SIMULTANEOUS ANALYSIS OF SEVEN COMPONENTS IN HSIAO-CHING-LUNG-TANG PREPARATION BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Chia-Chi Chen and Horng-Liang Lay*

Department of Plant Industry, National PingTung University of Science & Technology, PingTung, Taiwan

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To enhance quality control in "Hsiao-Ching-Lung-Tang", a simultaneous determination method using High Pressure Liquid Chromatography (HPLC) on seven marker substances was developed. The marker substances were paeoniflorin and paeonol from Paeoniae Radix; cinnamic acid and cinnamaldehyde from Cinnamomi Ramulus; glycyrrhizin from Glycyrrhizae Radix; and gomisin A and schizandrin from Schizandrae Fructus. Sample extracts were analyzed using a reverse-phase column (Inertsil 5 ODS-2, 4.6 I.D. × 250 mm.) at 30°C and then eluted with a solvent of mixture of 5% and 60% acetonitrile aqueous solution (adjusted to pH 2.8 with phosphoric acid) in gradients at a flow-rate of 1.0 mL/min, and were detected at UV245 nm. Relative coefficients of variations of intra- and inter-day analysis were less than 5% and the relative errors were below 4.2% and 4.1%, respectively. All the recoveries were between 94.7 and 122.3%. This method could be applied for the simultaneous determination of seven marker substances in "Hsiao-Ching-Lung-Tang" and as a comparison to commercial preparations.

Key words: Hsiao-Ching-Lung-Tang, paeoniflorin, paeonol, cinnamic acid, cinnamaldehyde, glycyrrhizin, gomisin A, schizandrin

Introduction

Since 2001, the Department of Health (DOH), Executive Yuan of the Republic of China has been promoting the High Pressure Liquid Chromatography (HPLC) method for quantitative analysis of ingredients in Chinese medicinal preparation[1]. The HPLC analysis is compulsory when manufacturers want to renew their licenses[1]. The goal of analytical method development is to discover more marker substances and simultaneously quantify them into one HPLC method. In this study, we selected one of the most popular traditional Chinese medicine called "Hsiao-Ching-Lung-Tang" ("Treatment of Typhoid" by Chang Chong-Ging, Han Dynasty), which contains Ephedrae Herba, Paeoniae Radix, Schizandrae Fructus, Zingiberis Rhizoma, Glycyrrhizae Radix, Cinnamomi Ramulus, Pinelliae Rhizoma, and Asari Herba[2].

*Correspondence to: Horng-Liang Lay, Department of Plant Industry, National PingTung University of Science & Technology, No.1, Hseuh Fu RD., Nei Pu Hsiang, PingTung 912, Taiwan, Tel: +886-8-7740365, Fax: +886-8-7740415, E-mail: layhl@mail.npust.edu.tw

This traditional mixture preparation is known to exogenous attack of wind, cold and dampness evils manifested by severe chill, fever, absence of sweat, asthma and cough with white and thin expectoration[2]. Asari Herba was used to relieve rheumatism and cold, warming lung and relieving sputum; however, it contains aristolochic acid and is harmful to the liver and kidney; therefore it is not advised for long-term usage[3]. Pinelliae Rhizoma contains polysaccharides and has anti-inflammation properties, which can inhibit the growth and proliferation of PC12 and induce apoptosis of SH-SY5Y and PC12 cells[4,5]. Zingiberis Rhizoma has effect on sweating and skin relaxation, body warming, stop vomiting, detoxication, stomachic, and anti-bacteria characteristics[6]. Gingerol, a component in ginger, is a bioactive component that has effects on lowering fever, prohibiting antidiuretic, cardiac, and prostate biosynthesis[7,8]. Pharmaceutic uses of Hsiao-Ching-Lung-Tang on allergic asthma, rhinitis were studied domestically[9,10]; however, only the components glycyrrhizin and paeoniflorin has been investigated[11]. The objective of this study was to assay the seven marker substances simultaneously using HPLC method.

Seven marker substances including paeoniflorin, paeonol (Paeoniae Radix), cinnamic acid, cinnamaldehyde (Cinnamomi Ramulus), glycyrrhizin (Glycyrrhizae Radix), and gomisin A, schizandrin (Schizandrae Fructus) were resolved and quantitatively measured using a reversed-phase HPLC approach. This method has demonstrated to be facile in the routine analysis for quality control by quantitatively determination of the active ingredients in the formula for the commercial preparation Hsiao-Ching-Lung-Tang from different manufacturers.

Materials and Methods

I. Materials

The crude drugs for Hsiao-Ching-Lung-Tang preparation are Ephedrae Herba (Specimen No. EH93001), Paeoniae Radix (Specimen No. PR93001), Schizandrae Fructus (Specimen No. SF93001), Zingiberis Rhizoma (Specimen No. ZR93001), Glycyrrhizae Radix (Specimen No. GR93001), Cinnamomi Ramulus (Specimen No. CR93001), Pinelliae Rhizoma (Specimen No. PR93001), and Asari Herba (Specimen No. AH93001). Each material was obtained from a local herbal market and pulverized through a #8 mesh sieve (2.36 mm). The original of each herbal was identified using microscopic examination. Voucher specimens were deposited in the department of Plant Industry, National Pingtung University Science and Technology. The samples of commercial concentrated Hsiao-Ching-Lung-Tang were obtained from four manufactures.

II. Chemicals and reagents

The structures of seven marker substances are shown in Fig. 1. Glycyrrhizin was purchased from Sigma Chemical Co. (St. Louis, Mo, USA). Cinnamic acid, cinnamaldehyde, and propyl-4-hydroxy benzoate were procured from Fluka (Buchs, Switzerland). Paeoniflorin was acquired from Nacalai tesque (Kyoto, Japan). Paeonol, gomisin A, and schizandrin were purified and identified in our laboratory.

The 95% ethanol was purchased from Taiwan Tobacco and Wine Board (R.O.C.). Acetonitrile and methanol (HPLC grade) were obtained from Mallinckrodt, Inc. (USA). *n*-hexane, ethyl acetate (EA), methanol, *n*-butanol (*n*-BuOH), and chloroform were purchased from Riedel-de Haën (Germany).

Phosphoric acid was purchased from Kanto Chemical (Japan). Ultra-pure distilled water with a resistivity greater than 18.2 MΩ/cm^2 was obtained from a Millipore mini-Q system (Bedford, MA, USA). Samples for HPLC were filtered separately through a 0.45 μm Millipore membrane filter (Bedford, MA, USA). All other reagents were of analytical grade.

III. Preparation of standard solutions and internal standard solution

The standard solutions were prepared by dissolving the amount of each marker substance as indicated in the parenthesis in 70% methanol aqueous solution to obtain the desired concentration: paeoniflorin (400.0 μg/mL), cinnamaldehyde (160.0 μg/mL), cinnamic acid (120.0 μg/mL), paeonol (80.0 μg/mL), glycyrrhizin (480.0 μg/mL), schizandrin (64.0 μg/mL), and gomisin A (40.0 μg/mL).

The internal standard solution (70.0 μg/mL) was prepared by dissolving 3.5 mg of propyl-4-hydroxy benzoate in 70% methanol aqueous solution to obtain a total volume of 50 mL.

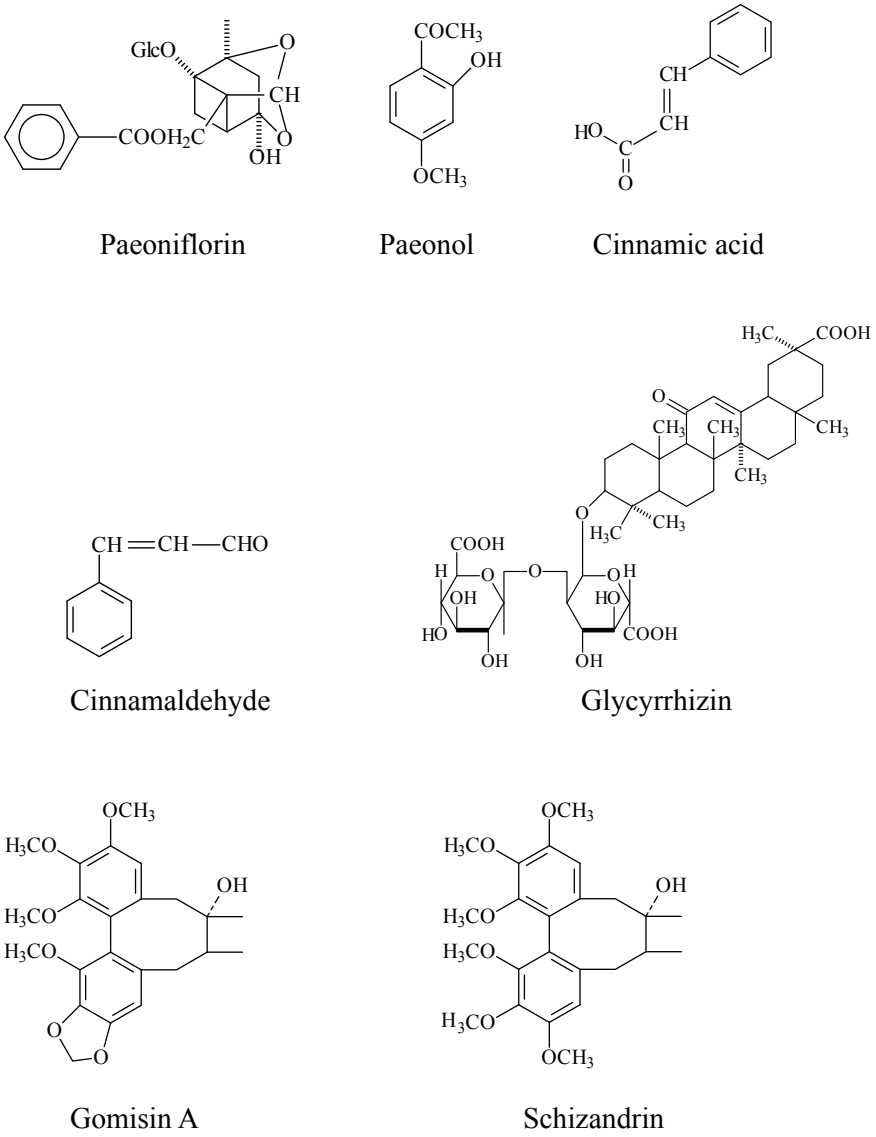

Fig. 1. Structures of the marker substances in Hsiao-Ching-Lung-Tang.

IV. Standard decoction preparation for HPLC

According to the notes[2], the standard decoction of Hsiao-Ching-Lung-Tang contains Ephedrae Herba 4.0 g, Paeoniae Radix 4.0 g, Schizandrae Fructus 1.5 g, Zingiberis Rhizoma 4.0 g, Glycyrrhizae Radix 4.0 g, Cinnamomi Ramulus 4.0 g, Pinelliae Rhizoma 4.0 g, and Asari Herba 1.5 g. To prepare a standard decoction, 27 g of the pulverized Chinese crude drugs listed above and 540 mL (i.e. 20 times) of water were put into a 1000 mL rounded flask and heated until only about 270 mL (i.e. 10 times) remained. Then, the hot solution was filtered by four layers of gauze, and allowed to cool and then was adjusted to 270 mL by adding water. One milliliter sample of the solution was removed and adjusted to 5 mL by adding 70% methanol solution and an appropriate amount of the internal standard solution was added simultaneously. After filtering (0.45 μm), the standard decoction was prepared and used for subsequent HPLC analysis.

V. Commercial concentrated preparations for HPLC

The four kinds of commercial product were pulverized and mixed individually. Accurately one third of the daily dosage (2 g) of each commercial product was put into an 100 mL flask, to which 40 mL of 70% methanol was added. After 30 minutes of ultrasonic extraction at 30°C, each was filtered and adjusted to 40 mL by adding 70% methanol, and the appropriate amount of the internal standard solution was added at the same time. After filtering (0.45 μm), the sample solutions were completed and used for quantification.

VI. HPLC Instruments and conditions

HPLC separation was conducted by a Hitachi system equipped with a degasser DG-2410, pump L-7100, UV/Vis detector L-7420, photodiode array detector L-4500 and autosampler L-7200. Peak areas were calculated with a D-7000 HSM software.

A reversed phase column Inertsil 5 ODS-2 (Nacalai, 4.6 mm I.D. × 250 mm) was utilized. The column oven was set at 30°C. The mobile phases consisting of 5% and 60% acetonitrile aqueous solutions in gradient elution are shown in Table 1. Detection wavelength was set at UV 245 nm. The flow rate was 1.0 mL/min. The volume for each injection was 20 μL. Sample solutions were prepared as described above and injected into the HPLC column for analysis.

VII. Calibration method

The standard solution of each marker substance was prepared from stock solutions by 70% methanol

Table 1. Gradient elution program using mobile phase A and B.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	90	10
25	77	23
50	57	43
55	40	60
68	40	60
75	25	75
85	0	100
90	0	100
98	90	10

Flow rate: l.0 mL/min

A: 5% acetonitrile (adjusted to pH 2.8 with phosphoric acid).

B: 60% acetonitrile (adjusted to pH 2.8 with phosphoric acid)

to give concentrations of paeoniflorin: 12.5, 25.0, 50.0, 100.0, and 200.0 μg/mL; cinnamic acid: 5.0, 10.0, 20.0, 40.0, and 80.0 μg/mL; cinnamaldehyde: 3.75, 7.5, 15.0, 30.0, and 60.0 μg/mL; paeonol: 2.5, 5.0, 10.0, 20.0, and 40.0 μg/mL; glycyrrhizin: 15.0, 30.0, 60.0, 120.0, and 240.0 μg/mL; schizandrin: 2.0, 4.0, 8.0, 16.0, and 32.0 μg/mL; gomisin A: 1.25, 2.5, 5.0, 10.0, and 20.0 μg/mL.

Each standard solution contained the internal standard solution (propyl-4-hydroxy benzoate) at 70.0 μg/mL. All standard solutions were filtered and 20 μL of each was injected into the HPLC column for analysis. The calibration curve was plotted by using the ratio of the peak areas (standard solution/internal standard solution) as the y-axis, and concentrations as the x-axis. Linear regression method was used to evaluate the equation of y = ax + b and the correlation coefficient (r).

VIII. Validation

(I) Precision

Standard stock solutions were diluted with 70% methanol into three different concentrations (Table 3). Intra-day test (injecting each concentration three times within 24 hours), and an inter-day test (injecting each concentration four times over 7 days with each injection separated by at least 24 hours) were run to check reproducibility. The standard deviation (S.D.) and relative standard deviation (R.S.D.) were calculated.

(II) Accuracy

Each standard stock solution of a series of various concentrations was spiked into ethanol extraction of Hsiao-Ching-Lung-Tang, and then refluxed at 80°C for 3 hrs. Internal standard solution was added to each solution to afford a concentration of 70.0 μg/mL. Then the solution was filtered and subjected to HPLC analysis in triplicates. The recovery (%) was calculated by the equation of [(C3-C2)/C1]×100%, in which C1 represents the amount of each standard spiked, C2 represents the amount of each marker in ethanol solution of Hsiao-Ching-Lung-Tang, and C3 represents the total amount of each marker in the solution.

Results and Discussion

I. Separation of marker substances by HPLC

All marker substances and internal standard propyl-4-hydroxy benzoate were successfully separated into a single run HPLC for the standard decoction solution of Hsiao-Ching-Lung-Tang. By using gradient elution, paeoniflorin, cinnamic acid, cinnamaldehyde, paeonol, glycyrrhizin, schizandrin, gomisin A, and propyl-4-hydroxy benzoate were resolved and eluted at 16.79, 53.72, 56.83, 61.39, 70.11, 77.86, 83.02, and 66.03 min, respectively (Fig. 2). The peak purity of marker substances in the Hsiao-Ching-Lung-Tang was qualified by HPLC with photodiode array detector. High purity of each peak was shown for each marker substance (Fig. 2).

The standard decoction of Hsiao-Ching-Lung-Tang was compared to four blank solutions, which were prepared with the deletion of one material of Puerariae Radix, Paeoniae Radix,Cinnamomi Ramulus, Glycyrrhizae Radix, respectively. As shown in Fig. 3B to 3E, no peak of the deleted material were observed at retention times corresponding to the respective marker substances. Apparently, there was no interaction among components of Hsiao-Ching-Lung-Tang. Therefore, the above

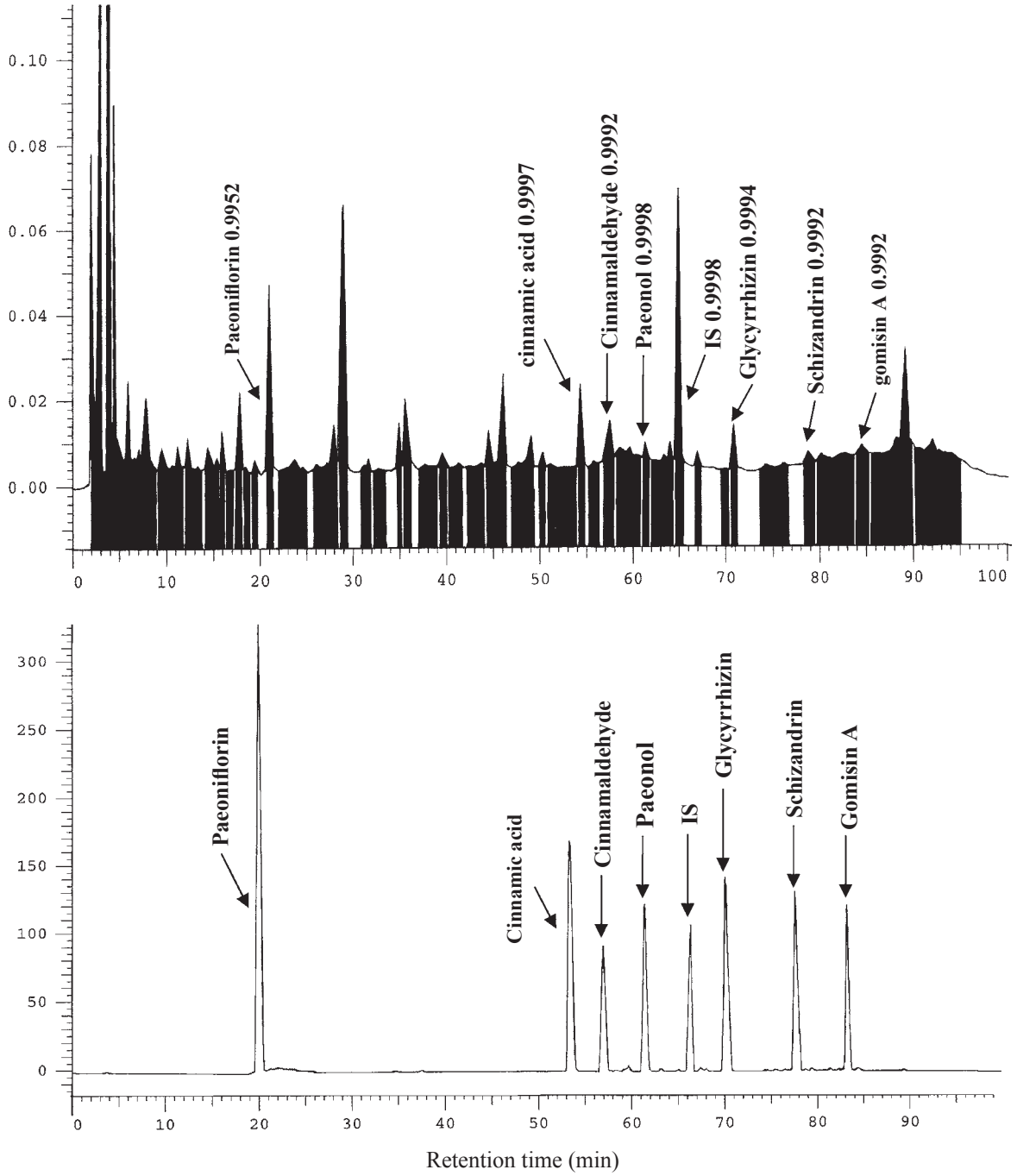

Fig. 2. HPLC Chromatograms of (A) peak purities of each marker in standard decoction of Hsiao-Ching-Lung-Tang, (B) marker substances.

conditions can be used for quantification of the marker substances (Fig. 3).

II. Calibration line

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

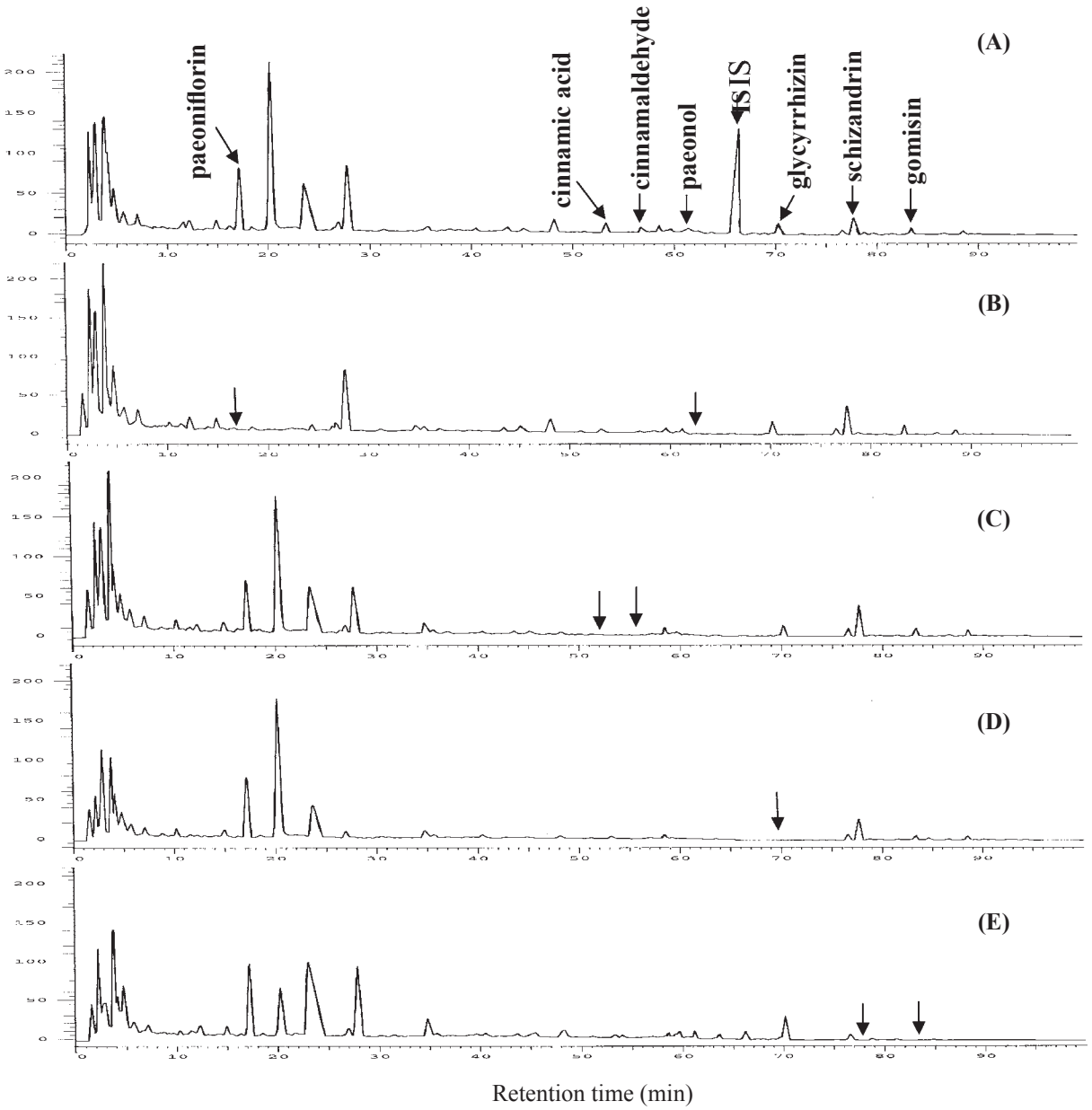

Fig. 3. Chromatograms of marker substances in standard decoction of Hsiao-Ching-Lung-Tang made from incomplete materials.

A: Standard decoction of Hsiao-Ching-Lung-Tang containing internal standard.

B: Standard decoction of Hsiao-Ching-Lung-Tang without Paeoniae Radix.

C: Standard decoction of Hsiao-Ching-Lung-Tang without Cinnamomi Ramulus.

D: Standard decoction of Hsiao-Ching-Lung-Tang without Glycyrrhizae Radix.

E: Standard decoction of Hsiao-Ching-Lung-Tang without Schizandrae Fructus.

III. Precision and accuracy

The relative standard deviations of the intra-day and inter-day analysis were 0.3~4.5% and 0.4~4.1%. The C.V. values were less than 5%, suggesting that the method had satisfactory reproducibility (Table 3).

Recoveries of the analysis were shown in

Table 2. Calibration curves of marker substances.

Compound	Concentration range μg/mL	Regression equation	r (n=5)
Paeoniflorin	12.5~200.0	y=0.007x+0.018	0.9995
Cinnamic acid	5.0~80.0	y=0.014x+0.017	0.9999
Cinnamaldehyde	3.8~60.0	y=0.009x+0.005	0.9996
Paeonol	2.5~40.0	y=0.011x+0.002	0.9999
Glycyrrhizin	15.0~240.0	y=0.004x-0.011	0.9998
Schizandrin	2.0~32.0	y=0.017x+0.007	0.9999
Gomisin A	1.3~20.0	y=0.020x+0.004	0.9998

Table 3. Reproducibilities of intra-day and inter-day analysis of standards in low, medium, and high concentrations.

Compound	Concentration (μg/mL)	Mean ± S.D. (R.S.D. %)	
		intra-day (n=3)	inter-day (n=4)
Paeoniflorin	200.0	199.0 ± 0.7 (0.4)	201.7 ± 3.1 (1.5)
	50.0	49.9 ± 0.4 (0.9)	49.3 ± 0.4 (0.8)
	12.5	11.5 ± 0.1 (1.0)	12.7 ± 0.3 (2.5)
Cinnamic acid	80.0	79.4 ± 1.7 (2.2)	80.3 ± 0.8 (1.2)
	20.0	20.0 ± 0.3 (1.3)	20.9 ± 0.5 (2.5)
	5.0	4.9 ± 0.1 (2.2)	5.4 ± 0.5 (1.0)
Cinnamaldehyde	60.0	59.7 ± 0.2 (0.4)	60.6 ± 1.4 (2.3)
	15.0	14.9 ± 0.3 (1.9)	16.1 ± 0.1 (0.7)
	3.8	3.6 ± 0.0 (0.9)	3.5 ± 0.1 (3.2)
Paeonol	40.0	39.9 ± 1.7 (4.2)	39.3 ± 0.9 (2.2)
	10.0	10.5 ± 0.2 (2.3)	11.2 ± 0.1 (0.6)
	2.5	2.7 ± 0.2 (0.8)	2.6 ± 0.1 (4.1)
Glycyrrhizin	240.0	241.4 ± 0.8 (0.3)	239. 5 ± 3.7 (1.5)
	60.0	60.7 ± 0.6 (1.0)	59.3 ± 0.9 (0.1)
	15.0	14.2 ± 0.0 (0.1)	16.5 ± 0.1 (0.4)
Schizandrin	32.0	31.7 ± 0.1 (0.3)	32.1 ± 0.1 (1.5)
	8.0	8.0 ± 0.1 (1.0)	8.1 ± 0.2 (2.1)
	2.0	1.9 ± 0.1 (3.1)	2.1 ± 0.1 (0.8)
Gomisin A	20.0	19.9 ± 0.2 (0.8)	21.3 ± 0.6 (2.9)
	5.0	5.0 ± 0.0 (0.8)	5.6 ± 0.4 (0.7)
	1.3	1.2 ± 0.1 (4.5)	1.2 ± 0.0 (1.1)

S.D.: Standard deviation.

R.S.D.: Relative standard deviation.

Table 4. Recovery of seven marker substances from Hsiao-Ching-Lung- Tang.

Compound	Concentration (μg/mL)	Recovery
		Mean ± S.D. (R.S.D. %)
Paeoniflorin	200.0	100.6 ± 3.2 (3.2)
	50.0	94.7 ± 3.2 (3.4)
	12.5	105.5 ± 2.2 (2.1)
Cinnamic acid	80.0	103.6 ± 1.3 (1.3)
	20.0	98.7 ± 1.7 (1.7)
	5.0	99.4 ± 2.5 (2.5)
Cinnamaldehyde	60.0	109.4 ± 1.7 (1.5)
	15.0	111.1 ± 3.7 (3.3)
	3.8	97.7 ± 3.5 (3.5)
Paeonol	40.0	109.6 ± 4.0 (3.7)
	10.0	122.3 ± 1.2 (1.0)
	2.5	97.4 ± 1.2 (1.2)
Glycyrrhizin	240.0	101.1 ± 2.7 (2.6)
	60.0	106.5 ± 1.5 (1.4)
	15.0	111.0 ± 5.0 (4.5)
Schizandrin	32.0	112.6 ± 4.8 (4.3)
	8.0	98.3 ± 2.6 (2.5)
	2.0	117.5 ± 4.0 (3.4)
Gomisin A	20.0	105.5 ± 1.2 (1.1)
	5.0	98.1 ± 3.8 (3.9)
	1.3	96.4 ± 4. 1(4.2)

S.D.: Standard deviation.
R.S.D.: Relative standard deviation

Table 3. All of the recoveries are greater than 94.7% (Table 4). Good recoveries were shown irrespective of concentration.

IV. Analysis of the commercial concentrated preparations

HPLC chromatograms and the contents of marker substances in commercial concentrated preparations, as shown in Fig. 4 and Table 5, were marked quite different from each other. Results from quantitative tests revealed that seven marker substances from different pharmaceutical factories differed as much as 69 folds. Among the factories, the product of A factory had the highest content. The contents of the seven marker substances in one third of the daily dosage (2 g) each commercial preparation were of paeoniflorin was from 1,544.4 to 4,314.6 μg, cinnamic acid was 116.9 to 378.7 μg,

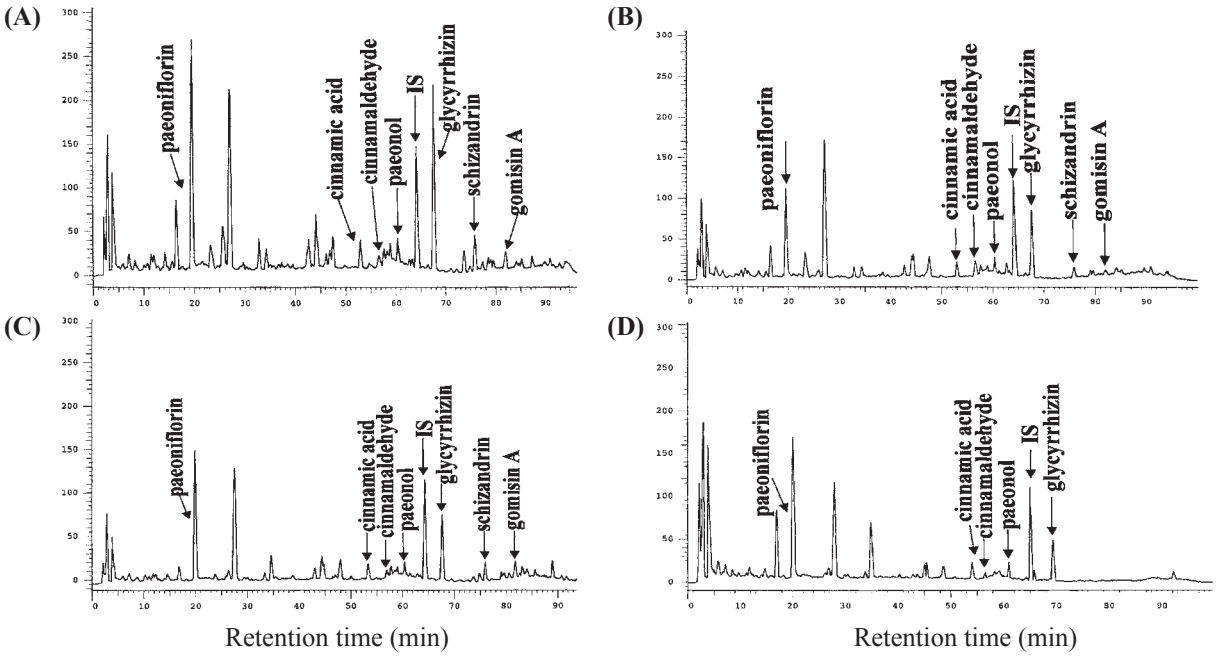

Fig. 4. HPLC Chromatograms of each maker in four various commercial preparations of Hsiao-Ching-Lung-Tang.

Table 5. Contents of marker substances in four various commercial preparations of Hsiao-Ching-Lung-Tang

	Commercial preparations (μg)			
	A	B	C	D
Paeoniflorin	4,314.5 ± 0.3	1,544.5 ± 1.9	2,011.4 ± 1.5	1,936.0 ± 1.0
Cinnamic acid	378.7 ± 0.9	154.0 ± 1.4	122.0 ± 1.2	116.6 ± 0.9
Cinnamaldehyde	526.9 ± 0.1	369.0 ± 2.7	185.5 ± 4.3	73.5 ± 1.9
Paeonol	283.4 ± 2.6	130.9 ± 0.2	166.5 ± 3.3	4.1 ± 2.3
Glycyrrhizin	6,497.3 ± 0.1	2,396.5 ± 0.1	2,027.5 ± 0.5	774.9 ± 0.1
Schizandrin	278.9 ± 2.0	97.2 ± 2.0	58.6 ± 4.7	--
Gomisin A	4.0 ± 2.5	2.3 ± 1.2	1.2 ± 3.2	--

Data represented as mean ± C.V. value (%) in one third of the daily dosage (2 g) each commercial preparation.

cinnamaldehyde was 73.5 to 526.9 μg, paeonol was 4.1 to 283.4 μg, glycyrrhizin was 774.9 to 6,497.3 μg, schizandrin was 58.6 to 278.9 μg, gomisin A was 1.2 to 4.0 μg. However, schizandrin and gomisin A were not detected in the products of D factory. This might be due to the difference in the origin of the source, the inconsistencies of the refining process of herbal medicine, as well as the discrepancy in excipients, temperature, humidity of additives or storage, or variations in manufacturing process in extraction, concentration, drying, and granulation.

This study established a precise and reliable quantification method for the simultaneous determination of seven marker substances in Hsiao-Ching-

Lung-Tang. The method can be used for quality control of manufacturing process of concentrated preparation of Hsiao-Ching-Lung-Tang in the future.

Conclusions

A multi-component HPLC method was developed for the simultaneous quantification of seven marker substances in of Hsiao-Ching-Lung-Tang. A matrix of 5% acetonitrile and 60% acetonitrile, which were both adjusted to pH 2.8 with phosphoric acid, was used as the mobile phase in a gradient elution program, with an ODS column for the stationary phase. The detection wavelength was set at 245 nm. The internal standard used to determine the calibration line resulted in a precise and reliable quantification method. The results of the quantitative analysis showed that the method can be used to establish the standards for quality control to ensure accuracy, efficiency and manufacturing process of Hsiao-Ching-Lung-Tang in the future.

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高效液相層析法對小青龍湯中七種成分同時分析之研究

陳嘉琪、賴宏亮 *

國立屏東科技大學農園生產系，屏東，台灣

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使用HPLC進行小青龍湯之成分分析，開發出包含白芍中paeoniflorin，paeonol、桂枝中cinnamic acid，cinnamaldehyde、甘草中glycyrrhizin及五味子中gomisin A，schizandrin等七種指標成分同時定量分析方法。小青龍湯之檢品溶液通過保持在30°C恒溫之HPLC層析管（Inertsil ODS-2, 4.6 mm I.D.×250 mm），移動相採用5%及60% acetonitrile之混和溶液，並用磷酸調整pH值為2.8，進行梯度沖提法，以1.0 mL／分之流速沖提。七種指標成分之檢測使用UV偵測器，偵測波長設定245 nm。本法之回收率，同日間及異日間的變異係數均在5%以下，相對誤差各低於4.2%及4.1%，回收率在94.7~122.3%之間。這個分析法對於小青龍湯製劑中七種指標成分是安定且值得信賴之定量法。

關鍵字：小青龍湯、芍藥苷、牡丹酚、桂皮酸、桂皮醛、甘草酸、五味子酯A、五味子素

*聯絡人：賴宏亮，國立屏東科技大學農園生產系，屏東縣內埔鄉912學府路1號，電話：08-7740365，傳真：08-7740415，電子郵件信箱：layhl@mail.npust.edu.tw