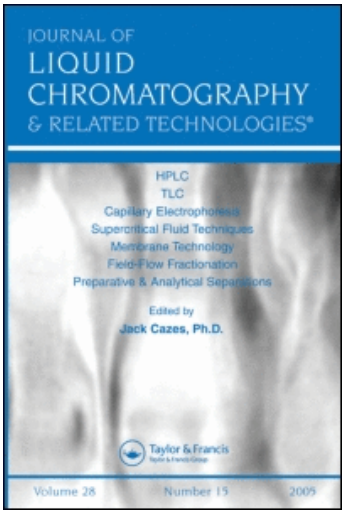

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DETERMINATION OF PARISHIN, PARISHINS B AND C IN TRADITIONAL CHINESE MEDICINAL PREPARATIONS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

High performance liquid chromatographic methods were established for the determination of parishin, parishins B and C in three kinds of the traditional Chinese medicinal prescriptions, including Gastrodiae Rhizoma: Shuen-Feng-Iun-Chi-San, Ban-Shia-Bai-Ju-Tian-Ma-Tang and Tsan-Siang-Tian-Ma-Tang. The samples were analyzed with an Inertsil ODS-2 reversed phase column by gradient elution with varied 0.1% (v/v) phosphoric acid-acetonitrile as mobile phases and detected at UV 222 nm. Ethoxybenzamide was used as an internal standard. Regression equations were derived showing linear relationships (correlation coefficients: 0.9976-0.9999) between the peak area ratios of each marker (parishin, parishins B and C) to ethoxybenzamide and concentration. The recoveries of parishin, parishins B and C from the Chinese medicinal preparations were 102.0-104.1%, 102.9-105.0% and 103.2-104.0%, respectively.

The relative standard deviations of three marker constituents ranged between 0.90-2.47% (intraday) and 1.05-4.41% (interday). The contents of parishin, parishins B and C measured 1.58-2.85 mg/g, 1.58-3.25 mg/g and 1.45-1.72 mg/g, respectively.

INTRODUCTION

Gastrodiae Rhizoma (Tianma) is the dried rhizome of *Gastrodia elata* Blume (Orchidaceae) and is a commonly used Chinese herb. It exerts sedative and anticonvulsant actions and is used to treat vertigo, blackout, headache and hemiplegia.[1] Because traditional medicine is usually prepared by decoction, their active constituents may be contained in the polar fraction.

Therefore, we isolated three highly polar constituents: parishin (tris[4-(β-D-gluco-pyranosyloxy) benzyl] citrate), parishins B and C (1,2- and 1,3-bis[4-(β- D-glucopyranosyloxy) benzyl]citrate)[2] and used these as markers to evaluate the quality of Gastrodiae Rhizoma by high performance liquid chromatography.[3] The analytical method, however, for determination of these markers in Chinese medicinal prescriptions, has not been reported.

In this study, we selected three kinds of Chinese medicinal prescriptions, Shuen-Feng-Iun-Chi-San (hereafter abbreviated as P1), Ban-Shia-Bai-Ju-Tian-Ma-Tang (P2) and Tsan-Siang-Tian-Ma-Tang (P3), all contained in Gastrodiae Rhizoma and applied HPLC to develop suitable methods to determine the contents of three marker constiuents.

For determination of the markers, we employed the documented mobile phase,[3] which was a mixture of methanol and 0.1% (v/v) phosphoric acid. However, poor peak patterns and interference resulted and the precessing time was extensive. Therefore, several other kinds of mobile phases were investigated and a method using a phosphoric acid-acetonitrile eluent was found to be a more feasible way to perform the analyses.

EXPERIMENTAL

Materials

The materials used to prepare the traditional Chinese medicinal prescription were as follows:[4,5]

Shuen-Feng-Iun-Chi-San (P1): Gastrodiae Rhizoma, Ginseng Radix (3.75 g each), Atractylodis Ovatae Rhizoma (15 g), Linderae Radix (11.25 g), and Chaenomelis Lagenariae Fructus, Citri Viridi Pericarpium, Glycyrrhizae Radix, Aquilariae Lignum, Perillae Folium, Angelicae Dahuricae Radix (2.25 g each).

Ban-Shia-Bai-Ju-Tian-Ma-Tang (P2): Gastrodiae Rhizoma, Hordei Germinatus Fructus, Zingiberis Recens Rhizoma, Messa Medicata Fermentat (2.0 g each), Pinelliae Rhizoma, Citri Reticulatae Pericarpium, Atractylodis Ovatee Rhizoma, Atractylodis Rhizoma, Hoelen (3.0 g each), Astragali Radix, Ginseng Radix, Alismatis Rhizoma (1.5 g each) and Phellodendri Cortex, Zingiberis Rhizoma (1.0 g each).

Tsan-Siang-Tian-Ma-Tang (P3): Gastrodiae Rhizoma, Amomi Amari Fructus, Angelicae Radix, Aquilariae Lignum (2.0 g each), Angelicae Tuhuo Radix, Sileris Radix, Notopterygii Rhizoma, Pinelliae Rhizoma (3.0 g each), Glycyrrhizae Radix, Aconiti Tuber (1.0 g each) and Bombyx Batryticatus (1.5 grams).

All materials were obtained from markets in Taipei and pulverized. Three different commercial brands of concentrated herbal preparations of Ban-Shia-Bai-Ju-Tian-Ma-Tang were also purchased from the market.

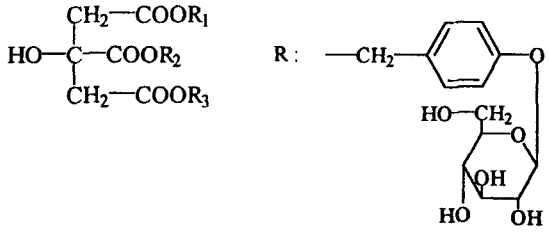
Chemicals and Reagents

Structures of the marker components are shown in Figure 1. Parishin, parishins B and C were isolated from the rhizome of *Gastrodia elata* and the internal standard ethoxybenzamide was obtained from Nacalai Tesque (Kyoto, Japan).

Acetonitrile (HPLC grade) was purchased from Labscan (Dublin, Ireland). Phosphoric acid was of analytilcal reagent grade. Ultrapure distilled water with a resistivity greater than 18 MΩ was used.

Instruments

HPLC was conducted with a Hitachi Model L-6200 Intelligent pump system equipped with a Hitachi Model L-3000 Photo Diode Array Detector and a Shimadzu SIL-9A autoinjector. An Inertsil ODS-2 reversed phase column (5μm, 250 X 4.6 m.m. I.D.) was used. Peak areas were calculated with a Shiunn Haw computing integrator.



Parishin B: R_1=R_2=R, R_3=H
Parishin C: R_1=R_3=R, R_2=H
Parishin: R_1=R_2=R_3=R

Figure 1. Structures of marker constituents.

Liquid Chromatography

The mobile phases for analysis of those prescriptions were the mixtures of 0.1% (v/v) phosphoric acid (A) and acetonitrile (13) with gradient elution (in linear shape) as follows:

Prescription	Flow Rate (mL/min)	Mobile Phase							
P1	1.0	**Time(min):**	0	10	12	20	40	50	55
		A/B:	90/10	87/13	88/12	88/12	83/17	83/17	90/10
P2	0.8	**Time(min):**	0	30	55	60	65		
		A/B:	88/12	88/12	80/20	80/20	88/12		
P3	0.8	**Time(min):**	0	15	20	36.7	42	52	62	70
		A/B:	90/10	88/12	88/12	83/17	100/0	83/17	90/10	90/10

Each mobile phase was filtered through a 0.45 μm Millipore filter and was degassed prior to use. The wavelength for detection was at 222 nm in each prescription. A constant operating temperature (room temperature) was maintained. The internal standard, ethoxybenzamide (10.3 mg), was dissolved in 10mL of methanol to yield the internal standard solution.

Preparation of Standard Solution

To prepare a standard solution containing parishin, parishins B and C, an appropriate amount of internal standard solution was added to an accurately weighed amount of parishin, parishins B and C standard dissolved in 70%

methanol to give various concentrations within the range 20.4-408.0, 21.6-432.0 and 20-200.0 μg/mL, respectively. Calibration graphs were plotted subsequent to linear regression analysis of the peak area ratios with concentrations.

Preparation of Sample Solution

Standard Decoction

Amounts of individual crude drugs equivalent to a daily dose of three kinds of Chinese medicinal prescriptions were weighed and pulverized; a twenty-fold weight of water was added and the mixture of each prescription was boiled, respectively, for more than 30 min to halve the original volume. After filtration while hot, 100 mL of the filtrate was concentrated, under reduced pressure, to dryness.

The concentrated extract was dissolved in 10 mL of 70% methanol to yield a ten-fold extract. This solution (0.98 mL) and a suitable amount of internal standard (0.02 mL) were mixed to give a final concentration of 20.6 μg/mL of ethoxybenzamide.

Blank Decoction

Amounts of individual crude drugs, equivalent to a daily dose of three kinds of Chinese medicinal prescriptions without Gastrodiae Rhizoma, were treated according to the method described above for the preparation of standard decoction.

Concentrated Herbal Preparation Ban-Shia-Bai-Ju-Tian-Ma-Tang from the Market

An amount of the concentrated herbal preparation equivalent to a daily dose was weighed accurately and extracted with 30 mL of 70 % methanol for 60 min in an ultrasonic bath. After extraction, the samples were filtered while hot. The filtrate was concentrated, under reduced pressure, to dryness.

The residues were dissolved in 5 mL of 70 % methanol to get a ten-fold extract and a suitable amount of internal standard was added to yield a concentration of 20.6 μg/mL of ethoxybenzamide. The solutions were immediately filtered (0.45 μm Millipore) and analysed by HPLC.

Table 1

Intraday and Interday Relative Standard Deviations (n=5) of Marker Constituents for Chinese Medicinal Prescriptions

Prescription	Marker Constituent	Intraday R.S.D. (%)	Interday R.S.D. (%)
P1	Parishin	1.06	2.98
	Parishin B	1.39	1.05
	Parishin C	0.90	1.14
P2	Parishin	1.10	2.60
	Parishin B	1.45	2.51
	Parishin C	2.47	1.45
P3	Parishin	1.40	4.41
	Parishin B	1.58	3.80
	Parishin C	1.58	3.80

Table 2

The Regression Equations and their Correlation Coefficient (r) of Marker Consituents for Chinese Medicinal Prescriptions

Prescription	Marker Constituent	Slope	Intercept	r
P1	Parishin	98.42	7.44	0.9997
	Parishin B	122.95	-6.98	0.9980
	Parishin C	108.57	4.96	0.9990
P2	Parishin	110.15	0.84	0.9999
	Parishin B	155.67	6.11	0.9976
	Parishin C	121.33	6.38	0.9998
P3	Parishin	91.65	-0.87	0.9997
	Parishin B	150.59	-0.22	0.9979
	Parishin C	101.06	2.18	0.9999

Solutions for Recovery Study

Three different concentration of markers; 51, 102 and 204 μg/mL for parishin, 54, 108 and 216 μg/mL for parishin B and 50, 100 and 200 μg/mL for parishin C, were added to each prescription. To each solution, a suitable amount of internal standard was added to give a final concentration of 20.6

μg/mL of ethoxybenzamide. All samples were filtered through a Millipore filter and injected for HPLC analysis to calculate the concentration of parishin, parishins B and C from their calibration graphs.

RESULTS AND DISCUSSION

The detection wavelength was chosen at 222 nm because the absorbances of the three marker constituents are higher at this wavelength. For the selection of internal standards, parabens, acetaminophen, bucetin, etc., were tried, but many overlapped with other constituents in the prescription. Ethoxybenzamide was ultimately chosen as the internal standard because there is no interference at the same retention time and it also absorbs strongly at 222 nm.

Photodiode array detection was also used in this experiment, so that UV spectra of the marker constituents could be compared with those of the reference standards.

To assess the precision of these methods, we injected standard solutions of parishin, parishins B and C at concentrations of 102, 108 and 100 μg/mL, respectively, five times on the same day. The results of peak area of relative standard deviations (R.S.D.'s) were 1.06, 1.39 and 0.90% for P1, 1.10, 1.45 and 2.47% for P2 and 1.40, 1.58 and 1.41% for P3, respectively. The R.S.D.'s obtained from a 5-day period were 2.98, 1.05 and 1.14% for P1, 2.60, 2.51 and 1.45% for P2 and 4.41, 3.80 and 4.08% for P3, respectively (Table 1). These values indicate that the precision is reliable.

To ensure the specificity and selectivity of the method, we prepared blank decoctions of each prescription for interference test. The chromatograms of P1, P2 and P3 are shown in Figures 2, 3 and 4, respectively. The retention times of the marker constituents, parishins B, C and parishin, in P1, P2 and P3 were 21.8, 31.0 and 45.4 min., 21.3, 32.8 and 45.4 min and 29.1, 36.8 and 61.2 min, respectively. No peaks were detected at their retention times in three blank decoctions.

The recoveries of parishin, parishins B and C were 104.3%, 103.3% and 102.0% for P1, 105.0%, 103.2% and 104.1% for P2, 102.9%, 104.0% and 102.6% for P3, respectively (Table 3). The R.S.D.'s of parishin, parishins B and C ranged from 0.89 to 2.86%, 0.56 to 2.37% and 0.37 to 1.19%, respectively. The contents of each marker constituent in standard decoctions are shown in Table 4.

Table 3

Recovery of Parishin, Parishins B and C from Chinese Medicinal Prescriptions

Prescription	Marker Constituent	Amount Added (μg/mL)	Amount Measured (μg/mL)	Recovery (%)	Mean ± S.D. (%)	R.S.D. (%)
		51.0	51.2	100.4		
	Parishin	102.0	104.3	102.2	102.0±1.2	1.19
		204.0	210.7	103.3		
		54.0	56.8	105.2		
P1	Parishin B	108.0	112.6	102.2	104.3±1.4	1.38
		216.0	227.7	105.4		
		50.0	51.9	103.8		
	Parishin C	100.0	101.1	101.1	103.3±1.7	1.66
		200.0	210.4	105.2		
		51.0	53.1	104.0		
	Parishin	102.0	105.7	103.6	104.1±0.4	0.37
		204.0	213.4	104.6		
		54.0	57.0	105.6		
P2	Parishin B	108.0	109.1	101.1	105.0±3.0	2.86
		216.0	234.1	108.4		
		50.0	51.2	102.4		
	Parishin C	100.0	103.7	103.7	103.2±0.6	0.56
		200.0	103.6	103.6		
		51.0	53.5	102.8		
	Parishin	102.0	105.6	103.5	102.6±0.8	0.81
		204.0	211.2	101.5		
		54.0	54.9	101.6		
P3	Parishin B	108.0	111.7	103.5	102.9±0.9	0.89
		216.0	223.7	103.6		
		50.0	53.7	107.4		
	Parishin C	100.0	101.6	101.6	104.0±2.5	2.37
		200.0	205.8	102.9		

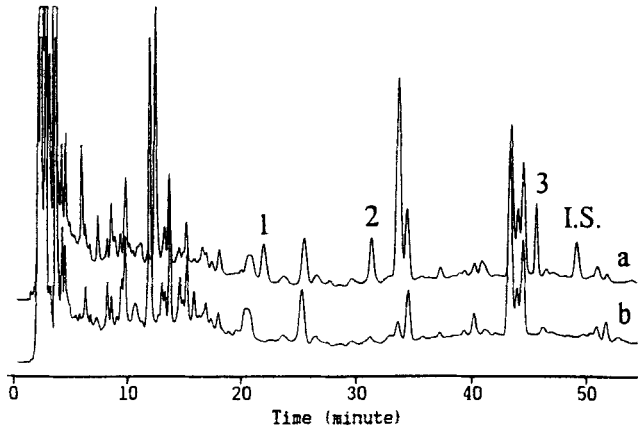


Figure 2. Chromatograms of parishin, parishins B and C in Shuen-Feng-Iun-Chi-San (P1): a) standard decoction; b) blank decoction. Peaks: 1 = parishin B; 2 = parishin C; 3 = parishin; I.S. = internal standard (ethoxybenzamide).

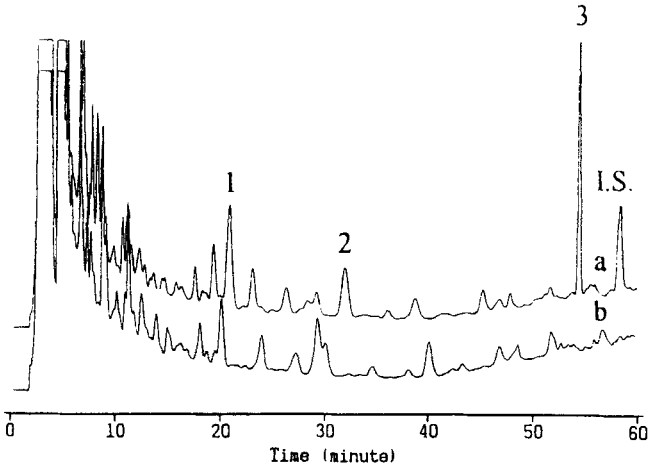


Figure 3. Chromatograms of parishin, parishins B and C in Ban-Shia-Bai-Ju-Tian-Ma-Tang (P2): a) standard decoction; b) blank decoction. Marker peak as in Figure 2.

The contents range of three marker constituents parishin, parishins B and C in Gastrodiae Rhizoma, extracted by 70% methanol, reported previously[2] were 3.66-15.01, 1.21-6.46 and 0.83-2.68 mg/g, respectively. Therefore, the contents of parishins B and C in three standard decoctions corresponded to the

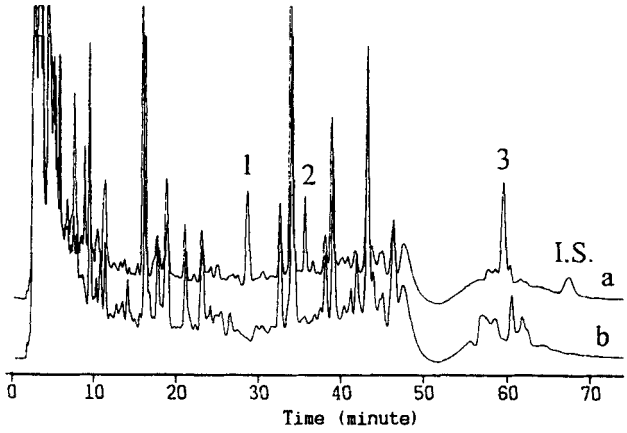


Figure 4. Chromatograms of parishin, parishins B and C in Tsan-siang-Tian-Ma-Tang (P3): a) standard decoction; b) blank decoction. Marker peaks as in Figure 2.

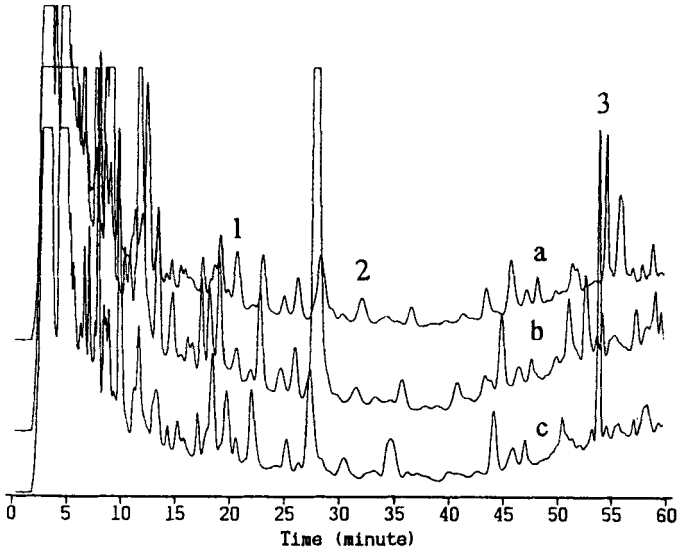


Figure 5. Chromatograms of parishin, parishins B and C in commercial preparations (a-c) of Ban-Shia-Bai-Ju-Tian-Ma-Tang. Marker peaks as in Figure 2.

Table 4

Contents of Marker Constituents in Standard Decoctions of Chinese Medicinal Prescriptions

Sample	Marker Constituents		
	Parishin Mean±S.D.[a] (mg/g)	Parishin B Mean±S.D.[a] (mg/g)	Parishin C Mean±S.D.[a] (mg/g)
P1	1.58±0.11	1.58±0.05	1.63±0.06
P2	2.85±0.09	3.25±0.09	1.72±0.01
P3	2.59±0.05	2.87±0.10	1.45±0.05

[a] n=3.

Table 5

Contents of Marker Constituents in Commercial Concentrated Herbal Preparation of Ban-Shia-Bai-Ju-Tian-Ma-Tang

Comm'l Product	Marker Constituents		
	Parishin Mean±S.D.[a] (mg/g)	Parishin B Mean±S.D.[a] (mg/g)	Parishin C Mean±S.D.[a] (mg/g)
A	1.38±0.02	0.81±0.01	0.26±0.01
B	0.17±0.00	0.37±0.02	0.30±0.01
C	1.14±0.01	0.97±0.01	0.52±0.00

[a] n=3.

contents of crude drug, but the content of parishin (1.58-2.85 mg/g) was lower than that of crude drug (3.66-15.01 mg/g). The extraction rates of these marker constituents in different extraction solvents were found to differ in the earlier study. The extraction rates of parishins B and C by 70% methanol were slightly lower than with water, but, parishin increased markedly. Thus, the difference of parishin between crude drug and decoction might be come from selection of extraction solvents. Chinese medicinal prescriptions usually contain many crude drugs. In this study, more than ten were combined in each prescription. Thus, they possess very complex chemical compositions. However, as mentioned above, convenient and effective methods for analyzing

markers in each preparation were established. All three commercial products of Ban-Shia-Bai-Ju-Tian-Ma-Tang (Fig. 5) did not show satisfactory separations because a peak appeared at the same retention time (58.3 min) as the intemal standard. It may be the constituent which comes from another crude drug in commercial products.

Fortunately, as shown in Table 2, the calibration graphs for parishin, parishins B and C, showed good linear relationships between peak-area ratio and concentration. The contents of marker constituents in commercial preparations were directly calibrated by the calibration curves and are shown in Table 5.

The contents of all marker constituents were lower than those found in standard decoctions. The difference might result from the manufacturing process. We also calculated the contents of marker constituents in standard decoctions of P2 by calibration graphs mentioned above without using internal standard.

The contents of parishin, parishins B and C were 2.89, 3.29 and 1.71 mg/g, respectively. They were nearly the same as the contents of P2 in Table 4; therefore, this method was also acceptable for determining the marker constituents in P2.

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