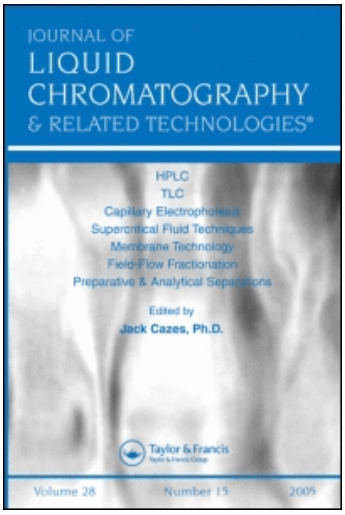

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DETERMINATION OF SIX BIOACTIVE COMPONENTS IN YU-PING-FENG-SAN BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

High performance liquid chromatography was employed to simultaneously determine the contents of six bioactive marker substances (cimifugin, *prim-O*-glucosylcimifugin, atractylon, atractylenolides I, II and III) in Yu-Ping-Feng-San. The separation was performed on a LiChrospher 100 RP-18e column by gradient elution with acetonitrile-water (v/v) (0 min, 16:84; 15 min, 20:80; 20 min, 50:50; 35 min, 55:45; 40 min, 75:25; 45 min, 80:20; 65 min, 85:15) as the mobile phase at a flow-rate of 1.0 mL/min, with detection at 220 nm or 277 nm. Benzophenone was used as the internal standard and six regression equations revealed linear relationships between the peak-area ratios (marker substances/internal standard) and concentrations. The repeatability and reproducibility (relative standard deviation) of the method were in the ranges of 0.03-1.07% and 0.13-1.21%, respectively.

INTRODUCTION

Chinese medicinal prescriptions, composed of crude drugs and usually decocted with boiling water, are very commonly used by Chinese herbal doctors to remedy diseases. The components of crude drugs vary according to the season, location, processing, and dispensing methods. Therefore, the composition, effectiveness, and safety of Chinese medicine manufacturing industry products are very important. There is an urgent need to establish a quantitative analysis method for the products used in Chinese medicine prescriptions. Yu-Ping-Feng-San (YPFS), a Chinese tonic medicinal prescription, has been used by traditional Chinese herbal doctors since the Yuan dynasty to boost qi. It fortifies the spleen, checking sweating, or against the external pathogens.[1] YPFS is composed of the root of *Astragalus mongolicus* Bunge (Leguminosae), the rhizome of *Atractylodes ovata* De Candolle (Compositae), and the root of *Saposhnikovia divaricata* (Turcz.) Schischk (Umbelliferae).

High performance liquid chromatographic (HPLC) methods have been developed for the determination of chromones and glycosides in the root of *S. divaricata*,[2] and gas chromatography or HPLC were also developed for the determination of atractylon, acetylene compounds, and atractylenolide III in the rhizome of *Atractylodes ovata.*[3-6] To date, the quantitative analysis of YPFS has not been reported. Therefore, the establishment of a rapid and simple HPLC analysis for quality control is a necessary and important task for the Good Manufacture Practice (GMP) pharmaceutical industry.

We report, herein, a simultaneous quantitative analysis using HPLC of the six bioactive constituents, (principle antihypertensive constituents [cimifugin and *prim-O*-gluco-sylcimifugin][7,8] of *S. divaricata,* the free radical scavenger [atractylon],[9] and the principle anti-inflammatory constituents [atractylenolides I, II, and III][10] of *Atractylodes ovata*) as marker substances in pulverized YPFS and concentrated YPFS.

EXPERIMENTAL

In accordance with "The Illustrated Chinese Materia Medica,"[11] the materials used to prepare a daily dose of YPFS are rhizome of *Atractylodes ovata* (6 g), root of *S. divaricata* (3 g), and root of *Astragalus mongolicus* (3 g).

Each dispensed material was purchased from the crude drug market in Taipei. Four different brands of concentrated YPFS and pulverized YPFS were purchased from four different GMP pharmaceutical industries in Taiwan.

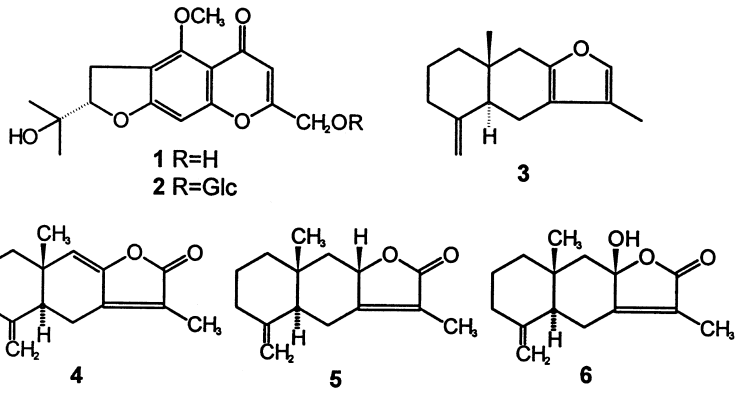

Figure 1. Structures of marker substances. 1: cimifugin, 2: *prim-O*-glucosylcimifugin, 3: atractylon, 4: atractylenolide I, 5: atractylenolide II, 6: atractylenolide III.

Chemicals and Reagents

The six marker substances were isolated by our institute, and their structures are shown in Figure 1. Cimifugin and *prim-O*-glucosylcimifugin were isolated from the root of *Saposhnikovia divaricata* (Turcz.) Schischk.[12] Atractylon, atractylenolides I, II, and III were isolated from the rhizome of *Atractylodes ovata* De Candolle.[13-14] Peak identification and purity checking of all the marker substances were done with photodiode-array detector. Benzophenone was purchased from Aldrich (St. Louis, MO, USA) for use as an internal standard. Acetonitrile was HPLC grade (Mallinckrodt, St. Louis, MO, USA). Ultrapure water, produced using the Milli-Q RG system (Millipore, Bedford, MA, USA) with a resistivity greater than 18 MΩ × cm, was used.

HPLC Analysis

The HPLC system consisted of a Waters (Milford, MA, USA) Model 600E multi-solvent delivery system equipped with a Waters Model 477 autoinjector and a Waters Model 996 photodiode-array detector. Peak areas were calculated with Waters Millennium 2010 chromatography manager software.

The mobile phase was composed of acetonitrile-water (v/v) with gradient elution (0 min, 16:84; 15 min, 20:80; 20 min, 50:50; 35 min, 55:45; 40 min, 75:25; 45 min, 80:20; 65 min, 85:15). The solvents were filtered through a 0.45-μm FP Vericel (PVDF) membrane filter (Gelman Sciences, Michigan, USA) and degassed with helium gas prior to use. A LiChrospher 100 RP-18e

reversed-phase column (250 × 4 mm I.D.) and a LiChrospher 100 RP-18e guard column (4 × 4 mm I.D.)(Merck, Darmstadt, Germany) were used. The flow-rate was 1.0 mL/min with UV absorbance detection at 220nm or 277nm. The analysis involved 20 μL of sample solution. The operating temperature was maintained at 35°C.

Preparation of Standard Solution

Cimifugin, *prim-O*-glucosylcimifugin, atractylon, atractylenolides I, II, and III were accurately weighed and dissolved in methanol to give serial concentrations in the ranges 50-0.5, 50-0.5, 50-0.5, 20-0.2, 50-0.5, and 50-0.5 μg/mL, respectively. Benzophenone had been added to each standard solution to give a concentration of 50 μg/mL. Different wavelengths were selected to quantify the amount of atractylenolide I at 277 nm or cimifugin, *prim-O*-glucosylcimifugin, atractylenolides II, III, and atractylon at 220nm. Calibration graphs were plotted by linear regression of the peak-area ratios (marker substance/internal standard) with the concentration of each marker substance.

Standard Decoction of YPFS

Amounts of crude drugs equivalent to a daily dose of YPFS were mixed and pulverized by blender, a twenty-fold mass of water was added, and the mixtures were boiled for more than 30 min to halve the original volume. After filtration, while hot, a suitable amount of internal standard was added to the filtrate to give a concentration of 50 μg/mL of benzophenone. The solution was then filtered through the 0.45 μm filter and analysed by HPLC.

Test Solution of Pulverized YPFS

A daily dose of pulverized YPFS was weighed and extracted with a twentyfold mass of methanol for 30 min in an ultrasonic bath. After filtration, a suitable amount of internal standard was added to the filtrate to give a concentration of 50 μg/mL of benzophenone. The solution was then filtered through the 0.45 μm filter and analysed by HPLC.

Test Solution of Concentrated YPFS

A daily dose of each brand of concentrated YPFS from four GMP pharmaceutical industries in Taiwan was weighed. It was then ultrasonicly extracted with a twentyfold mass of methanol for 30 min. After filtration, the

appropriate amount of internal standard was added to the filtrate to give a concentration of 50 μg/mL of benzophenone. The solution was then filtered through the 0.45 μm filter and analysed by HPLC.

Recovery Tests

A daily dose of both the concentrated YPFS and the pulverized YPFS from the market was weighed accurately and extracted with methanol for 30 min in an ultrasonic bath. The filtrate was spiked with standard solutions to introduce various concentrations of cimifugin (12.5 μg/mL), *prim-O*-glucosylcimifugin (12.5 μg/mL), atractylenolide I (5.0 μg/mL), atractylenolide II (12.5 μg/mL), atractylenolide III (12.5 μg/mL), or atractylon (12.5 μg/mL), and internal standard was then added to give a concentration of 50 μg/mL of benzophenone. Each sample was filtered through a 0.45 μm filter and injected for HPLC analysis to calculate the recovery.

RESULTS AND DISCUSSION

The dosage forms of traditional Chinese medicine prescription products are decoction, pill, powder, ointment, syrup, and tincture. Commonly used “san” is a preparation of crude drugs pulverized into a fine powder to be taken orally with water. Recently, commercially prepared concentrated prescription products have replaced the traditional prescriptions used in Chinese hospitals due to the commercial products' convenience and ease of preparation. However, there is not a reasonable method to determine the comparability between traditional and concentrated prescriptions. In this paper, we used the HPLC method to compare the different content of six active components between those two kinds of Chinese medicine prescriptions.

Analytical Conditions

Chinese medicinal prescriptions are a combination of crude drugs. Several active components, from polar to nonpolar, will be present in one prescription. YPFS contained a large array of active components from hydrophilic glycoside (i.e. *prim-O*-glucosylcimifugin) to hydrophobic sesquiterpenoids (i.e., atractylon) making the analysis difficult.

In addition to the difference in polarity between six active components, the great differences between their concentrations in the water extracts of YPFS made simultaneous determination of each compound with isocratic mobile phase very difficult. Therefore, according to Wang et al. report,[2] we used a methanol-

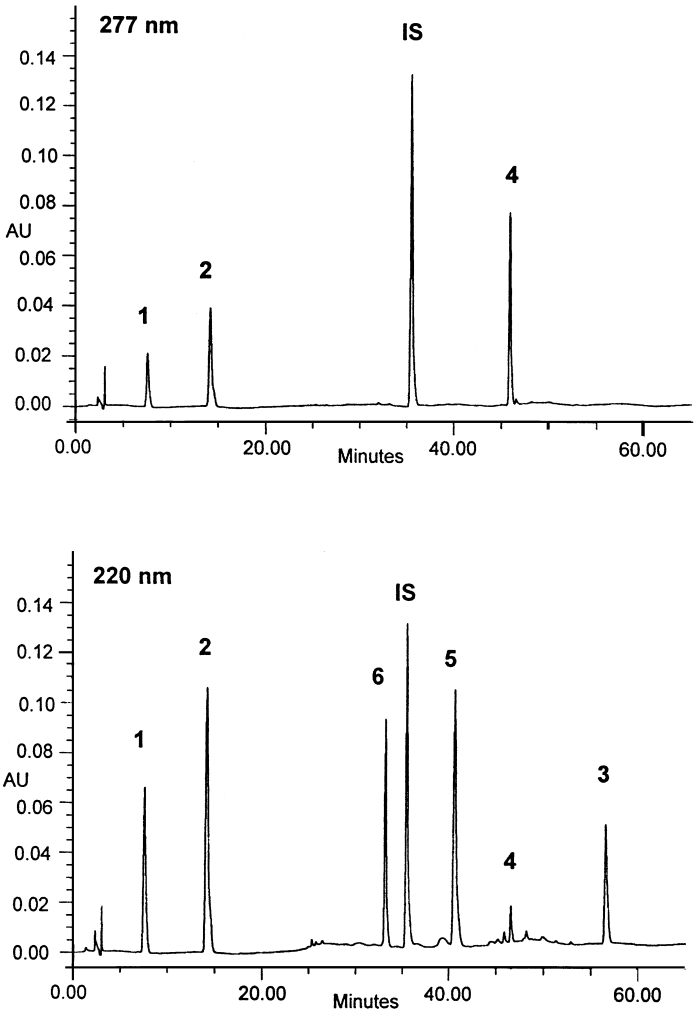

Figure 2. HPLC chromatogram of marker substances. Column, LiChrospher 100 RP-18e, 250x4mm I.D.; guard column, LiChrospher 100 RP-18e, 4x4mm I.D., mobile phase, acetonitrile-water (0 min, 16:84; 15 min, 20:80; 20 min, 50:50; 35 min, 55:45; 40 min, 75:25; 45 min, 80:20; 65 min, 85:15); flow-rate, 1.0 mL/min. Benzophenone was used as the internal standard. 1: cimifugin, 2: *prim-O*-glucosylcimifugin, 3: atractylon, 4: atractylenolide I, 5: atractylenolide II, 6: atractylenolide III.

Table 1

Calibration Equation of Marker Substances

Marker Substance	Calibration Equation	Correlation Coefficient (r)*
Prim-O-glucosycimi-fugin	Y=0.0022X+0.0209	0.9959
Cimifugin	Y=0.0441X-0.0062	0.9999
Atractylenolide I	Y=0.0199X-0.0062	0.9995
Atractylenolide II	Y=0.0406X+0.0014	0.9974
Atractylenolide III	Y-0.0232X+0.0115	0.9981
Atractylon	Y=0.0196X-0.0084	0.9989

* n = 3.

water gradient system to analyze YPFS. However, methanol use in HPLC has the disadvantage of producing a relatively highly viscous mixture with water, giving rise to much higher pressures than other mobile phases. High back pressure may cause the damage of column and HPLC system. Acetonitrile is very expensive, but viscosity properties cause no problems. Thus we used an acetonitrile-water gradient system to replace the methanol-water gradient system. Because the polarity of acetonitrile is lower than methanol, the elution power in reversed-phase HPLC increased and shortened the analysis time.

The selection of marker substances in this study was based on the bioactivities of each crude drug mentioned previously. The UV absorbance maxima of most marker substances, except for atractylenolide I, are within the range of 210-230nm. Thus we selected 220nm as the detection wavelength. For the detection of atractylenolide I, the wavelength must change to 277nm for the best resolution and sensitivity. Therefore, use of a photodiode-array detector was the best choice for simultaneous quantitative analysis of the six marker substances (Figure 2). Each marker substance had a remarkable correlation coefficient of calibration equation (Table 1). The retention times of the marker substances and internal standard were 7.7, 14.4, 33.2, 36.6, 41.4, 45.9, and 56.7 min, for *prim-O*-glucosylcimifugin, cimifugin, atractylenolide III, atractylenolide II, atractylenolide I, and atractylon, respectively. On inspection of the three-dimensional chromatograms, these constituents all demonstrated high purity. The relative standard deviation of intra-day and inter-day marker components in the pulverized YPFS and the standard decoction of YPFS are between 0.03-1.07% and 0.13-1.21%, respectively (Table 2). This indicated that the repeatability and reproducibility of this method were satisfactory.

Table 2

The Interday and Intraday Relative Standard Deviations for the Marker Substances in Pulverized YPFS and a Standard Decoction of YPFS

Marker Substance	Pulverized YPFS* Mean (mg/mL)	Interday** R.S.D.(%)	Intraday** R.S.D.(%)	Standard Decoction of YPFS* Mean (mg/mL)	Interday** R.S.D.(%)	Intraday** R.S.D. (%)
Prim-O-glucosylcimifugin	0.56	1.08	0.93	1.50	0.97	0.78
Cimifugin	1.08	0.17	0.14	2.55	1.21	0.92
Atractylenolide III	1.04	1.18	1.07	0.95	0.94	0.43
Atractylenolide II	1.41	0.26	0.13	0.29	0.55	0.23
Atractylenolide I	0.66	0.13	0.03	0.06	0.76	0.38
Atractylon	9.33	0.13	0.11	0.25	0.18	0.15

*n = 3.
** R.S.D.: relative standard deviation.

Table 3

Recovery of the Marker Substances in a Standard Decoction of YPFS

Marker Substance	Added (M=μg/mL)	Found (μg/mL)	Recovery Mean(%)±S.D.*	R.S.D. (%)
Prim-O-glucosylcimifugin	12.5	13.05	104.39±1.13	1.08
Cimifugin	12.5	11.93	95.46±0.17	0.17
Atractylenolide III	12.5	11.12	88.95±1.05	1.18
Atractylenolide II	12.5	11.64	93.13±0.25	0.26
Atractylenolide I	5.0	4.43	88.59±0.12	0.13
Atractylon	12.5	8.69	69.50±0.09	0.13

*n = 5. S.D.: standard deviation. R.S.D.: relative standard deviation.

Otherwise, the recovery of each marker substance in the standard decoction of YPFS was more than 88.59%, except for atractylon (Table 3), but in the interday variety of the content of atractylon in the pulverized YPFS and the standard decoction of YPFS it did not change significantly. Atractylon is unstable in a pure state and it will auto-oxidize to produce atractylenolides II and III.[15] The poor recovery of atractylon may be due to the volatile and auto-oxidative property of atractylon when decocted with boiling water.

Table 4

Contents of Marker Substances in Four Brands of Pulverized YPFS and Concentrated YPFS (mg/ Daily Dose)

Marker Substance	Pulverized YPFS				Concentrated YPFS			
	No. 1	No. 2	No. 3	No. 4	No. 1	No. 2	No. 3	No. 4
Prim-O-glucosylcimifugin	11.62	5.19	25.41	16.84	4.41	5.64	2.25	2.52
Cimifugin	16.24	27.33	12.73	8.96	6.66	0.45	2.43	3.99
Atractylenolide III	5.36	8.20	11.56	10.68	7.35	1.20	1.56	1.83
Atractylenolide II	0.73	3.00	0.78	1.73	4.50	0.99	1.32	1.26
Atractylenolide I	---	0.79	0.44	0.40	0.99	0.75	0.69	1.35
Atractylon	10.72	1.40	---	0.50	0.48	0.36	0.54	0.60

"---" undetectable.

Quantitative Determination of the Bioactive Constituents of YPFS

Comparison of the contents of the marker substances in commercial pulverized and concentrated products with the standard decoction of YPFS showed significant variety (Table 4). This is probably due to the different origins of the crude drugs, seasonal variations, and varied storage conditions, manufacturing process methods, and dosage forms.

The amounts of atractylon and atractylenolide I in the standard decoction and commercial concentrated YPFS were lower than those in pulverized YPFS. When MeOH was used as a solvent and ultrasound was used to extract the traditional pulverized YPFS, the amounts of both atractylon and atractylenolide I increased (Figure 3). This was caused by the poor solubility, the volatility, and the oxidative properties of atractylon during boiling water extraction. On the other hand, the amounts of *prim-O*-glucosylcimifugin and cimifugin increased by boiling water extraction.

The traditional Chinese medicine dosage form "san" is a pulverized drug preparation, but modern, commercially available concentrated Chinese medicine prescriptions are usually extracted with boiling water and the decoction is then sprayed or freeze-dried. However, some of the poorly water-soluble constituents remain in the residue and are discarded. We found that all the constituents of YPFS could be extracted by boiling water but were more efficiently extracted by MeOH. This clarification of the difference in pharmacological effects between the two kinds of extraction is noteworthy.

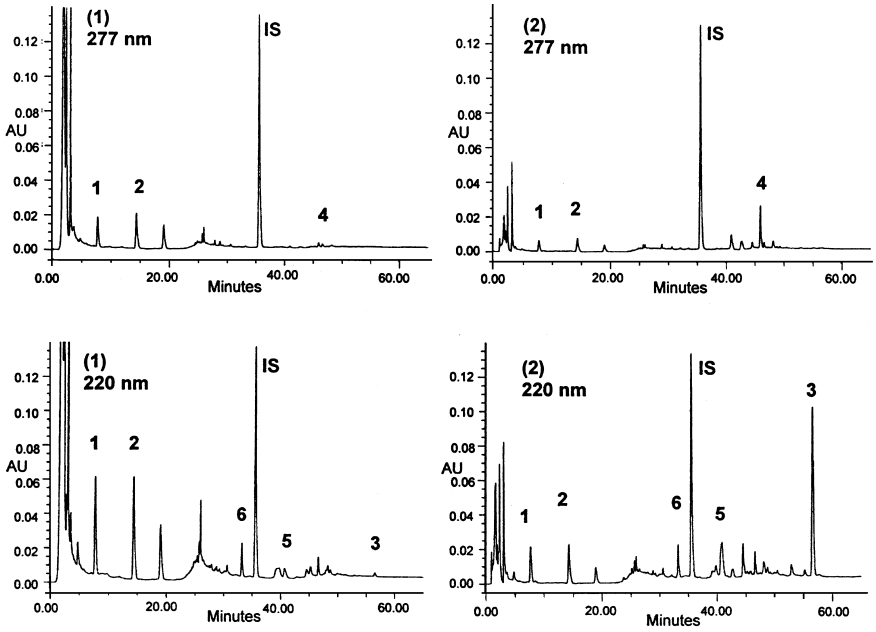

Figure 3. Compare the difference between (1) concentrated YPFS and (2) pulverized YPFS extract. Condition as in Figure 2.

This HPLC method can rapidly and simply quantify the six bioactive components, *prim-O*-glucosylcimifugin, cimifugin, atractylon and atractylenolides I, II, III present in YPFS and can be used for quality control of commercial YPFS.

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