Simultaneous Determination of Chlorogenic Acid, Forsythin and Arctiin in Chinese Traditional Medicines Preparation by Reversed Phase-HPLC

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A reversed phase (RP)-HPLC method was established for simultaneous determination of chlorogenic acid, arctiin and forsythin in Yinqiao Jiedu Granules, which was a commonly used Chinese herbal medical preparation for treatment of rheum ailments. The determination was based on a gradient elution (A: 1% acetic acid, pH=3.0, B: methanol) on a C18 column and an automatic wavelength switching program, where 325 nm was used for chlorogenic acid and 280 nm for arctiin and forsythin, respectively. Good linearities were obtained over the range of 2—200 mg·l^{-1} for the 3 objective compounds. The spike recoveries were within 96.0—97.9%.

Key words chlorogenic acid; arctiin; forsythin; reversed phase (RP)-HPLC; herbal medical preparation; Yinqiao Jiedu Granules

Herbal materials are traditionally used as medicines, especially in Asia and Africa. As a common herbal medical preparation for treating rheum ailments, Yinqiao Jiedu Granules is made from 9 medical herbal materials, including *Flos Lonicerae*, *Fructus Forsythia*, *Fructus Arctii*, *Radix Platycodonis*, *Herbal Menthae*, *Herbal Schizonepetae*, *Radix glycyrrhizae*, *et al.*[1] Its active components are complicate, but the main ones are chlorogenic acid (CGA) from *Flos Lonicerae*, arctiin (ARC) from *Fructus Arctii*, and forsythin (FOR) from *Fructus Forsythia*. As a well-known natural antioxidant, CGA has attracted great attention for long time.[2] Early to 1969, Chassevent[3] reviewed its physiological and pharmacological activities. The ubiquitously accepted biomedical effects of CGA include antigenic and allergenic activities,[4] antimicrobial activity,[5,6] *etc.* ARC and FOR were also intensively researched, such as, the anti-tumor-promoting and anti-mutation activities,[7,8] hepatoprotective effect[9] and neuroprotective effect[10] of ARC, and the anti-inflammatory activity of FOR.[11,12]

In the updated Chinese Pharmacopoeia,[1] the quality control of Yinqiao Jiedu Granules is only based on qualitative identification using thin layer chromatography (TLC), but no method was recommended for quantitative determination of the active compounds. On the other hand, HPLC is used to quantify CGA, ARC, and FOR in their related herbal sources, respectively. The involved TLC method for qualitative identification of Yinqiao Jiedu Granules demands complicate pre-treating and developing steps and the reference materials of *Fructus Forsythia*, *Fructus Arctii*, and *Herbal Schizonepetae*, respectively, Therefore, it's very necessary for Yinqiao Jiedu Granules to establish a more reliable quality evaluation method, which should be based on quantitation of the main active components.

Among many approaches of quantitative measurement, chromatography and its hyphenated techniques have been the most effective and commonly used method for active compounds in medical herbs. TLC was the earliest used for routine qualitative and quantitative analysis of herbal medicines,[1,13] Cimpan and Gocan[14] reviewed HPLC techniques in medicinal plants analysis. Chromatography, especially HPLC, was also widely applied for determination of CGA in various kinds of samples (foods,[15—20] plant materials,[21—28] and biomedical materials[29,30]), ARC,[31—33] and FOR,[34] respectively. Tsai and coworkers[29] used HPLC with microdialysis to investigate the pharmacokinetics of unbound CGA. Nose and colleagues[31,32] researched structural transformation of ARC in rat gastrointestinal tract using HPLC. However, simultaneous determination of CGA, ARC and FOR in herbal medicines or medicinal preparation has been seldom reported. Luan and coworkers[35] applied a signal multiplier spectrophotometric method to determine CGA, FOR and baicalin in an injection made from medicinal herbs. Obviously, the sample matrix in their work was much simpler than that in the granular samples. Li and coworkers[36] measured baicalin, CGA and FOR in Chinese medicinal preparation by capillary electrophoresis. In this work, a RP-HPLC method was developed and successfully applied to the simultaneous determination of CGA, FOR and ARC in Yinqiao Jiedu Granules. The established method is promising of application for quality control of Yinqiao Jiedu Granules.

Experimental

Reagents and Materials The standards of CGA, ARC and FOR were purchased from the Chinese National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Methanol was purchased from the Tianjin Shield Company (Tianjin, China). Other reagents were of analytical grade or better from commercial availability. Double distilled water was used throughout.

Apparatus The chromatographic separation was carried out using a Waters 510 HPLC system (MA, U.S.A.), consisting of two Waters 510 delivery pumps with a pump control module (PCM), a temperature control module (TCM), a Waters 486 Tunable Absorbance Detector (wavelength range 190—600 nm). A personal computer with a chromatography manager Millenium 2010 was used to control the equipments, and to record the chromatograms. A Rheodyne 7725*i* manual injector (CA, U.S.A.) was used.

A Johnsson C18 column (150×4.6 mm, Nucleosil C18 packings in 5 μm, Dalian, China) was used for separation.

An SB3200 Ultrasonic Generator (50 kHz, 120 W) from the Shanghai Branson Ultrasonics Co. Ltd. (Shanghai, China) was used to extract the objective compounds in the samples, as well as to degas the mobile phase and sample solutions.

Conditions for Chromatographic Separation The mobile phase was methanol and 1% acetic acid (pH=3.0) at a flowrate of 1.0 ml/min in a gradient mode. The column was thermostated at 30 °C. A wavelength program was used for the detection, in which the detection wavelengths were automatically switched from 325 nm to 280 nm at a proper time. The injection volume was 20 μl.

Experimental Procedure The chromatography system was equilibrated by the mobile phase. When same retention times and peak areas of repetitive

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Table 1. Effect of Ultrasonication Time on the Extraction Completeness

	Ultrasonication time (min)					
	10	20	25	30	40	50
Measured CGA ($mg \cdot g^{-1}$)	0.2544	0.3016	0.3254	0.3594	0.3601	0.3617
Measured ARC ($mg \cdot g^{-1}$)	0.9912	1.1032	1.2451	1.4682	1.4657	1.4700
Measured FOR ($mg \cdot g^{-1}$)	0.0476	0.0584	0.0701	0.0789	0.0776	0.0785

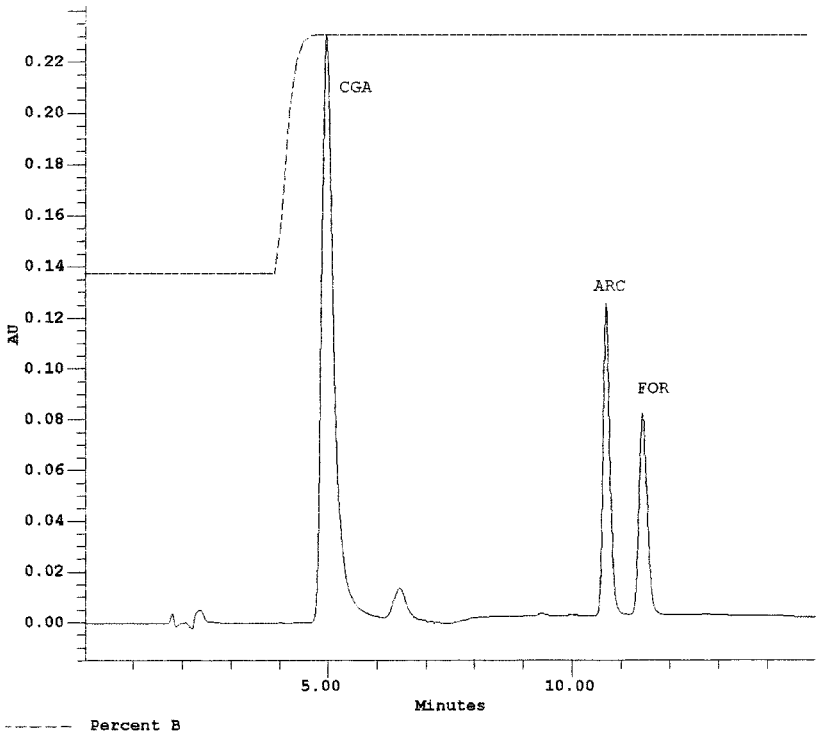

Fig. 1. A Chromatogram of the Standard Mixture of CGA, ARC and FOR
The broken line was the gradient curve of solvent B (methanol).

injections of standard solution (not less than 3 times) were observed, separation of sample could then be carried out. If not specified, the average result of triplicate injections was reported.

Sample Pretreatment The tested pharmaceutical samples were all from commercial availability.

An aliquot of 3-g ground sample (pass through 50 mesh screen) was ultrsonicated for 30 min in a stoppered conical beaker with 25 ml methanol as extracting solvent. The extract was filtered sequentially with filter paper and 0.45 μm filter membrane. The final filtrate was subject to chromatographic analysis.

Results and Discussion

Extraction of the Active Components Reflux and ultrasonication (both with methanol as extractant) were tested for extraction of the active components from the samples. Ultrasonication was found advantageous with respects to two facts, *i.e.*, clearness of the extract solution and reproducibility of the measurements. Experimental results in Table 1 showed that 30 min were sufficient for complete extraction. Thus in this work, 30-min. ultrasonication was used to extract the active components in the samples.

Parameters for Chromatographic Separation and Detection The great polarity difference among the objective compounds made the separation intractable. The pH value of mobile phase was a key factor for CGA retention, and relatively lower pH was beneficial for resolution of CGA. Under isocratic elution modes, it was in a dilemma to compromise resolution and sensitivity or the peak shapes. Gradient elution mode was therefore used. 1% acetic acid (pH=3.0) (A) and methanol (B) were used as eluents. The gradient procedure was carried out as follows: in the beginning, A : B was kept as 70 : 30 for 4.0 min, and then B up to 50% in 1.0 min by a linear gradient, and 50% B kept till the end of the run.

The maximal absorption wavelengths for CGA, ARC and FOR were 325 nm, 280 nm and 278 nm, respectively. In order to acquire the maximal detection sensitivities for all the 3 compounds, a wavelength program was used to automatically switch the detection wavelengths from 325 nm to 280 nm during the separation run, where 280 nm was set for both ARC and FOR at the 7th minute.

Linearities and Sensitivities of the Method Figure 1 was a chromatogram of the standard solution mixture under the chosen conditions. Over the range of 2—200 $mg \cdot l^{-1}$, good linearities were observed for CGA, ARC and FOR, respectively. The regression equations were as follows:

CGA $A=50936C+3662$ ($r=0.9999$, $n=6$)

ARC $A=14932C+2180$ ($r=0.9999$, $n=6$)

FOR $A=11234C+1943$ ($r=0.9999$, $n=6$)

In the equations, C was the compound concentration ($mg \cdot l^{-1}$), and A the corresponded peak area ($\mu V \cdot s$).

The limits of detection, defined as signal-to-noise ratio of 3, were 0.20 $mg \cdot l^{-1}$ CGA, 0.30 $mg \cdot l^{-1}$ ARC, and 0.30 $mg \cdot l^{-1}$ FOR.

Robustness and Recoveries of the Method Five 3-g samples from a same source were ultrasonicated and separated in parallel using the developed method, and the measured results were given in Table 2. The RSDs for CGA, ARC and FOR measurements were all less than 3.2%. The obtained average contents of the objective compounds would be used as the "real value" to calculate the spike recoveries in the followed tests.

Table 2. The Results of Parallel Measurements of CGA, ARC and FOR in Yinqiao Jiedu Granules Sample

Active compounds	Means ($mg \cdot g^{-1}$)	RSD (%) ($n=5$)
CGA	0.3679	3.11
ARC	1.4584	2.85
FOR	0.0785	2.81

Another four 3-g samples from the same source were spiked in different amounts of standard CGA and FOR, respectively, and also treated and separated using the developed method. Another four 1-g samples from the same source spiked in different amounts of ARC were also treated and separated as the same procedure at the meantime. The obtained recoveries were (97.4±0.95)%, (96.0±1.5)%, and (97.9±1.2)% for CGA, ARC, and FOR, respectively (Table 3).

Applications of the Method The developed HPLC method was applied to determine CGA, ARC and FOR in Yinqiao Jiedu Granules samples from different sources. Figure 2 showed a typical chromatogram of the samples. Although complicate matrix featured the chromatogram, no sig-

Table 3. The Results of Recoveries

Active compounds	Sample weights (g)	Active compounds in samples (mg)	Spiked compounds (mg)	Measured compounds (mg)	Recoveries (%)	Mean±S.D. (%)
CGA	3.0066	1.1061	2.5000	3.5545	97.9	97.4±0.95
	3.0108	1.1077	1.5000	2.5507	96.2	
	3.0093	1.1071	1.0000	2.0907	98.4	
	3.0155	1.1094	0.5000	1.5956	97.2	
ARC	1.0247	1.4944	2.5000	3.9017	96.3	96.0±1.5
	1.0185	1.4854	1.5000	2.9450	97.3	
	1.0175	1.4839	1.0000	2.4418	96.4	
	1.0214	1.4896	0.5000	1.9593	93.9	
FOR	3.0066	0.2360	0.5000	0.7273	98.3	97.9±1.2
	3.0108	0.2363	0.3000	0.5336	99.1	
	3.0093	0.2362	0.2000	0.4322	98.0	
	3.0155	0.2367	0.1000	0.3330	96.3	

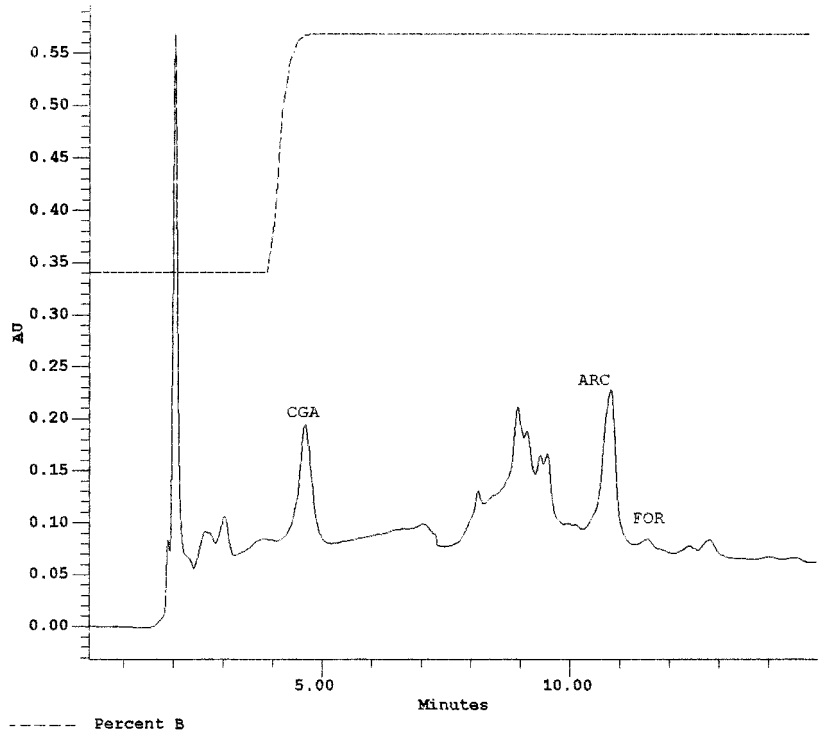

Fig. 2. A Typical Chromatogram of Yinqiao Jiedu Granules Sample

The broken line was the gradient curve of solvent B (methanol).

Table 4. The Measurement Results of CGA, ARC and FOR in the Yinqiao Jiedu Granules Samples

Sample sources[a)]	Lots No.	Active compounds	Present method		Modified reference method	
			Mean ($mg \cdot g^{-1}$)	RSD% ($n=4$)	Mean ($mg \cdot g^{-1}$)	RSD% ($n=3$)
I	000803	CGA	0.3679	3.11	0.3247	5.0
		ARC	1.4584	2.85	1.3822	4.2
		FOR	0.0785	2.81	0.0744	3.5
II	010307	CGA	0.4085	3.16	0.4112	3.9
		ARC	1.2315	2.84	1.2554	3.6
		FOR	0.0763	3.52	0.0771	4.2

a) I: Zhanjiang Xiangyan Pharmaceutics Manufacturer, China. II: Zhongshan Manufacturer of Traditional Chinese Medicines, China.

nificant interferences with the quantification were found at the retention times of the objective compounds. The measured results were listed in Table 4.

To validate the established method, the Granules sample were also analyzed using those pharmacopoeia reference methods for quantitative measurement of CGA, ARC and FOR in their herbal sources, respectively.[1)] However, because of the more complicated matrix in the Granules sample than that in the herbal materials, those pharmacopoeia methods couldn't be directly applied to the determination of the Granules sample, and must be necessarily modified. On the used Johosson C_{18} column, CGA was separated with nitrile : 0.4% phosphoric acid in 10 : 90 (V : V) (the pharmacopoeia method in 13 : 87) and detected at 325 nm, FOR was separated with nitrile : water in 20 : 80 (V : V) (the pharmacopoeia method in 25 : 75) and detected at 280 nm, ARC was separated with methanol : water in 40 : 60 (V : V) (the pharmacopoeia method in 48 : 52) and detected at 278 nm. The obtained results were also given in the Table 4, which showed agreement between the presented method and those modified reference methods. Obviously, the presented method was much simpler for the simultaneous measurement of their Granules sample.

Conclusion

The presented reversed phase (RP)-HPLC method, which involved a gradient elution and a detection wavelength program, for simultaneous determination of CGA, ARC and FOR, was rather simple, robust, and accurate. It was satisfactorily applied to simultaneously measure CGA, ARC and FOR in the Yinqiao Jiedu Granules from different sources. It is promising of being used in quality control of Yinqiao Jiedu Granules.

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