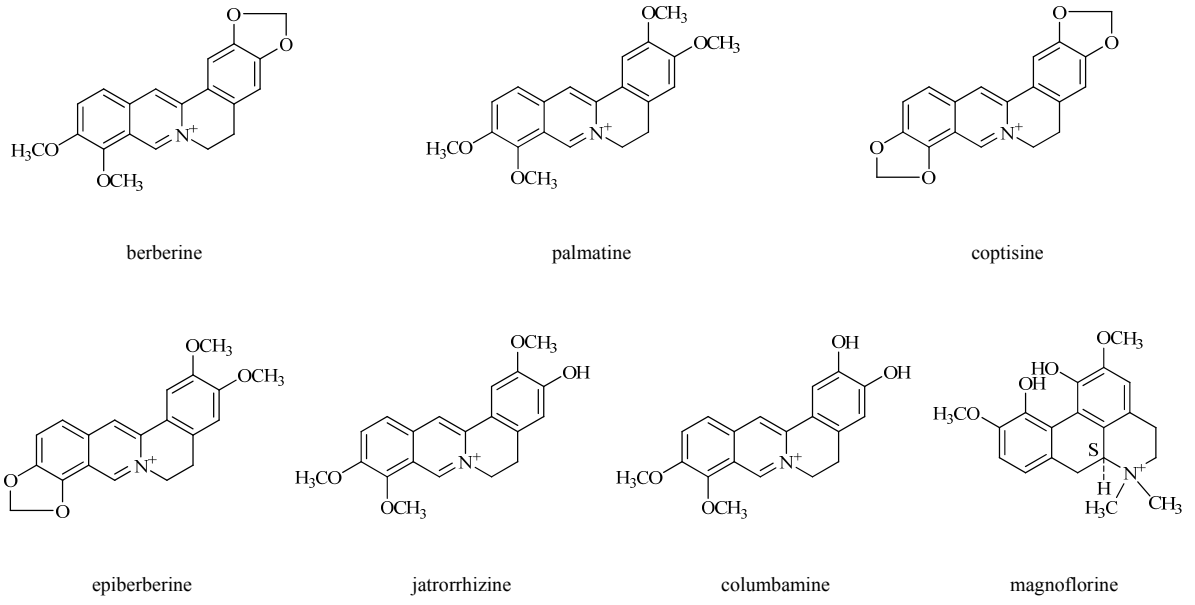

Fig. 1 Chemical structures of seven alkaloids

reference substances was assumed as provided by the suppliers. Epiberberine and columbamine were supplied by Chongqing Academy of Chinese Materia Medica. Samples of FZTC were provided by Institute of Materia Medica, Guangdong Pharmaceutical University (batch No: 20090607, 20090907, and 20081028). All comprised crude drugs were purchased from Zhixin Chinese Herbal Medicine Co., Ltd. (China).

Analytical and chromatographic conditions

The HPLC system Dionex UltiMate 3000 HPLC System (Dionex, Germany), equipped with Chromeleon software (Dionex) and comprised a quaternary pump, an online vacuum degasser, an auto-sampler, and a thermostated column compartment and DAD, was used for chromatographic analysis. All separations were carried out on a Dionex Acclaim C_{18} column (250 mm × 4.6 mm, 5.0 μm) and the column temperature was maintained at 30 ℃. The isocratic mobile phase, pumped at a flow rate of 1.0 min/mL, consisted of acetonitrile-potassium dihydrogen phosphate solution (0.015 mol/L, 40:60, including 1.7 g/L sodium dodecyl sulfate and phosphoric acid used to regulate pH value to 3.0), which was freshly prepared, filtered through a 0.22 μm filter, and degassed by sonication for 15 min prior to use. The injection volume was 20 μL and the detection wavelength was 270 nm.

Preparation of reference solutions

The reference chemicals of seven alkaloids, berberine (12.08 mg), palmatine (4.62 mg), coptisine (5.37 mg), epiberberine (3.19 mg), jatrorrhizine (1.50 mg), columbamine (1.55 mg), and magnoflorine (2.13 mg), were separately and accurately weighed, put into 10 mL volumetric flasks, and dissolved in methanol to make the reference stock solutions, respectively. The stock solutions were then further diluted with methanol to the concentration required in the experiments. All the solutions were stored at 4 ℃, with the longest period of 5 d, and brought to room temperature before use. Calibration reference working solutions were freshly prepared by appropriate dilution of the stock solutions, giving final concentration in the range of 6.04－60.40 μg/mL for berberine, 2.31－23.10 μg/mL for palmatine, 2.69－26.85 μg/mL for coptisine, 1.60－15.95 μg/mL for epiberberine, 0.75－7.50 μg/mL for jatrorrhizine, 0.78－7.75 μg/mL for columbamine, and 1.07－10.65 μg/mL for magnoflorine.

Preparation of samples and negative control samples

The accurately weighed powder (0.4 g, 40-mesh) was extracted with 50 mL methanol in an ultrasonic bath for 40 min (250 W, 40 kHz). The solution was filtered through filter paper and then a 0.45 μm membrane filter prior to HPLC analysis. The concentration of the interest analytes in FZTC samples was calculated via integration of the peak area, and comparison with calibration curves was generated for the individual analytes using reference samples.

As negative controls (NCs), samples of FZTC without *Coptidis Rhizoma* were prepared to validate the specificity of the method. The preparation of NCs strictly conformed to the prescription of FZTC described in patent (Guo, 2005).

The typical chromatograpm of the reference solution and the real sample solution were shown in Fig. 2. It indicated that a good separation was obtained under the described condition and no interfering peaks were found at the retention time of analytes.

Calibration curves, limits of detection (LOD) and quantification (LOQ)

Methanol stock solution containing seven accurately weighed reference compounds was prepared and diluted to appropriate concentration ranges for the

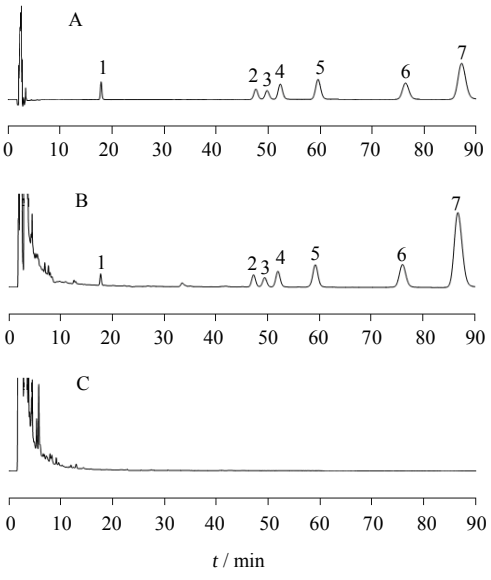

Fig. 2 Representative chromatograms of mixed reference substances (A), FZTC (B), and negative sample of FZTC (C)

1: magnoflorine 2: columbamine 3: jatrorrhizine 4: epiberberine 5: coptisine 6: palmatine 7: berberine

construction of calibration curves. The calibration curve of each compound was performed with seven appropriate concentration. The calibration curve was plotted by using the ratio of peak areas of reference solution as *Y*-axis, and concentration as *X*-axis. Linear regression was used to evaluate the equation of $Y = aX + b$ and R^2 was the correlation coefficient (Lay *et al*, 2010). The LOD and LOQ values under the present chromatographic conditions were determined at a signal-to-noise (S/N) ratio of 3 and 10, respectively.

R^2, linear range, regression equation, LOD, and LOQ of linear calibration curves generated using reference samples of the seven compounds were shown in Table 1. Good correlation between compound concentration (*X*) and peak area (*Y*) was obtained over a wide concentration range with correlation coefficients $R^2 > 0.9991$ in all cases. The LOD and LOQ values of each analyte were in the ranges of 0.05－0.17 and 0.27－0.76 μg/mL, respectively, indicating the high sensitivity of the optimized HPLC conditions.

Precision and accuracy

Intra-day variations for six times within 1 d and inter-day variations for consecutive 6 d (using the reference solutions) were chosen to determine the precision of the developed method. The intra- and inter-day precisions of analyte detection were in the ranges of 0.69% － 1.42% and 0.5% － 1.7%, respectively, indicating that the developed method is sufficiently precise for the quantitative evaluation of the target analytes in FZTC. The intra- and inter-day accuracy for each marker substance is listed in Table 2.

Repeatability

The analytic repeatability was examined by the injection of six different samples (No. 20090907), which were prepared with the same sample preparation procedure. The repeatability of the solution at medium concentration was in the range of 0.24%－0.92% (Table 3).

Stability

For the stability testing, the same real sample (No. 20090907) was analyzed within 24 h at room temperature. Stabilities of the solution shown in RSD of retention time and peak area were all within 1.55% and no significant difference was observed, indicating that the solution was stable (Table 3).

Recovery

Accurate amounts of seven references were added to FZTC sample and then processed and analyzed in triplicate to calculate recoveries. The recovery was calculated

Table 1 Calibration curves, LOD, and LOQ of seven alkaloids

Alkaloids	Calibration curves	R^2	Linear range / (μg·mL^{-1})	LOD / (μg·mL^{-1})	LOQ / (μg·mL^{-1})
magnoflorine	$Y = 0.5415X - 0.0355$	0.9999	1.07－10.65	0.07	0.27
columbamine	$Y = 1.1181X - 0.1508$	0.9995	0.78－7.75	0.05	0.39
jatrorrhizine	$Y = 1.0614X - 0.2498$	0.9991	0.75－7.50	0.09	0.38
epiberberine	$Y = 0.9146X - 0.3258$	0.9996	1.60－15.95	0.10	0.40
coptisine	$Y = 0.8203X - 0.4671$	0.9998	2.69－26.85	0.17	0.67
palmatine	$Y = 1.0072X - 0.8743$	0.9998	2.31－23.10	0.14	0.58
berberine	$Y = 0.9865X - 0.8133$	0.9999	6.04－60.40	0.19	0.76

Table 2 Precision and accuracy (n = 6)

Alkaloids	Nominal concentration / (μg·mL^{-1})	Observed concentration / (μg·mL^{-1})		RSD / %		Accuracy / %	
		Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
magnoflorine	4.26	4.30 ± 0.03	4.34 ± 0.06	0.69	1.28	101.0	101.9
columbamine	3.10	3.17 ± 0.04	3.08 ± 0.05	1.11	1.63	102.2	99.3
jatrorrhizine	3.00	3.11 ± 0.04	3.00 ± 0.07	1.42	1.70	103.8	100.6
epiberberine	6.38	6.59 ± 0.07	6.48 ± 0.09	1.05	1.33	103.3	101.5
coptisine	10.74	11.00 ± 0.11	10.92 ± 0.12	0.97	1.14	102.4	101.7
palmatine	9.24	9.44 ± 0.12	9.45 ± 0.05	1.24	0.50	102.2	102.3
berberine	24.16	24.22 ± 0.34	24.43 ± 0.40	1.39	1.66	100.2	101.1

Table 3 Repeatability and stability (n = 6)

Analytes	Repeatability		Stability	
	Average contents / (mg·g^{-1})	RSD / %	Average peak area	RSD / %
magnoflorine	0.346	0.72	1.470	1.05
columbamine	0.441	0.82	4.083	1.55
jatrorrhizine	0.363	0.92	3.315	0.73
epiberberine	0.855	0.75	5.881	1.42
coptisine	1.588	0.51	9.956	0.46
palmatine	1.652	0.35	12.504	0.22
berberine	6.526	0.24	50.915	0.08

by the formula of $(C_3-C_2)/C_1 \times 100\%$, in which C_1 represents the amount of each reference spiked, C_2 represents the amount of each marker in methanol solution of FZTC, and C_3 represents the total amount of each marker in the solution (Lay *et al*, 2010).

The average recovery of the investigated compounds spiked into FZTC ranged between 96.6% and 101.1%, and RSD values were all < 2% (Table 4). The developed method is therefore sufficiently accurate and reliable for the measurement of the target analyte.

Quantitative analysis

In order to investigate the application for practical analysis, the proposed method was applied to the simultaneous determination of seven alkaloid components in FZTC of different production batches. The results were summarized in Table 5, indicating that the concentration of analytes showed a few differences among different production batches. The content of berberine in FZTC was the highest (6.526, 9.532, and 5.344 mg/g, respectively), averagely reaching 55% of total

Table 4 Statistic results of recovery for extract from analytes in FZTC (n = 3)

Analytes	Added amount / mg	Found amount / mg	Calculated recovery / %	Average recovery / %	RSD / %
magnoflorine	0.149	0.150	100.5		
	0.128	0.130	101.3	101.0	0.91
	0.107	0.108	101.2		
columbamine	0.109	0.111	99.8		
	0.093	0.095	99.2	99.6	0.89
	0.078	0.077	99.9		
jatrorrhizine	0.105	0.104	101.0		
	0.090	0.090	99.7	99.8	1.35
	0.075	0.076	98.7		
epiberberine	0.223	0.224	100.3		
	0.191	0.189	98.7	100.1	1.77
	0.160	0.161	101.2		
coptisine	0.376	0.372	99.1		
	0.322	0.323	100.3	100.1	1.25
	0.269	0.271	100.9		
palmatine	0.323	0.327	101.3		
	0.277	0.280	101.1	101.1	0.94
	0.231	0.233	101.1		
berberine	0.846	0.838	99.1		
	0.725	0.721	99.4	99.7	1.21
	0.604	0.607	100.4		

Table 5 Contents of seven alkaloids in FZTC (n = 3, mg·g^{-1})

Batch number	Magnoflorine	Columbamine	Jatrorrhizine	Epiberberine	Coptisine	Palmatine	Berberine
20090907	0.346	0.441	0.363	0.855	1.588	1.652	6.526
20090607	0.272	0.369	0.363	0.254	0.739	1.439	5.344
20081028	0.545	0.686	0.532	1.167	2.231	2.435	9.532

contents of seven alkaloids in FZTC; while magnoflorine was the lowest (0.346, 0.272, and 0.545 mg/g, respectively). The distinction of total contents of the seven alkaloids in the three bathes could be ascribed to the use of *Coptidis Rhizoma* with different quality. Previous pharmacological studies (Kong *et al*, 2004; Tang *et al*, 2006; Hsieh *et al*, 2007) have shown that, berberine, which is considered to be the main active ingredient of *Coptidis Rhizoma*, had a conclusive effect in regulation of lipid metabolism with clear mechanism. What's more, some studies reported that magnoflorine could regulate lipid metabolism to some extent by other mechanisms different from berberine (Hung *et al*, 2007). It is well known that the therapeutic effects of CHM are usually attributed to multiple bioactive compounds. Thus, it is interesting that both berberine and magnoflorine may be the active compounds contributing to the lipid-lowering effect of FZTC. Therefore, it is very significant to develop an effective, accurate, and reliable method for analysis of FZTC. The proposed method in this paper is particularly suitable for the routine analysis of FZTC and its quality control.

Discussion

Optimization of sample preparation method

In order to obtain satisfactory extraction efficiency, the solvent, method, and time of extraction were optimized. Anhydrous and aqueous methanol or hydrochloric acid-methanol solutions were examined as extraction solvent, with anhydrous methanol proved to be the best solvent choice, allowing efficient extraction of all target analytes in high yield and with fewer impurities.

The suitable extracting condition was established as follows: Samples were extracted by ultrasonic extraction using anhydrous methanol as the extraction solvent, and the process lasted for 40 min, 40 kHz.

Optimization of chromatographic conditions

The selection of fast HPLC conditions was guided by the requirement for obtaining chromatograms with better resolution of adjacent peaks within a short time. Because of the similar interaction with the column which results from their similar chemical structures, it is challenging to develop a chromatograph and separate seven alkaloids simultaneously in FZTC.

Different types of column were tested, such as Dikma Platisil ODS (250 mm × 4.6 mm, 5 μm), Welch Xtimate C_{18} HPLC column (150 mm × 4.6 mm, 3 μm), and Dionex Acclaim C_{18} column (250 mm × 4.6 mm, 5 μm); The Dionex Acclaim C_{18} column (250 mm × 4.6 mm, 5 μm) gave the best results. Different mobile phases were tried, such as methanol-water and acetonitrile-water with different modifiers, including phosphate buffer, formic acid, acetic acid, phosphoric acid, triethylamine acid, and ammonium carbonate buffer. The acetonitrile-water system showed more powerful separation ability for the investigated compounds than the methanol-water system. When phosphate buffer and ion-pairing solution were added into the mobile phase, the symmetries of all the chromatographic peaks were improved remarkably. A solvent system consisting of acetonitrile and 0.015 mol/L potassium dihydrogen added phosphate with sodium dodecyl sulfate (phosphoric acid was used to regulate pH 3.0), which provides lower pressure and greater baseline stability, was ultimately selected as mobile phase system.

Apart from the elution system, column temperature and flow rate were also important influence factors on separation for multi-compound. After extensive optimization of the separation, under the conditions described above, all the reference compounds could be eluted with baseline separation in approximate 85 min; The investigated compounds in samples were also well separated. Although these compounds have different UV absorption characteristics, 270 nm was found to provide strong UV absorption for all components.

Conclusion

The RP-LC separation of basic analytes such as

protoberberine alkaloids often results in broad and tailing bands, which are caused by acidic sites present in the column packing. The use of phosphate buffers and ion-pairing solution in mobile phase is necessary. In this work, sodium dodecyl sulfate was used to obtain the best results. The method which was accurate and reliable for the simultaneous determination of seven active components (berberine, palmatine, coptisine, epiberberine, jatrorrhizine, columbamine, and magnoflorine) in FZTC was developed by RP-HPLC method coupled with DAD. This proposed method is promising to be the routine analysis for FZTC and its quality control with simplicity, accuracy, and reliability.

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