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Simultaneous Analysis of Ten Components in Patch Preparation of Wan-Yin-Gau by High Performance Liquid Chromatography

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ABSTRACT

A facile HPLC method for the reduction and quantitative measurement of ten marker substances, the active ingredients in formula preparation of Wan-Yin-Gau, was established under the gradient elution in the reversed-phase mode. These marker substances included cinnamic acid, cinnamaldehyde (Cinnamomi Cortex), isoimperatorin (Angelicae Dahuricae Radix and Notopterygii Rhizoma), ferulic acid (Angelicae Sinensis Radix), paeoniflorin (Paeoniae Radix), glycyrrhizin (Glycyrrhizae Radix), harpagoside (Scrophulariae Radix), emodin, sennoside A, and sennoside B (Rhei Rhizoma). The ingredients in the water-based and oil-based patches from different manufactures were also analyzed for quality evaluation.

Extracted samples were analyzed with reverse-phase column (Inertsil 5 ODS-2, 4.6 i.d. × 250 mm) at 30°C, eluted with a mixture of 20% and 70% acetonitrile aqueous solution in gradient manner at a flow rate of 1.0 mL/min, and detected at 250 nm.

Relative coefficients of variation of intra- and inter-day analysis were less than 5%. All the recoveries were 96.29~103.46%. This method could be applied for the simultaneous determination of ten marker substances in "Wan-Yin-Gau".

Key words: Wan-Yin-Gau, cinnamic acid, cinnamaldehyde, isoimperatorin, ferulic acid, paeoniflorin, glycyrrhizin, harpagoside, emodin, sennoside A, sennoside B

INTRODUCTION

A number of analytical methods for Chinese medicinal preparations have been established in our laboratory[(1-8)] recently. However, the method for analyzing the Wan-Yin-Gau, a very popular Chinese medicinal patch preparation well-known for reducing swelling and relieving pain, etc., remains unavailable for its complexity since. The patch preparation contains active ingredients from a variety of Chinese crude drugs including Aconiti Radix, Aconiti Kusnezoffii Radix, Rehmanniae Radix, Ampelopsis Radix, Bletillae Tuber, Cinnamomi Cortex, Angelicae Dahuricae Radix, Angelicae Sinensis Radix, Paeoniae Radix, Notopterygii Rhizoma, Sophorae Radix, Momordicae Semen, Linderae Radix, Glycyrrhizae Radix, Angelicae Tuhou Radix, Scrophulariae Radix, and Rhei Rhizoma. In this study, ten marker substances including cinnamic acid, cinnamaldehyde (Cinnamomi Cortex), isoimperatorin (Angelicae Dahuricae Radix and Notopterygii Rhizoma), ferulic acid (Angelicae Sinensis Radix), paeoniflorin (Paeoniae Radix), glycyrrhizin (Glycyrrhizae Radix), harpagoside (Scrophulariae Radix) and emodin, sennoside A, sennoside B (Rhei Rhizoma) are resolved and quantitatively measured through a reversed-phase HPLC approach. The method developed will be demonstrated to be facile in the routine quality analysis control by quantitatively determining the active ingredients in the formula for the water-based and oil-based patches from different manufactures.

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MATERIALS AND METHODS

I. Materials

The crude drugs for Wan-Yin-Gau preparation are Aconiti Radix, Aconiti Kusnezoffii Radix, Rehmanniae Radix, Ampelopsis Radix, Bletillae Tuber, Cinnamomi Cortex, Angelicae Dahuricae Radix, Angelicae Sinensis Radix, Paeoniae Radix, Notopterygii Rhizoma, Sophorae Radix, Momordicae Semen, Linderae Radix, Glycyrrhizae

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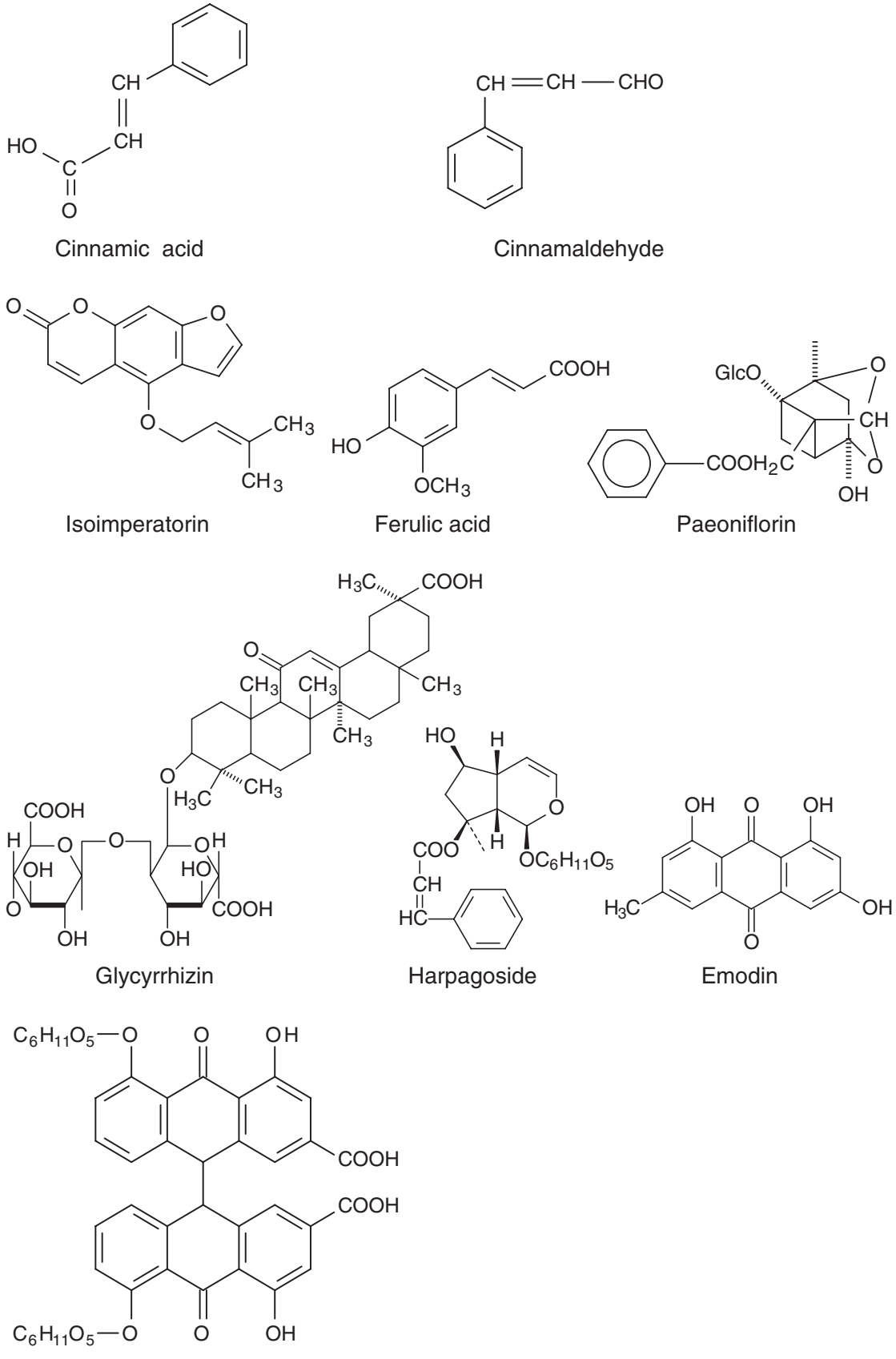

Figure 1. Structures of the marker substances in Wan-Yin-Gau.

Radix, Angelicae Tuhou Radix, Scrophulariae Radix, and Rhei Rhizoma. Each material was obtained from local herbal market and pulverized through a #8 mesh sieve (2.36 mm). The origin of 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.

Oil-based and water-based patch of Wan-Yin-Gau were obtained from Sheng Chun Tang Pharmaceutical Co., Ltd. in Taiwan.

II. *Chemicals and Reagents*

Structures of the ten marker substances are shown in Figure 1. Emodin (98%), ferulic acid (99%), glycyrrhizin (98%), and internal standard propylparaben (99%) were purchased from Sigma (St. Louis, Mo, USA). Cinnamic acid (99%) and cinnamaldehyde (98%) were purchased from Fluka (Switzerland). Harpagoside (98%), sennoside A (98%) and sennoside B (98%) were purchased from Extrasynthese (Genay, France). Paeoniflorin (99%) was purchased from Nacalai Tesque (Japan). The isoimperatorin was obtained from Professor Tian-Shung Wu of National Cheng Kung University.

Ethanol (95%) was purchased from Taiwan Tobacco and Wine Board (Taiwan, ROC). Acetonitrile and methanol (HPLC grade) were obtained from Mallinckrodt (USA), and phosphoric acid from Kanto (Japan). Ultrapure distilled water with a resistivity greater than 18 MΩ was obtained from a Millipore mini-Q system (Bedford, MA, USA). Samples for HPLC were filtered through a 0.45 μm Millipore membrane filter (Bedford, MA, USA). All other reagents were analytical grade.

III. *HPLC Instruments and Conditions*

HPLC was conducted 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 reverse phase column Inertsil 5 ODS-2 (Nacalai, 4.6 mm i.d. × 250 mm) was used. The column oven was set at 30˚C. The mobile phases consisting of 20% and 70% acetonitrile aqueous solutions in gradient elution are shown in Table 1. The detection wavelength was set at 250 nm. The flow rate was set at 1.0 mL/min. The volume for each injection was 20 μL.

IV. *Preparation of Standard and Internal Standard Solutions*

Standard solutions were prepared by dissolving each marker substance as indicated in the parenthesis in 70% methanol solution to obtain the desired concentration: paeoniflorin (80.0 μg/mL), ferulic acid (192.0 μg/mL), sennoside B (1200.0 μg/mL), sennoside A (100.0 μg/mL), harpagoside (200.0 μg/mL), cinnamic acid (76.0 μg/mL), cinnamaldehyde (796.0 μg/mL), glycyrrhizine (176 μg/mL), emodin (80.0 μg/mL), and isoimperatorin (48.0 μg/mL).

Internal standard solution (120 μg/mL) was prepared by dissolving 30.0 mg of propylparaben in 70% methanol solution to obtain a total volume of 250 mL.

V. *Extraction Conditions*

According to Cang-Yang-Jing-Yan-Quan-Shu (Ming Dynasty, 1569), 255 g of Wan-Yin-Gau is consisted of 15.0 g of Aconiti Radix, 15.0 g of Aconiti Kusnezoffii Radix, 15.0 g of Rehmanniae Radix, 15.0 g of Ampelopsis Radix, 15.0 g of Bletillae Tuber, 15.0 g of Cinnamomi Cortex, 15.0 g of Angelicae Dahuricae Radix, 15.0 g of Angelicae Sinensis Radix, 15.0 g of Paeoniae Radix, 15.0 g of Notopterygii Rhizoma, 15.0 g of Sophorae Radix, 15.0 g of Momordicae Semen, 15.0 g of Linderae Radix, 15.0 g of Glycyrrhizae Radix, 15.0 g of Angelicae Tuhou Radix, 15.0 g of Scrophulariae Radix, and 15.0 g of Rhei Rhizoma. In order to obtain better extraction rate of the ten marker substances from Wan-Yin-Gau, solvents such as sesame oil, 50% ethanol, ethanol and water were used. The above-mentioned Chinese crude drugs (127.5 g) were extracted by four different methods as follows.

A. Add 1,000 mL of sesame oil and store the mixture at room temperature (25˚C) for one day, and then reflux at 150˚C for 3 hr.
B. Add 1,000 mL of 50% ethanol, and then reflux at 90˚C for 3 hr.
C. Add 1,000 mL of ethanol, and then reflux at 80˚C for 3 hr.
D. Add 1,000 mL of water, and then reflux at 100˚C for 3 hr.

The extract from method A was partitioned with *n*-hexane and methanol. The methanol layer was evaporated under vacuum and adjusted to 50 mL by adding 80% methanol. A suitable amount of internal standard propylparaben was added to the solution to give a concentration of 60.0 μg/mL. The extracts from method B, C and D were evaporated under vacuum and adjusted to 50 mL by adding

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

Time (min)	Mobile phase A[a] (%)	Mobile phase B[b] (%)
0	92	8
20	90	10
30	82	18
40	72	28
50	60	40
60	50	50
70	30	70
100	30	70
110	0	100
120	92	8

Flow rate 1.0 mL/min.
[a]20% acetonitrile (adjusted to pH 3.0 with phosphoric acid).
[b]70% acetonitrile (adjusted to pH 3.0 with phosphoric acid).

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80% methanol, respectively. A 1.0 mL aliquot of the solution was diluted to 5 mL by 70% methanol solution, and internal standard propylparaben was added to each solution to give a concentration of 60.0 μg/mL. All of them were subjected to HPLC for quantification.

VI. *Preparation of Sample Solution from Oil-based Patch*

Three pieces of oil-based patch of Wan-Yin-Gau were extracted with 500 mL of *n*-hexane by refluxing at 75˚C for 3 hr, the extract was partitioned with methanol. The methanol layer was added with a suitable amount of internal standard propylparaben to give a concentration of 60.0 μg/mL.

VII. *Preparation of Sample Solution from Water-based Patch*

Three pieces of water-based patch of Wan-Yin-Gau were extracted with 500 mL of methanol by refluxing at 75˚C for 3 hr. The extract was evaporated under vacuum and adjusted to 25 mL by adding 80% methanol. Internal standard propylparaben was added to the solution to give a concentration of 60.0 μg/mL.

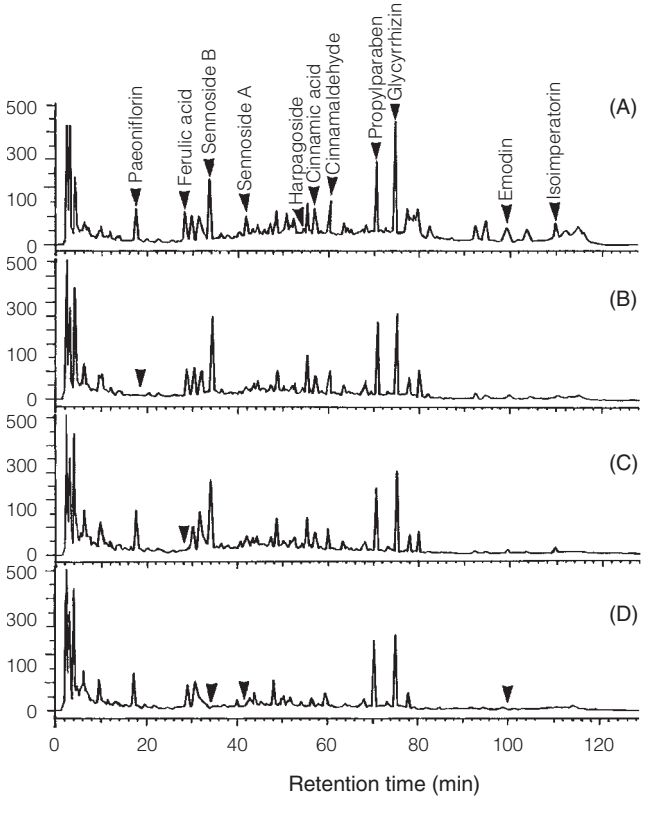

Figure 2. Chromatograms of marker substances in ethanol extract of Wan-Yin-Gau made from incomplete materials.
A: Ethanol extract of Wan-Yin-Gau containing internal standard, propylparaben.
B: Ethanol extract of Wan-Yin-Gau without Paeoniae Radix.
C: Ethanol extract of Wan-Yin-Gau without Angelicae Sinensis Radix.
D: Ethanol extract of Wan-Yin-Gau without Rhei Rhizoma.

VIII. *Calibration Method*

The standard solutions of each marker substance was prepared from the stock solutions by adding 80% methanol to give concentrations of paeoniflorin and emodin: 2.5, 5.0, 10.0, 20.0, 40.0 μg/mL; ferulic acid: 6.0, 12.0, 24.0, 48.0, 96.0 μg/mL; sennoside B: 18.75, 35.0, 75.0, 150.0, 300.0, 600.0 μg/mL; sennoside A: 3.12, 6.25, 12.5, 25.0, 50.0 μg/mL; harpagoside: 6.25, 12.5, 25.0, 50.0, 100.0 μg/mL; cinnamic acid: 2.38, 4.75, 9.5, 19.0, 38.0 μg/mL; cinnamaldehyde: 24.88, 49.75, 99.5, 199.0, 398.0 μg/mL; glycyrrhizine: 5.5, 11.0, 22.0, 44.0, 88.0 μg/mL; and isoimperatorin: 1.5, 3.0, 6.0, 12.0, 24.0 μg/mL.

Each standard solution contained the internal standard (propylparaben) at 60 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 each concentration as the x-axis. Linear regression method was used to evaluate the linear equation and the correlation coefficient.

IX. *Validation*

(I) *Precision*

Standard stock solutions were diluted with 80% methanol to three different concentrations (Table 2). Intra-day (injecting each concentration three times within 24 hr) and inter-day test (injecting each concentration four times over 7 days with each injection separated by at least 24 hr) were run to check reproducibility. The standard deviation (SD) and relative standard deviation (RSD) were calculated.

(II) *Accuracy*

Each standard stock solution of a series of concentrations was spiked into an ethanol solution of Wan-Yin-Gau, and then refluxed at 80˚C for 3 hr. Internal standard solution was added to each solution to afford a concentration of 60 μg/mL. Then the solution was filtered and subjected to HPLC analysis in triplicates. The recovery

Table 2. Calibration curves of marker substances

Compound	Concentration range (μg/mL)	Regression equation	r
Paeoniflorin	2.5~40.0	y=0.0023x—0.0002	0.9998
Ferulic acid	6.0~96.0	y=0.0293x—0.0047	0.9998
Sennoside B	18.7~600.0	y=2.6663x + 0.0468	0.9994
Sennoside A	3.1~50.0	y=0.0057x + 0.0007	0.9998
Harpagoside	6.2~100.0	y=0.0081x + 0.0028	0.9999
Cinnamic acid	2.4~38.0	y=0.0119x + 0.0035	0.9999
Cinnamaldehyde	24.9~398.0	y=0.0504x + 0.0537	0.9997
Glycyrrhizin	5.5~88.0	y=0.0504x + 0.0329	0.9995
Emodin	2.5~40.0	y=0.0144x + 0.0268	0.9992
Isoimperatorin	1.5~24.0	y=0.0053x + 0.0019	0.9999

(%) was calculated by the equation of ((C3-C2)/C1) × 100%, where C1 represents the amount of each standard spiked, C2 represents the amount of each marker in ethanol solution of Wan-Yin-Gau, and C3 represents the total amount of each markers in the solution.

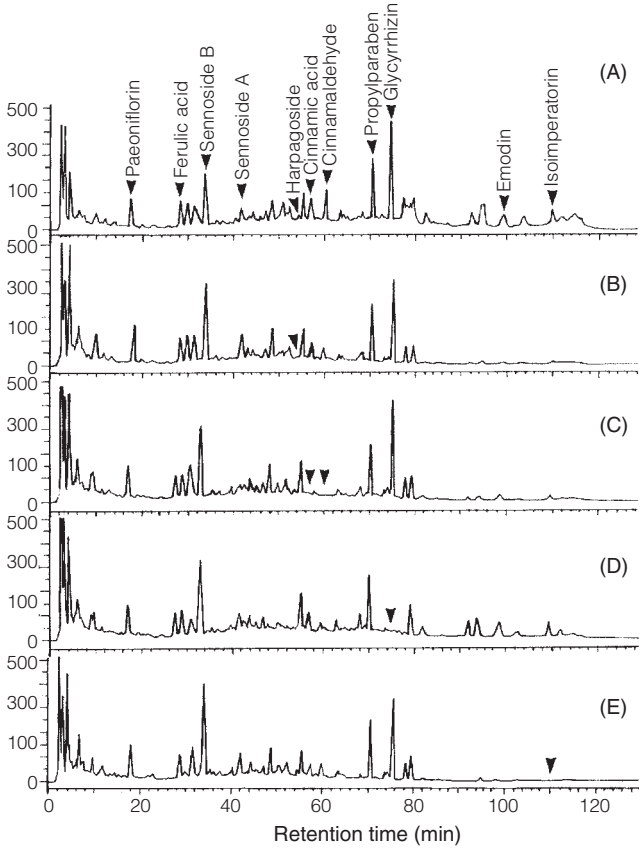

Figure 3. Chromatograms of marker substances in ethanol extractions of Wan-Yin-Gau made from incomplete materials.
A: Ethanol extract of Wan-Yin-Gau containing internal standard, propylparaben.
B: Ethanol extract of Wan-Yin-Gau without Scrophulariae Radix.
C: Ethanol extract of Wan-Yin-Gau without Cinnamomi Cortex.
D: Ethanol extract of Wan-Yin-Gau without Glycyrrhizae Radix.
E: Ethanol extract of Wan-Yin-Gau without Angelicae Dahuricae Radix and Notopterygii Rhizoma.

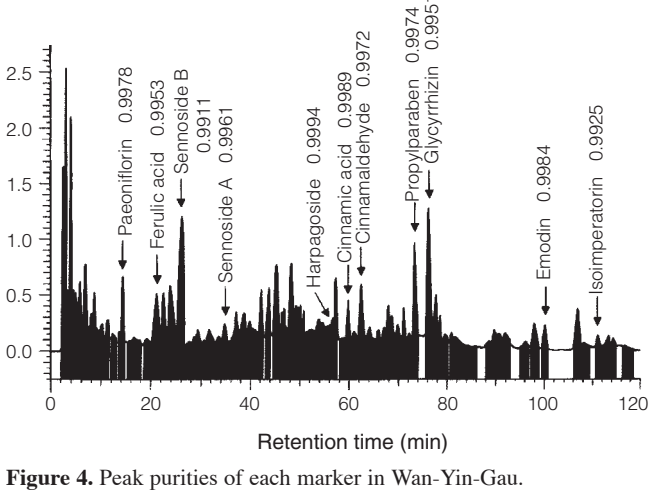

Figure 4. Peak purities of each marker in Wan-Yin-Gau.

RESULTS AND DISCUSSION

I. *Separation of Marker Substances by HPLC*

All marker substances and internal standard, propylparaben, were successfully separated in a single run HPLC for the ethanol extracts of Wan-Yin-Gau. By using gradient elution, paeoniflorin, ferulic acid, sennoside B, sennoside A, harpagoside, cinnamic acid, cinnamaldehyde, glycyrrhizin, emodin, isoimperatorin and propylparaben were resolved and eluted at 16.29 min, 28.21 min, 37.50 min, 40.71 min, 54.19 min, 55.63 min, 58.49 min, 74.64 min, 100.91 min, 109.25 min and 69.75 min, respectively (Figures 2 and 3).

The peak purity of marker substances in the Wan-Yin-Gau was qualified by HPLC with photodiode array detector. High purity of each peak was shown for each marker substance (Figure 4). The ethanol extract of Wan-Yin-Gau was compared to the seven kinds of blank solutions, which were prepared with the deletion of one material of Paeoniae Radix, Angelicae Sinensis Radix, Rhei Rhizoma, Scrophulariae Radix, Cinnamomi Cortex, Glycyrrhizae Radix, Angelicae Dahuricae Radix and Notopterygii Rhizoma, and Schizandrae Fructus, respectively. As shown in Figure 2B to 2D and Figure 3B to 3E, no peak of the deleted material was observed at retention times corresponding to the respective marker substances. Apparently, there was no interaction among components of Wan-Yin-Gau. Therefore, the above conditions can be used for quantification of the marker substances.

II. *Calibration Curve*

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 0.9998~0.9999.

III. *Extraction Methods*

The contents of all marker substance extracted with four kind of extraction methods are shown in Table 3. The results indicated that method B (Add 1,000 mL of 50% ethanol, and then reflux at 90˚C for 3 hr) and C (1,000 mL of ethanol, and then reflux at 80˚C for 3 hr) provided higher yield of the ten marker substances.

IV. *Precision and Accuracy*

The relative standard deviations of the intra-day and inter-day analyses were 0.10~2.63% and 0.11~3.60%, suggesting that both method had very good reproducibility

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(Table 4).

Recoveries of the analysis were obtained as shown in Table 5. All of the recoveries are greater than 91.08%.

Table 3. The relative extraction ratio of 10 maker substances of Wan-Yin-Gau

	A[a]	B[b]	C[c]	D[d]
Paeoniflorin (%)	1.3	100.0	84.9	68.1
Ferulic acid (%)	0.5	100.0	73.0	57.0
Sennoside B (%)	0.0	100.0	28.9	50.5
Sennoside A (%)	5.4	30.5	100.0	77.2
Harpagoside (%)	0.4	100.0	28.1	6.1
Cinnamic acid (%)	1.4	86.6	100.0	28.7
Cinnamaldehyde (%)	—[e]	100.0	30.4	—
Glycyrrhizin (%)	3.3	86.4	100.0	23.9
Emodin (%)	3.8	100.0	71.9	0.9
Isoimperatorin (%)	14.3	100.0	73.0	32.4

[a]Addition of 1000 mL of sesame oil and store the mixture either at room temperature (25˚C) for one day, and then reflux at 150˚C for 3 hr.
[b]Addition of 1000 mL of 50% ethanol, and then reflux at 90˚C for 3 hr.
[c]Addition of 1000 mL of ethanol, and then reflux at 80˚C for 3 hr.
[d]Addition of 1000 mL of water, and then reflux at boiling temperature for 3hr.
[e] Not detected.

Table 4. Reproducibilities of intra-day and inter-day analysis of Wan-Yin-Gau

Compound	Concentration	Mean ± SD (RSD %)	
	(μg/mL)	intra-day (n = 5)	inter-day (n = 4)
	40.000	39.71 ± 0.15(0.37)	39.30 ± 0.17(0.42)
Paeoniflorin	10.000	9.58 ± 0.09(0.99)	11.21 ± 0.37(3.34)
	2.500	2.77 ± 0.09(3.33)	2.60 ± 0.02(0.95)
	96.000	95.89 ± 0.46(0.48)	96.21 ± 0.89(0.92)
Ferulic acid	24.000	23.38 ± 0.12(0.55)	24.55 ± 0.23(0.14)
	6.000	5.55 ± 0.04(0.71)	6.29 ± 0.06(0.93)
	600.00	578.00 ± 4.97(0.86)	612.50 ± 7.53(1.23)
Sennoside B	150.00	163.00 ± 0.90(0.55)	159.09 ± 0.64(0.40)
	18.75	19.61 ± 0.18(0.92)	17.54 ± 0.13(0.73)
	50.000	51.08 ± 0.26(0.50)	52.00 ± 0.53(1.02)
Sennoside A	12.500	11.28 ± 0.05(0.40)	13.63 ± 0.02(0.18)
	3.125	4.337 ± 0.05(1.18)	4.251 ± 0.02(0.50)
	100.000	105.55 ± 0.26 (0.25)	104.81 ± 0.56 (0.53)
Harpagoside	25.000	23.89 ± 0.06 (0.24)	26.74 ± 0.17(0.62)
	6.250	7.773 ± 0.01 (0.15)	7.428 ± 0.21(2.87)
	38.000	39.28 ± 0.14 (0.35)	39.36 ± 0.28 (0.72)
Cinnamic acid	9.500	10.70 ± 0.04 (0.37)	8.66 ± 0.02(0.23)
	2.375	2.229 ± 0.03 (1.24)	2.441 ± 0.02(0.87)
	398.000	404.00 ± 1.29 (0.32)	405.82 ± 6.42(1.59)
Cinnamaldehyde	99.500	98.27 ± 1.04 (1.06)	98.46 ± 1.55(1.57)
	24.875	25.235 ± 0.28 (1.10)	25.678 ± 0.09 (0.36)
	88.000	88.72 ± 0.47 (0.53)	88.61 ± 1.37(1.55)
Glycyrrhizin	22.000	23.22 ± 0.72 (3.09)	21.54 ± 0.55(2.54)
	5.500	5.23 ± 0.06 (1.06)	5.36 ± 0.16(2.98)
	40.000	40.67 ± 0.55 (1.35)	41.22 ± 0.26(0.62)
Emodin	10.000	9.81 ± 0.09 (0.87)	10.74 ± 0.51(4.71)
	2.500	2.72 ± 0.04 (1.44)	2.89 ± 0.13(4.33)
	24.000	23.33 ± 0.22 (0.96)	24.78 ± 0.53(2.13)
Isoimperatorin	6.000	5.29 ± 1.10 (1.86)	6.16 ± 0.16(2.61)
	1.500	1.83 ± 0.02 (0.83)	1.68 ± 0.07(4.24)

V. *Quantitation of Marker Substances in Water-based and Oil-based Patch Preparations of Wan-Yin- Gau*

The contents of marker substances in water-based and oil-based patch preparations, as shown in Table 6, were significantly different from each other. This was probably due to the different sources of the pharmaceutical excipients and different manufacturing processes.

In this report, we established a precise and reliable

Table 5. Recovery of ten marker substances from Wan-Yin-Gau

Compound	Concentration (μg/mL)	Recovery (%) Mean ± SD (RSD %)
	40.00	101.20 ± 2.63 (2.60)
Paeoniflorin	10.00	99.20 ± 3.15 (3.18)
	2.50	99.20 ± 4.75 (4.79)
	96.00	96.36 ± 3.11 (3.23)
Ferulic acid	24.00	99.29 ± 4.09 (4.12)
	6.00	99.91 ± 4.78 (4.78)
	600.00	101.54 ± 2.78 (2.74)
Sennoside B	150.00	102.34 ± 3.89 (3.80)
	18.75	103.25 ± 4.56 (4.42)
	50.00	100.35 ± 1.37 (1.37)
Sennoside A	12.50	100.62 ± 3.17 (3.15)
	3.13	99.95 ± 4.56 (4.56)
	100.00	96.40 ± 2.69 (2.79)
Harpagoside	25.00	103.46 ± 2.75 (2.66)
	6.25	99.18 ± 2.39 (2.41)
	38.00	96.29 ± 3.78 (3.93)
Cinnamic acid	9.50	103.19 ± 3.43 (3.32)
	2.38	102.13 ± 4.72 (4.62)
	398.00	100.20 ± 2.88 (2.87)
Cinnamaldehyde	99.50	102.70 ± 3.35 (3.26)
	24.88	102.85 ± 2.39 (2.32)
	88.00	100.51 ± 4.87 (4.85)
Glycyrrhizin	22.00	101.24 ± 3.68 (3.63)
	5.50	102.38 ± 4.55 (4.44)
	40.00	98.56 ± 2.32 (2.35)
Emodin	10.00	101.39 ± 3.68 (3.63)
	2.50	97.59 ± 3.43 (3.51)
	24.00	98.30 ± 3.86 (3.93)
Isoimperatorin	6.00	97.51 ± 4.90 (5.03)
	1.50	98.79 ± 3.38 (3.42)

Table 6. Contents of marker substances in water-base and oil-base Wan-Yin-Gau

	Water-base Patch[a]	Oil-base Patch
Paeoniflorin	0.53 ± 0.03	4.12 ± 0.05
Ferulic acid	—[b]	0.19 ± 0.01
Sennoside B	—	1.18 ± 0.02
Sennoside A	7.86 ± 0.28	0.02 ± 0.00
Harpagoside	—	—
Cinnamic acid	—	2.79 ± 0.02
Cinnamaldehyde	—	0.80 ± 0.01
Glycyrrhizin	—	5.03 ± 0.01
Emodin	—	—
Isoimperatorin	—	2.00 ± 0.08

[a]Data expressed as mean (mg/one patch) ± SD, n = 7.
[b]Not detected.

quantification method for the simultaneous determination of ten marker substances in Wan-Yin-Gau. The method can be used for quality control of manufacturing process of Wan-Yin-Gau patch in the future.

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