Simultaneous Determination of Chlorogenic Acid, Caffeic Acid, Ferulic Acid, Protocatechuic Acid and Protocatechuic Aldehyde in Chinese Herbal Preparation by RP-HPLC

Xin-Pu LI,[a,b] Jie YU,[b] Jin-Yin LUO,[b] Hong-Sheng LI,[b] Fu-Jie HAN,[b] Xing-Guo CHEN,*[,a] and Zhi-De HU[a]

[a] *Department of Chemistry, Lanzhou University; Lanzhou, 730000, P. R. China: and* [b] *Lanzhou Institute of Animal & Veterinary Pharmaceutics Sciences, CAAS; Lanzhou, 730050, P. R. China.*

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In the present study, a reversed phase high performance liquid chromatographic (RP-HPLC) method was established for simultaneous determination of chlorogenic acid, caffeic acid, ferulic acid, protocatechuic acid and protocatechuic aldehyde in a Chinese herbal preparation (Fufang-Pugongying-Mixture). The separation was performed on a Hypersil ODS-2 column by isocratic elution with methanol and 0.2 M acetate buffer (pH 3.6) (15 : 85, v/v) as the mobile phase at the flow-rate of 1.0 ml/min with operating temperature of 30 °C, and detection wavelength of 300 nm. A good linear regression relationship between peak-areas and concentrations was obtained over the range of 2—200 μg/ml for the five marker compounds mentioned above. The spike recoveries were within 96.72—104.07%. The variation coefficient (CV) values of the precision were in the range of 0.89—4.50%. Moreover the developed method has reference value for quantitative analysis of Taraxacum, Lonicera and Angelica.

Key words chlorogenic acid; caffeic acid; ferulic acid; protocatechuic acid; protocatechuic aldehyde; Fufang-Pugongying-Mixture

Herbal materials are traditionally used as medicines in Asia and Africa, and they have become recognized by scientific medical system and have become increasingly popular. Chinese herbal preparations have been used to prevent diseases in both humans and animals for centuries in China, most of them are composed of several herbs containing complex chemical components. So far Chinese herbal preparations cann't be built absolute quantitative standards like chemical preparations. The studies analyzing active components are basis of both quality control and scientific evaluation of Chinese herbal preparations.

Fufang-Pugongying-Mixture (FFPM) is a herbal preparation used for treatment of clinical mastitis in dairy cattle, and the cure rate of FFPM is 81.82%.[1,2] In recent years, FFPM has attracted the public's attention due to the possibility of developing the resistance population of the bacteria and concern about the residues on animal products caused by antibiotic agents in farm animals. The FFPM is composed of six herbs, *i.e.* Taraxacum Herb, Lonicera Bud, Scutellaria Radix, Angelica Radix, Salvia Radix, and Isatis Siccative Radix. The bio-active components are quite complicated in FFPM, including chlorogenic acid (CGA, in Taraxacum Herb and Lonicera Bud), caffeic acid (CFA, in Taraxacum Herb, Lonicera Bud and Salvia Radix), ferulic acid (FRA, in Angelica Radix), protocatechuic acid (PCA, in Salvia Radix) and protocatechuic aldehyde (PAD, in Salvia Radix). The pharmacology functions of CGA, CFA, FRA, PCA and PAD were studied extensively, and were proved to have various pharmacology activities, such as antibacterial,[3—5] antivirus,[6,7] anti-inflammatory,[8,9] immunity activity[10,11] and invigorate blood,[12,13] *etc.*, which could reflect those of FFPM treating clinical mastitis in dairy cattle. Therefore the five compounds were considered as a part of main active components, and were selected for analyzing FFPM.

Though Chinese government (Gansu Animal Husbandry Bureau) has granted a certificate for producing the FFPM, however, only the qualitative methods were available for quality control of FFPM. The thin-layer chromatography (TLC),[14—17] high performance liquid chromatography (HPLC),[18—27] and capillary electrophoresis (CE)[28—31] have been used to determine CGA, CFA, FRA, PCA and PAD in their intact materials or Chinese herbal preparations. To the best of our knowledge, there were no reports on simultaneous determination of the five marker compounds in Chinese herbal preparations or FFPM by HPLC. The HPLC with gradient elution mode was often used to determination of three and over constituents.[22,32,33] In this paper, a simple, practical and low-cost RP-HPLC method with isocratic elution mode for the quality control of FFPM was developed and successfully applied for the first time. With the developed method, the influence of different processing procedure on the FFPM quality was investigated, and contents of CGA, CFA and FRA in Taraxacum, Lonicera and Angelica were analyzed.

Experimental

Reagents and Materials The standards of CGA, CFA, FRA, PCA and PAD were purchased from the Chinese National Institute of Control of Pharmaceutical and Biological Products (Beijing, China), their structures were shown in Fig. 1. The intact herbal materials (air-dried and cut into small pieces) used for preparation of the FFMP were purchased from Huanghe herbal market (Gansu, China). Methanol of HPLC grade and other reagents of analytical grade were purchased from the Tianjin Second Reagent Factory (Tianjin, China). The distilled water was used throughout.

Apparatus The chromatographic separation was carried out on a Waters 510 HPLC system (MS, U.S.A.), consisting of two Waters 510 Pumps, a Waters Temperature Control Module, a Waters Automated Gradient Controller, and a Waters 486 Tunable Absorbance Detector (wavelength range from 190 to 600 nm). A personal computer with a chromatography manager PC-800 (Dalian, China) was used to record the chromatograms and to analyze the data. A Rheodyne 7725I manual injector (CA, U.S.A.) was used to add the samples to the HPLC. The separation was achieved on a Hypersil ODS-2 column (200×4.6 mm I.D. 5 μm particle size, Dalian, China).

Chromatography Conditions In this system the mobile phase was a

* To whom correspondence should be addressed. e-mail: chenxg@lzu.edu.cn

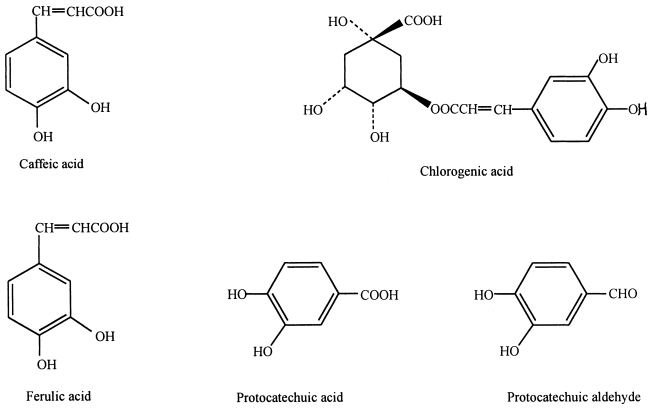

Fig. 1. Structures of Five Marker Compounds

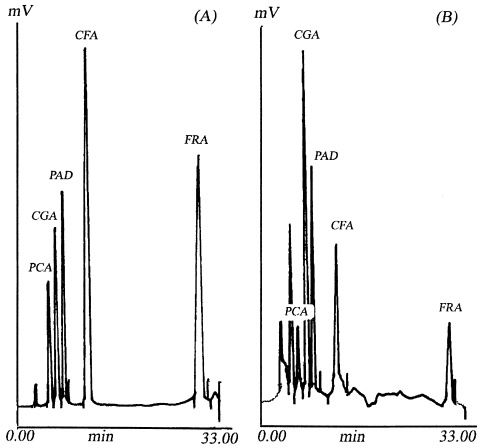

Fig. 2. Chromatograms of the Standard Mixture (A) of Five Marker Compounds and Fufang-Pugongying-Mixture Sample (B)

PCA: caffeic acid, CGA: chlorogenic acid, FRA: ferulic acid, PCA: protocatechuic acid, PAD: protocatechuic aldehyde. Analytical condition: column, Hypersil ODS-2; flow-rate, 1.0 ml/min; temperature, 30 °C; wavelength, 300 nm; mobile phase, methanol–0.2 M acetate buffer (pH 3.6) (15 : 85, v/v).

mixture of methanol and 0.2 M acetate buffer (pH 3.6) (15 : 85, v/v) at a flow rate of 1.0 ml/min. The column temperature was at 30 °C. The detection wavelength was set at 300 nm. The injection volume was 20 μl. The mobile phase was filtered through a 0.45 μm filter membrane, and was degassed prior to used. The chromatography system was equilibrated by the mobile phase. The separation of samples was conducted when the same retention time and the peak areas of the repetitive injections of the standard solution were obtained.

Preparation of Sample 150 g Taraxacum Herb, 100 g Lonicera Bud, 100 g Scutellaria Radix, 100 g Angelica Radix, 100 g Salvia Radix, and 100 g Isatis Radix, which equivalent to a daily dose of FFPM, were carefully weighted and mixed, a twentyfold mass of water was added. The mixture was boiled on electric heater, and was decocted continuously for 2 h without temperature control. The decoction was filtered through a colander when it was still hot, and the filtrate was concentrated to 500 ml, which was used as the FFPM sample for HPLC.

To investigate the influence of different processing procedure on the five marker compounds, the other three FFPM samples with different decocting way, time and temperature were prepared by follow means: the herbs of the FFPM formula were decocted and concentrated individually, and then all concentrates were mixed; The decocting time was decreased to 1 h; After the mixture of water and herbs was boiled, the decocting temperature was adjusted to 100 °C.

Sample Pretreatment: The FFPM sample (5 ml) was extracted with 40 ml ethanol for 10 min in an ultrasonic bath, and centrifuged (1500 rpm for 10 min). The 1.5 ml supernatant was blow-dried with nitrogen gas (N_2) at 50 °C. The dried residue was then dissolved in 2 ml methanol (50%), and filtered with 0.45 μm filter membrane. The final filtrate was subject to the chromatographic system.

Results and Discussion

Selection of the Mobile Phase Many studies have shown that acetic acid or acetate buffer is used extensively as composition of mobile phase in HPLC. They are less expensive, safer, and the 0.2 M concentration may provide theoretically suitable pH value for analyzing the five marker compounds in FFPM. So the 0.2 M acetic acid was first selected, and mixed with methanol as mobile phase. Having close retention times, the five marker compounds couldn't be separated. Then 0.2 M acetate buffer mixed with methanol as the mobile phase. The volume ratio of methanol and 0.2 M acetate buffer was 20 : 80. At pH 4.2, the five marker compounds couldn't be separated each other. At pH 3.6, except for PCA and CGA were not separated, other three marker compounds were separated effectively. CGA, CFA, FRA and PCA were organic acids, at pH 3.6 their dissociation degrees decreased and the retention times increased, which resulted in the separation improvement. Because the structure of PAD is similar to PCA, its change during separation may be similar to PCA. According to the result, the methanol and 0.2 M acetate buffer (pH 3.6) were used at volume ratios of 10 : 90, 15 : 85 and 20 : 80, respectively. Only when the volume ratio was at 15 : 85, the standard mixture of the five marker compounds obtained a nice separation within 35 min (Fig. 2A).

Selection of Column Temperature When column temperature reached 40 °C, the retention times of the five marker compounds curtailed obviously, but their peak shapes broadened affected separation efficiency. Column temperature was 25 °C, the retention times prolonged. In the test, 30 °C was suitable temperature for measurement.

Selection of Detection Wavelength In previous reports, UV wavelength used for determination of CGA, CFA, FRA, PCA and PAD were generally 326, 326, 320, 260 and 281 nm, respectively. In this experiment, 300 nm of UV wavelength was selected, and the five compounds were effectively detected.

Evaluation of Sample Pretreatment The extraction solvent for extracting markers in a sample was an important factor, which affects efficiencies of extraction and separation of the markers. Methanol and ethanol are good solvents for dissolving the five marker compounds in FFPM. If FFPM sample was extracted only by methanol for analysis, more interfering peaks disturbed analysis of the marker peaks. If FFPM sample was extracted only by ethanol for analysis, it was found that in the chromatograms before the marker peaks there was a large peak, which was so large that the marker peaks couldn't be analyzed by PC-800 manager. Therefore, in this study, ethanol was first used to extract FFPM sample, and the extraction was centrifuged, and then the supernatant was blow-dried with N_2. The dried residue was dissolved in 50% methanol for HPLC analysis. A very clear chromatogram of FFPM sample was obtained (Fig. 2B). The retention times of CGA, CFA, FRA, PCA and PAD were 6.71, 11.97, 31.30, 5.48 and 7.87, respectively.

Preparation of Standard Curve The standards of the five marker compounds were accurately weighed and dissolved in 50% methanol to give a serial concentrations in rang 2—200 μg/ml. The standard curves were prepared using the peak areas (uv. sec, *y*-axis) of ten different concentrations (μg/ml, *x*-axis) in triplicate assays, and were expressed by the

linear least-squares regression equation. The regression equations of the five marker compounds were as follow:

CGA $y=54710x-151702$ ($r^2=0.9993$)
CFA $y=94796x-45627$ ($r^2=0.9983$)
FRA $y=104468x+3420$ ($r^2=0.9985$)
PCA $y=28152x+6321$ ($r^2=0.9987$)
PAD $y=81214x-28575$ ($r^2=0.9975$)

As can be seen from the above equations, the correlation coefficients of the equations were greater than 0.99, and the five calibration curves yielded straight lines in a wide concentration range. The detection limits (signal-to-noise ratio=3) of CGA, CFA, FRA, PCA and PAD were 0.78, 0.10, 0.05, 0.39 and 0.20 μg/ml, respectively.

Validation of the Assay Method The method for quantitative analysis of FFPM was validated with regard to its specificity, accuracy and precision.

The specificity of the method was confirmed by reliability of the five marker peaks in FFPM sample. The standard mixture of the five marker compounds, FFPM sample and FFPM sample spiked with the five maker compounds were separated by mobile phases A (methanol–0.2 M acetate buffer (pH 3.6) (20 : 80, v/v)) and mobile phase B (methanol–0.2 M acetate buffer (pH 3.6) (6.5 : 93.5, v/v)), respectively. The retention times of the five marker compounds in FFPM sample changed consistently with their corresponding standards, and the peak areas of the five marker compounds in FFPM sample spiked increased identically with those of the spiking marker compounds, which can demonstrate that the developed method was specific for quantitative determination of the five marker compounds in FFPM sample.[34] Both mobile phase A and mobile phase B were used in the test, making up their weakness single-used, and achieved the test purpose.

The accuracy of the method was determined by spike recoveries of the five marker compounds in FFPM sample. An appropriate amount of FFPM sample was extracted with eight-time mass ethanol, the ethanol extraction was divided twelve portions (three of them as a control), the nine portions (except the control) were spiked with five marker compounds in three different concentrations (0.05, 0.10, 0.15 mg/ml, three portions in each concentration). The twelve portions were blow-dried, and were dissolved in methanol (50%) for analysis. The spike recoveries for the five markers were between 96.72—104.07% (Table 1).

The precision of the method was validated by both intra- and inter-day variances. To determine intra-day variance, the five assays were carried out on same FFPM samples at different times during the day. Inter-day variance was determined by analyzing the FFPM samples over five consecutive days. As shown in Table 2, the CV values of Intra-day and inter-day variances were between 0.89—2.08% and 1.36—4.50%, respectively.

Evaluation of Processing Procedure Since Chinese herbal preparations are generally prepared from the combination of several herbs, they often have combined effects, which differ from the sum of effects of the individual constituents of the herbs, and the time and temperature of processing procedure may affect medicine quality.[35] The FFPM samples with different procession procedure were analyzed by the developed method. The results indicated that the decocting way and time produced complex influence to contents of five marker compounds, it was difficult to indicate which decocting way or time was the better. But the decocting temperature produced identical effect to the five markers, which indicated that decocting temperature should be higher than 100 °C which contributed to increasing the contents of the five marker compounds.

Table 1. Spike Recoveries of CGA, CFA, FRA, PCA and PAD in FFPM Samples

Marker compound	Content in sample (mg/ml)	Spiked concentration (mg/ml)	Recovery[a] mean±S.D. (%)
CGA	1.349	0.10±0.05	99.56±4.98
CFA	0.111	0.10±0.05	104.07±3.88
FRA	0.098	0.10±0.05	98.07±4.60
PCA	0.544	0.10±0.05	96.72±2.82
PAD	0.550	0.10±0.05	99.81±3.65

CGA: chlorogenic acid, CFA: caffiec acid, FRA: ferulic acid, PCA: protocatechuic acid, PAD: protocatechuic aldehyde, FFPM: Fufang-Pugongying-Mixture. *a*) Means±standard deviation from nine determinations.

Table 2. CV Values of Intra-Day and Inter-Day Variances of FFPM Samples

Marker compound	CV[a] (%)	
	Intra-day variance	Inter-day variance
CGA	1.65	2.57
CFA	2.08	1.36
FRA	1.47	2.98
PCA	0.89	4.50
PAD	0.98	3.22

CGA: chlorogenic acid, CFA: caffiec acid, FRA: ferulic acid, PCA: protocatechuic acid, PAD: protocatechuic aldehyde, FFPM: Fufang-Pugongying-Mixture. *a*) Means of five determinations.

Table 3. Contents of CGA, CFA, FRA, PCA and PAD in FFPM Preparations with Three Batch Numbers

Marker compound	Content[a]±S.D. (mg/ml)		
	Batch I	Batch II	Batch III
CGA	1.738±0.017	1.627±0.083	1.565±0.060
CFA	0.126±0.003	0.109±0.004	0.131±0.005
FRA	0.113±0.003	0.104±0.004	0.108±0.003
PCA	0.853±0.045	0.592±0.024	0.701±0.026
PAD	0.372±0.006	0.269±0.008	0.223±0.009

CGA: chlorogenic acid, CFA: caffiec acid, FRA: ferulic acid, PCA: protocatechuic acid, PAD: protocatechuic aldehyde, FFPM: Fufang-Pugongying-Mixture. *a*) Means±standard deviation from five determinations.

Applications of the Method The developed method was applied to analyze the FFPM preparations with three-batch numbers. Because the three-batch FFPM preparations were produced by a same manufacturer (Animal Pharmaceutical factory, Institute of Traditional Chinese Veterinary Medicine, CAAS), and the herbal materials came from same sources, the significant difference can be avoided, shown in Table 3.

The contents of CGA, CFA in Taraxacum and Lonicera, and the content of FRA in Angelica vary along with different source.[36—38] The method was used to analysis of the Taraxacum, Lonicera and Angelica from same source, the results (Table 4) approached the previous reports, [36—38] it has reference value for quantitative analysis of Taraxacum, Lonicera and Angelica, or study of the content change of Taraxacum,

Table 4. Quantitantive Analysis of Taraxacum, Lonicera and Angelica

Herb name	Growth place	Content[a)]±S.D. (%)		
		Caffeic acid	Chlorogenic acid	Ferulic acid
Taraxacum	Dongbei China	0.0203±0.0005	0.4305±0.0209	
Lonicera	Pingyi Shandong China	0.0121±0.0003	4.032±0.0781	
Angelic	Minxian Gansu China			0.0475±0.0016

a) Means±standard deviation from five determinations.

Lonicera and Angelic with different collection place and time.

Comparing with other conventional methods, we built an analysis method more suiting for practical where analysis equipments or condition was limited. We selected economical testing method. The mobile phase had simple composition, and used isocratic elution mode instead of gradient elution mode that was often used to determination of more than two components; The way to change mobile phase, rather than usual photodiode array detector and interference trial, was applied to evaluate the reliability of detected peaks; Under the built analysis condition, the five marker compounds could be simultaneously build standard curves in same range of concentration, thus the operation steps were simplified.

Conclusions

A simple, practical and low-cost RP-HPLC method has been developed for the simultaneous determination of CGA, CFA, FRA, PCA and PAD in FFPM. The results demonstrated the method was highly specific, accurate and precision, it is promising of being used in quality control of FFPM. The method has reference value for quantitative analysis of Taraxacum, Lonicera and Angelica.

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