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Simultaneous Analysis of Nine Components in "Byi-Liang-Tang" Preparation by High Performance Liquid Chromatography

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ABSTRACT

Simultaneous determination of nine marker substances was established for the quality control of "Byi-Liang-Tang" by high performance liquid chromatography (HPLC). These marker substances include ferulic acid from Cnidii Rhizoma, paeoniflorin from Paeoniae Radix, glycyrrhizin from Glycyrrhizae Radix, cinnamic acid and cinnamaldehyde from Cinnamomi Cortex, puerarin and daidzin from Puerariae Radix, and baicalin and baicalein from Scutellariae Radix.

Extracted samples were analyzed with reverse-phase column (Inertsil 5 ODS-2, 4.6 mm i.d. × 250 mm) at 30˚C and eluted with a mixture of 20%, 50% and 90% acetonitrile (adjusted to pH 3.0 with phosphoric acid) aqueous solution in gradients at a flow-rate of 1.0 mL/min, and detected at 230 nm.

Relative coefficients of variations of intra- and inter-day analysis were less than 5% and the relative errors were below 2.63% and 1.81%, respectively. All the recoveries were 90~108.67%. This method could be applied for the simultaneous determination of nine marker substances in "Byi-Liang-Tang" and comparisons to commercially preparations.

Key words: Byi-Liang-Tang, ferulic acid, paeoniflorin, glycyrrhizin, cinnamic acid, cinnamaldehyde, puerarin, daidzin, baicalin, baicalein

INTRODUCTION

A number of analytical methods for Chinese medicinal preparations have been established in several laboratories including ours in recent years[(1-11)]. In this study, we selected Byi-Liang-Tang, one of the most popular Chinese medicines in Taiwan, which contains Cnidii Rhizoma, Paeoniae Radix, Glycyrrhizae Radix, Cinnamomi Cortex, Puerariae Radix, Scutellariae Radix, Ephedrae Herba, Zingiberis Rhizoma, Zizyphi Fructus, Platycodi Radix, Magnoliae Flos, and Gypsum Fibrosum[(12)]. This traditional mixture preparation is known to colonies cure nasal inflammation in conjunction with yellowish and fowl nasal discharge[(12)]. Literature search revealed that there is no analytical method reported for Byi-Liang-Tang. The goal of this study is to assay the nine marker substances simultaneously using a HPLC method. Markers included ferulic acid from Cnidii Rhizoma, paeoniflorin from Paeoniae Radix, glycyrrhizin from Glycyrrhizae Radix, cinnamic acid and cinnamaldehyde from Cinnamomi Cortex, puerarin and daidzin from Puerariae Radix, and baicalin and baicalein from Scutellariae Radix. After optimization, this analytical method was applicable to the commercial preparation Byi-Liang-Tang of concentrated.

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MATERIALS AND METHODS

I. *Materials*

The materials for Byi-Liang-Tang preparation are Cnidii Rhizoma (*Ligusticum chuanxiong* Hort), Paeoniae Radix (*Paeonia lactiflora* Pall), Glycyrrhizae Radix (*Glycyrrhiza glabra* L.), Cinnamomi Cortex (*Cinnamomum cassia* Presl), Puerariae Radix (*Pueraria lobota*), Scutellariae Radix (*Scutellaria baicalensis* Georgi), Ephedrae Herba (*Ephedra sinica* Stapf), Zingiberis Rhizoma (*Zingiber officinale* Rosc), Zizyphi Fructus (*Ziziphus jujube* Mill), Platycodi Radix (*Platycodon grandiflorum*), Magnoliae Flos (*Magnolia biondii* Pamp), and Gypsum Fibrosum. Each material was obtained from the local herbal market and pulverized through a #8 mesh sieve (2.36 mm). The origin of their crude drugs were identified by microscopic and TLC examination. Voucher specimens were deposited in the department of Plant Industry, National Pingtung University Science and Technology. For the commercially concentrated preparation was obtained from Chuang Song Zong Pharmaceutical Co., Ltd.

II. *Chemicals and Reagents*

The structures of the 9 marker substances are shown in Figure 1. Puerarin, daidzin, and internal standard propyl-4-

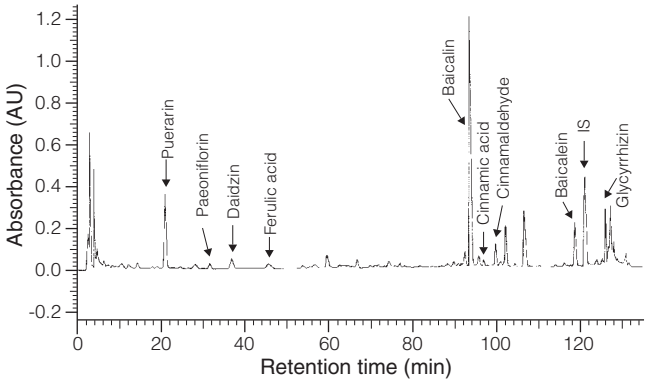

Figure 1. HPLC chromatogram of markers in standard solutions IS: Propyl-4-hydroxybenzoate.

hydroxybenzoate were purchased from Sigma (St. Louis, Mo, U.S.A.). Ferulic acid, cinnamic acid and cinnamaldehyde were purchased from Fluka (Switzerland), and glycyrrhizin was purchased from Yoneyama (Japan). Paeoniflorin, baicalin and baicalein were purified and identified from Paeoniae Radix and Scutellariae Radix in our laboratory.

Rice wine (19.5% ethanol) and 95% ethanol were from Taiwan Tobacco and Wine Board. Acetonitrile and methanol (HPLC grade) were obtained from Mallinckrodt (U.S.A.), and phosphoric acid was obtained from Kanto (Japan). Ultra-pure distilled water with a resistivity greater than 18 MΩ was obtained from a Millipore mini-Q system (Bedford, MA, U.S.A.). Samples for HPLC were filtered through a 0.45 μm Millipore membrane filter (Bedford, MA, U.S.A.). All other reagents were analytical grade.

III. Preparation of Standard and Internal Standard Solutions

(I) *Preparation of standard solution*

Ten mg, 10.0 mg, 2.0 mg, 2.0 mg, 10.0 mg, 2.0 mg, 40.0 mg, 10.0 mg, and 16.0 mg of puerarin, paeoniflorin, daidzin, ferulic acid, baicalin, cinnamic acid, cinnamaldehyde, baicalein, and glycyrrhizin were dissolved in 70% methanol to give sequential concentrations 400.0 μg/mL, 400.0 μg/mL, 80.0 μg/mL, 80 μg/mL, 400.0 μg/mL, 80 μg/mL, 1,600 μg/mL, 400.0 μg/mL and 640.0 μg/mL, respectively.

(II) *Preparation of internal standard solution*

Propyl-4-hydroxybenzoate (33.0 mg) was dissolved in 70% methanol to a volume of 250 mL (132 μg/mL).

IV. *Preparation of Standard Decoction*

The standard decoction of Byi-Liang-Tang contains 4.0 g of Puerariae Radix, 1.5 g of Cnidii Rhizoma, 1.5 g of Paeoniae Radix, 1.0 g of Glycyrrhizae Radix, 1.5 g of Cinnamomi Cortex, 1.5 g of Scutellariae Radix, 2.0 g of Ephedrae Herba, 2.0 g of Zingiberis Rhizoma, 2.0 g of Zizyphi Fructus, 1.5 g of Platycodi Radix, 1.5 g of Magnoliae Flos, and 1.5 g of Gypsum Fibrosum[12]. To prepare a standard decoction, 21.5 g of the pulverized Chinese crude drugs listed above and 430 mL of water were added into a 1-L round flask and heated until only about 215 mL remained. Then, the hot solution was filtered by four layers of gauze, allowed to cool, and adjusted to 215 mL by adding water. A 1.0 mL of the solution was removed and diluted to 5 mL by adding methanol, while the internal standard solution was added to each solution to afford a concentration of 66.0 μg/mL. After filtration through a 0.45 μm membrane filter, the sample was subjected to HPLC analysis.

V. *Preparation of Solution of Commercial Concentrated Preparation*

One third of the daily dosage (2 g) of commercially concentrated preparation was weighed accurately and transferred into a 125-mL flask, then 100 mL of 70% methanol was added. After ultrasonic extraction at 30˚C for 30 min, the extract was filtered and evaporated under vacuum and reconstituted with 10 mL of 70% methanol, the internal standard solution was added to each solution to afford a concentration of 66.0 μg/mL. After filtration through a 0.45 μm membrane filter, the solutions were subjected to HPLC analysis.

IV. *HPLC Instrumentation and Analytical Conditions*

HPLC was equipped with a Hitachi system including a degasser DG-2410, pump L-7100, UV/Vis detector L-7420,

Table 1. Gradient elution program

Time (min)	Mobile phase A (%)	Mobile phase B (%)	Mobile phase C (%)
0	100	0	0
10	97	3	0
25	96	4	0
40	92	8	0
45	92	8	0
50	85	15	0
55	80	20	0
65	75	25	0
70	70	30	0
75	70	30	0
85	50	50	0
95	40	60	0
105	40	60	0
115	20	80	0
120	0	0	100
125	0	0	100
130	100	0	0

A: 20% acetonitrile (adjusted to pH 3.0 with phosphoric acid).
B: 50% acetonitrile (adjusted to pH 3.0 with phosphoric acid).
C: 90% acetonitrile (adjusted to pH 3.0 with phosphoric acid).

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photodiode array detector L-4500 and autosampler L-7200. Peak areas were calculated with a D-7000 HSM software. A reversed-phase column Cosmosil 5C18 AR-II (Nacalai, 4.6 mm i.d. × 250 mm) was used. The column oven was set at 30˚C. The mobile phases consisting of 20%, 50% and 90% acetonitrile aqueous solutions in gradient elution are shown in Table 1. Detection wavelength was set at 230 nm. Flow rate was set at 1.0 mL/min. Sample solution prepared as described above was injected into the HPLC column for analysis.

VII. *Calibration*

The standard solutions of each marker substance described in section III(I) were diluted with 70% methanol to give sequential concentrations of daidzin, ferulic acid, and cinnamic acid: 2.5, 5.0, 10.0, 20.0, 40.0 μg/mL; puerarin, paeoniflorin, baicalin, and baicalein: 12.5, 25.0, 50.0, 100.0, 200.0 μg/mL; glycyrrhizin: 20.0, 40.0, 80.0, 160.0, 320.0 μg/mL; cinnamaldehyde: 50, 100.0, 200.0, 400.0, 800.0 μg/mL.

Each calibrator contains the internal standard solution, 66 μg/mL propyl-4-hydroxybenzoate. After filtering through a 0.45 μm membrane filter, 20 μL of each concentration was injected into the HPLC column for analysis. The calibration curve was drawn by plotting the ratio of the peak areas of each marker versus the internal standard. Linear regression was used to calculate the parameters of y = ax+b and the correlation coefficient.

RESULTS AND DISCUSSION

I. *Separation of Markers*

The HPLC chromatogram of a standard decoction of Byi-Liang-Tang is shown in Figure 2. Puerarin, paeoniflorin, daidzin, ferulic acid, baicalin, cinnamic acid, cinnamaldehyde, baicalein, glycyrrhizin, and the internal standards were well separated. And their respective retention times were 21.3 min, 31.6 min, 37.3 min, 45.7 min, 93.9 min, 97.0 min, 99.8 min, 118.9 min, 126.1 min, respectively and that of the internal standard, propyl-4-hydroxybenzoate is 121.2 min.

The peak purities of components in the Byi-Liang-Tang were evaluated with a photodiode array detector where good peak purities were shown irrespective of each component (Figure 2). Apparently, there was no interaction between the components of Byi-Liang-Tang; therefore, the above conditions can be used for the quantification of these marker substances.

II. *Calibration Lines*

The linear regression equations, correlation coefficients and concentration range of calibration lines for those marker substances were listed in Table 2. All calibration curves of the 5 ingredients were in good linear correlation with correlation coefficient of 0.9995-0.9999.

III. *Precision and Accuracy*

Using the standard solutions with various concentrations shown in Table 3, an intra-day test (repeat five times), and inter-day test (four repeats during 7 days, injection interval at least 24 hr) were used to check for reproducibility. Coefficients of variation (CV) values of different concentrations of the nine marker substances were less than 5% and the relative errors (RE) were below 2.63% and 1.81%, respectively, suggesting that this method had very good reproducibility.

A series of various concentrations of each standard were spiked into concentrated commercial preparation of Byi-Liang-Tang sample solution, then extracted to test solutions as described in section V. Internal standard solution was added to each solution to afford a concentra-

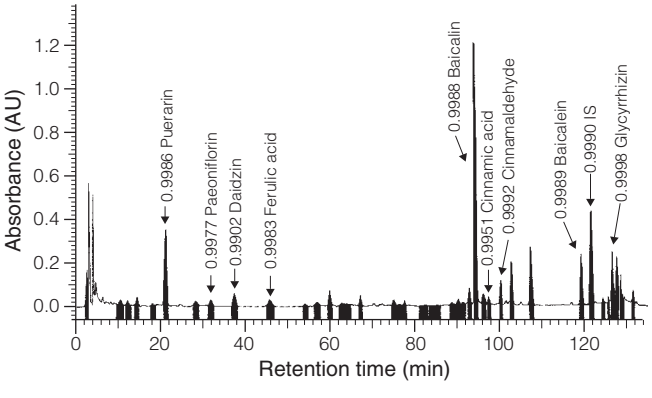

Figure 2. Peak purities of each marker in Byi-Liang-Tang IS: Propyl-4-hydroxybenzoate.

Table 2. Calibration curves of marker substances

Compound	Concentration μg/mL	Regression equation	r	n
Puerarin	12.5-200.0	y = 0.2442x + 0.028	0.9998	5
Paeoniflorin	12.5-200.0	y = 0.02x – 0.0107	0.9995	5
Daidzin	2.5-40.0	y = 0.0426x + 0.002	0.9998	5
Ferulic acid	2.5-40.0	y = 0.0539x + 0.0066	0.9997	5
Baicalin	50.0-800.0	y = 0.3208x + 0.0083	0.9998	5
Cinnamic acid	2.5-40.0	y = 0.1507x + 0.0418	0.9999	5
Cinnamaldehyde	50.0-800.0	y = 0.1507x + 0.0418	0.9997	5
Baicalein	12.5-200.0	y = 0.1624x – 0.081	0.9999	5
Glycyrrhizin	20.0-320.0	y = 0.0539x + 0.0066	0.9997	5

Table 3. Precision and accuracy of intra-day and inter-day analysis of marker substances

Compound	Concentration (μg/mL)	Intra-day (n = 3)		Inter-day (n = 4)	
		Precision CV (%)	Accuracy RE (%)	Precision CV (%)	Accuracy RE (%)
Puerarin	12.50	0.94	-5.92	0.94	-4.29
	50.00	0.32	0.20	1.47	-1.06
	200.00	0.68	-0.40	0.71	-3.49
Paeoniflorin	12.50	1.99	-3.56	3.91	-0.03
	50.00	0.03	0.57	2.14	0.37
	200.00	0.19	-3.47	2.90	-4.37
Daidzin	2.50	1.28	-4.05	2.71	-0.09
	10.00	0.28	-1.61	1.58	0.13
	40.00	0.74	-0.24	0.67	-3.24
Ferulic acid	2.50	0.14	-1.02	3.38	1.81
	10.00	0.02	1.87	1.95	-0.04
	40.00	0.39	1.51	0.68	-1.44
Baicalin	50.00	0.60	-4.66	1.47	-1.21
	200.00	0.30	-0.89	1.26	-0.51
	800.00	0.75	-0.52	0.23	-3.65
Cinnamic acid	2.50	0.24	-7.69	2.12	-2.78
	10.00	0.06	-0.09	0.23	-0.52
	40.00	0.12	-1.68	1.86	-8.24
Cinnamaldehyde	50.00	0.47	2.63	2.15	-3.45
	200.00	0.36	1.09	1.41	0.32
	800.00	0.99	-2.12	2.28	-8.69
Baicalein	12.50	0.90	-7.47	0.73	-1.21
	50.00	0.39	-3.42	1.46	-0.51
	200.00	0.53	-3.08	1.01	-3.65
Glycyrrhizin	20.00	0.94	-5.01	0.94	-1.82
	80.00	0.32	-0.36	1.47	-0.85
	320.00	0.68	-1.18	0.71	-3.63

Table 4. Recovery of marker substance in Byi-Liang-Tang

Compound	Concentration (μg/mL)	Recovery[a] (%)
Puerarin	12.50	90.00 ± 2.36
	50.00	99.12 ± 0.41
	200.00	104.26 ± 1.99
Paeoniflorin	12.50	100.74 ± 0.13
	50.00	103.06 ± 1.33
	200.00	99.58 ± 0.45
Daidzin	2.50	92.80 ± 0.22
	10.00	96.20 ± 0.31
	40.00	108.67 ± 2.05
Ferulic acid	2.50	90.00 ± 0.94
	10.00	100.90 ± 0.21
	40.00	108.75 ± 1.85
Baicalin	50.00	97.50 ± 0.01
	200.00	99.50 ± 0.91
	800.00	101.25 ± 1.68
Cinnamic acid	2.50	98.80 ± 1.47
	10.00	107.10 ± 3.28
	40.00	102.70 ± 0.26
Cinnamaldehyde	50.00	103.76 ± 0.15
	200.00	104.11 ± 0.23
	800.00	103.94 ± 0.68
Baicalein	12.50	98.51 ± 0.17
	50.00	104.80 ± 0.15
	200.00	103.35 ± 0.26
Glycyrrhizin	20.00	105.85 ± 1.19
	80.00	107.79 ± 2.30
	320.00	108.18 ± 1.31

[a]Mean ± SD, n = 3.

Table 5. Content of marker substance in commercial preparation of Byi-Liang-Tang

Compound	Commercial preparation[a]
Puerarin	38.48 ± 1.06
Paeoniflorin	5.71 ± 1.08
Daidzin	6.58 ± 1.30
Ferulic acid	2.76 ± 0.81
Baicalin	50.97 ± 1.11
Cinnamic acid	5.86 ± 1.99
Cinnamaldehyde	22.42 ± 1.95
Baicalein	25.35 ± 1.05
Glycyrrhizin	7.94 ± 1.73

[a]Data represented as mean (mg/g) ± SD, n = 7.

tion of 66.0 μg/mL. All samples were filtered through a 0.45 μm membrane filter, injected into the HPLC column for analysis and the recoveries were calculated. Recoveries of the analysis were as shown in Table 4. All of the recoveries are in the range of 90-108.67%.

IV. *Analysis of the Commercial Concentrated Preparation*

The commercial concentrated preparation showed similar chromatogram as the standard decoction. Therefore, it could be used for simultaneous quantification of the nine marker substances in commercial concentrated preparation. The results showed that the measured values of marker substances were 2.76-38.48 mg/g (Table 5).

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