SIMULTANEOUS ANALYSIS OF SEVEN COMPONENTS IN PATCH PREPARATION OF RUI-YUN-GAU BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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A facile HPLC method for the resolution and quantitative measurement of seven marker substances, the active ingredients in patch preparation of Rui-Yun-Gau, was established under the gradient elution in the reversed-phase mode. These marker substances included berberine (Coptidis Rhizoma and Phellodendri Cortex), and coptisine (Coptidis Rhizoma), emodin, and sennoside A (Rhei Rhizoma), harpagoside (Scrophulariae Radix), baicalin, and baicalein (Scutellariae Radix). The ingredients in the water-based and oil-based patches of the formula from different manufactures were also analyzed for quality evaluation.

Extracted samples were analyzed with a reversed-phase column (Inertsil 5 ODS-2, 4.6 i.d. × 250 mm) at 30 °C, eluted with a mixture of 10% acetonitrile and 60% acetonitrile aqueous solution (each adjusted to pH 2.8 with phosphoric acid) in gradient manner at a flow-rate of 1.0 mL/min, and detected at 240 nm.

Relative coefficients of variations of intra- and inter-day analysis were less than 5%. All the recoveries were 90.65~112.22%, therefore this method could be applied for the simultaneous determination of seven marker substances in "Rui-Yun-Gau".

Key words: Rui-Yun-Gau, HPLC.

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INTRODUCTION

Recently, a number of analytical methods for Chinese medicinal preparations have been established in our laboratory[1-11]. However, the method for analyzing the Rui-Yun-Gau, a very popular Chinese medicinal patch preparation is well-known to reduce swelling and relieve pain, etc. remains unavailable for its complexity. The patch preparation contains active ingredients from a variety of Chinese crude drugs including Coptidis Rhizoma, Phellodendri Cortex, Rhei Rhizoma, Scrophulariae Radix, Scutellariae Radix, and Momordicae Semen. Although a number of analytical methods for these Chinese crude drugs and these marker substances have been reported[12-19], but no analytical methods for Rui-Yun-Gau has been reported.

In this study, seven marker substances including berberine (Coptidis Rhizoma and Phellodendri Cortex), and coptisine (Coptidis Rhizoma), emodin, and sennoside A (Rhei Rhizoma), harpagoside (Scrophulariae Radix), baicalin, and baicalein (Scutellariae Radix) are resolved and quantitatively measured through a reversed-phase HPLC approach. The method developed demonstrated to be facile in the routine analysis for quality control by quantitatively determining the active ingredients in the water-based and oil-based patches of the formula from different manufactures.

MATERIALS AND METHODS

Materials

The crude drugs for Rui-Yun-Gau preparation are Coptidis Rhizoma, Phellodendri Cortex, Rhei Rhizoma, Scrophulariae Radix, Scutellariae Radix, and Momordicae Semen. Each material was obtained from local herbal market and pulverized through a #8 mesh sieve (2.36 mm). The origin of crude drugs were verified 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 patches of Rui-Yun-Gau were obtained from Sheng Chun Tang Pharmaceutical Co., Ltd. in Taiwan.

Chemicals and Reagents

The structures of seven marker substances are shown in Fig. 1. Emodin, sennoside A, and harpagoside were purchased from Extrasynthese (Genay, France). Internal standard thymol was purchased from Sigma Chemical Co. (St. Louis, Mo, USA). Berberine, coptisine, baicalin, and baicalein 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. (New Jersey, USA), and phosphoric acid from Kanto

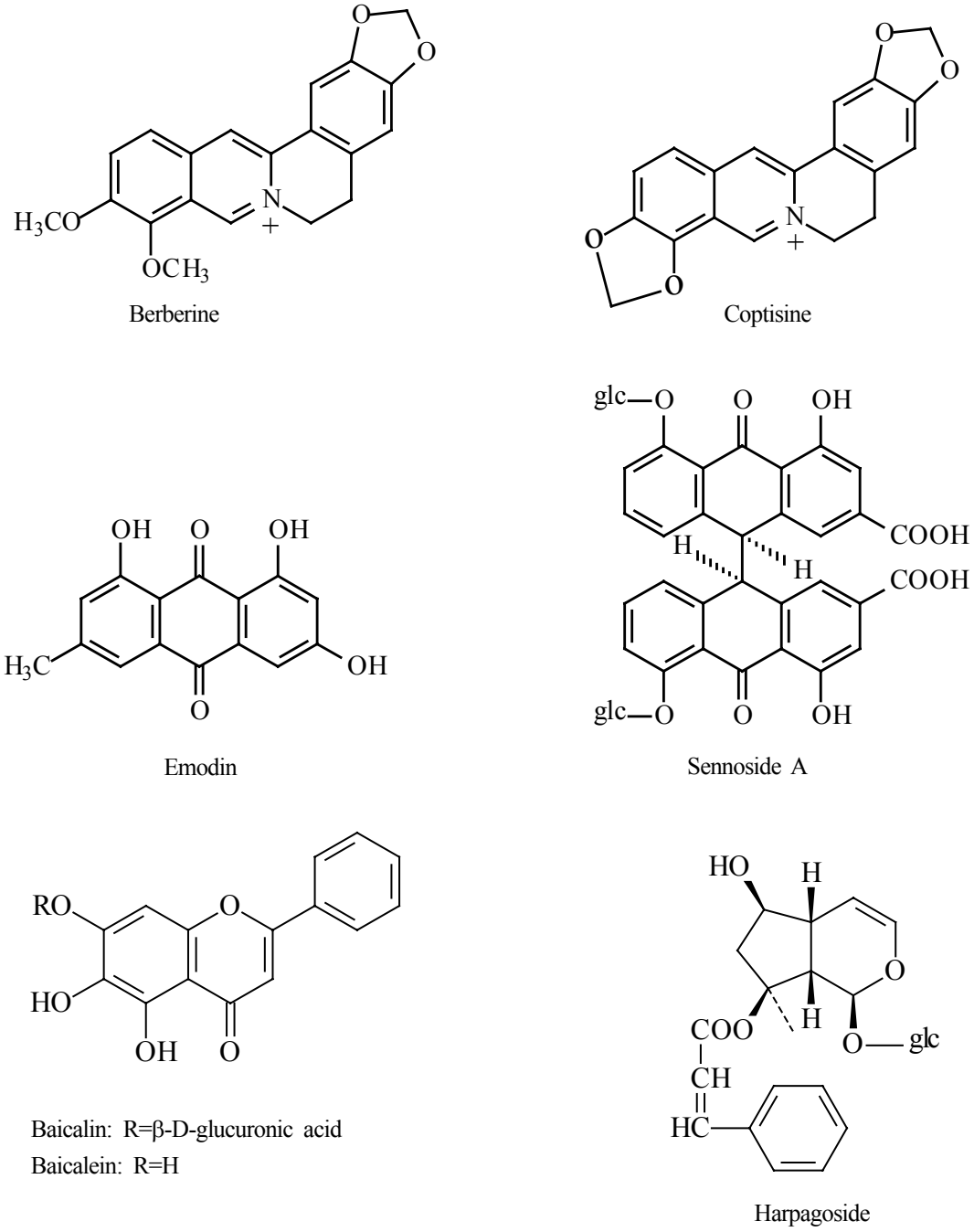


Fig. 1. Structures of the marker substances in Rui-Yun-Gau.

Chemical (Tokyo, Japan). Ultra-pure 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.

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 (GL Science, 4.6 mm i.d. × 250 mm) was used. The column oven was set at 30 °C. The mobile phases consisting of 10% and 60% acetonitrile aqueous solutions in gradient elution are shown in Table 1. The detection wavelength was set at 240 nm. The flow rate was set at 1.0 mL/min. The volume for each injection was 20 μL.

Preparation of Standard Solution 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 solution to obtain the desired concentration: coptisine (160.0 μg/mL), berberine (480.0 μg/mL), sennoside A (240.0 μg/mL), baicalin (700.0 μg/mL), harpagoside (70.0 μg/mL), baicalein (700.0 μg/mL), and emodin (80.0 μg/mL).

The internal standard solution (1,200 μg/mL) was prepared by dissolving 300.0 mg of thymol in 70% methanol solution to obtain a total volume of 250 mL.

Extraction Conditions

According to Yi Hsueh Cheng Chuan (Ming dynasty, 1515), Rui-Yun-Gau consists of each 3.75 g of Coptidis Rhizoma, Phellodendri Cortex, Rhei Rhizoma, Scrophulariae Radix, Scutellariae Radix, and Momordicae Semen. In order to obtain better extraction of seven marker substances from Rui-Yun-Gau, solvents such as sesame oil, 50% ethanol, ethanol and water were used. 22.5 g of above-mentioned Chinese crude drugs were extracted by four different methods as follow and denoted as A, B, C and D.

A. Addition of 450 mL of sesame oil and stored the mixture either at room temperature (25 °C) for one day,

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

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	100	0
40	100	0
45	90	10
55	85	15
60	80	20
80	75	25
90	70	30
95	50	50
110	0	100
115	100	0
120	100	0

Flow rate l.0 mL/min
A: 10% acetonitrile (adjusted to pH 2.8 with phosphoric acid).
B: 60% acetonitrile (adjusted to pH 2.8 with phosphoric acid).

and then refluxed at 150 °C for 3 hrs.

B. Addition of 450 mL of 50% ethanol, and then refluxed at 90 °C for 3 hrs.

C. Addition of 450 mL of ethanol, and then refluxed at 80 °C for 3 hrs.

D. Addition of 450 mL of water, and then refluxed at 100 °C for 3 hrs.

The extract obtained 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 thymol was added to the solution to give a concentration of 600.0 μg/mL. The extracts from method B, C and D were evaporated under vacuum and adjusted to 50 mL by adding 70% methanol. A 1.0 mL aliquot of the solution was diluted to 5 mL by 70% methanol solution, and internal standard thymol was added to each solution to give a concentration of 600.0 μg/mL. All of them were subjected to HPLC for quantification.

Preparation of Sample Solution from Oil-Based Patch

Three pieces of oil-based patch of Rui-Yun-Gau were dissolved with 500 mL of *n*-hexane by refluxing at 75 °C for three hours, the *n*-hexane solution was partitioned with methanol. Suitable amount of internal standard thymol was added to the methanol layer to give a concentration of 600.0 μg/mL.

Preparation of Sample Solution from Water-Based Patch

Three pieces of water-based patch of Rui-Yun-Gau were extracted with 500 mL of methanol by refluxing at 75 °C for three hours. The extract was evaporated under vacuum and adjusted to 25 mL by adding 80% methanol, and internal standard thymol was added to the solution to give a concentration of 600.0 μg/mL.

Calibration Method

The standard solution of each marker substance was prepared from the stock solution by adding 80% methanol to give concentrations of coptisine: 2.5, 5.0, 10.0, 20.0, 40.0, 80.0 μg/mL; berberine: 7.5, 15.0, 30.0, 60.0, 120.0, 240.0 μg/mL; sennoside A: 3.75, 7.5, 15.0, 30.0, 60.0, 120.0 μg/mL; harpagoside: 1.09, 2.19, 4.38, 8.75, 17.5, 35.0 μg/mL; emodin: 1.25, 2.5, 5.0, 10.0, 20.0, 40.0; baicalin, and baicalein: 10.94, 21.88, 43.75, 87.5, 175.0, 350.0 μg/mL, respectively.

Each standard solution contained the internal standard (thymol) at 600.0 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.

Validation

1. Precision

Standard stock solutions were diluted with 80% methanol to three different concentrations. 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.

2. Accuracy

Each standard stock solution of a series of various concentrations was spiked into an ethanol solution of Rui-Yun-Gau, and then refluxed at 80 °C for 3 hrs. Internal standard solution was added to each solution to afford a concentration of 600.0 μg/mL. Then the solution was filtered and subjected to HPLC for 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 Rui-Yun-Gau, and C3 represents the total amount of each markers in the solution.

3. Limit of detection test

To evaluate the HPLC method's limit of detection (LOD), the prepared various concentrations of each standard stock solution were spiked into ethanol solution of LOD were based on a signal to noise (S/N) ratio with 3:1 as the minimum of that observed in the standard.

RESULTS AND DISCUSSION

Separation of Marker Substances by HPLC

All marker substances and internal standard, thymol were successfully separated in a single run HPLC for the ethanol extracts of Rui-Yun-Gau. By using gradient elution, coptisine, berberine, sennoside A, baicalin, harpagoside, baicalein, emodin and thymol were resolved and eluted at 31.80 min, 53.35 min, 65.85 min, 80.75 min, 87.76 min, 102.99 min, 114.67 min, and 110.52 min, respectively (Fig. 2).

The ethanol extract of Rui-Yun-Gau was compared to the four kinds of blank solutions, which were pre-pared with the deletion of one or two materials of Coptidis Rhizoma, Phellodendri Cortex, Rhei Rhizoma, Sc-rophulariae Radix, and Scutellariae Radix, respectively. As shown in Fig. 3B to 3E, no peak of the deleted ma-terial were observed at retention times corresponding to the respective marker substances. Apparently, there was no interaction among components of Rui-Yun-Gau. Therefore, the above conditions can be used for quanti-fication of the marker substances.

Calibration Curve

The linear regression equations, correlation coefficients and concentration range of calibration lines for those

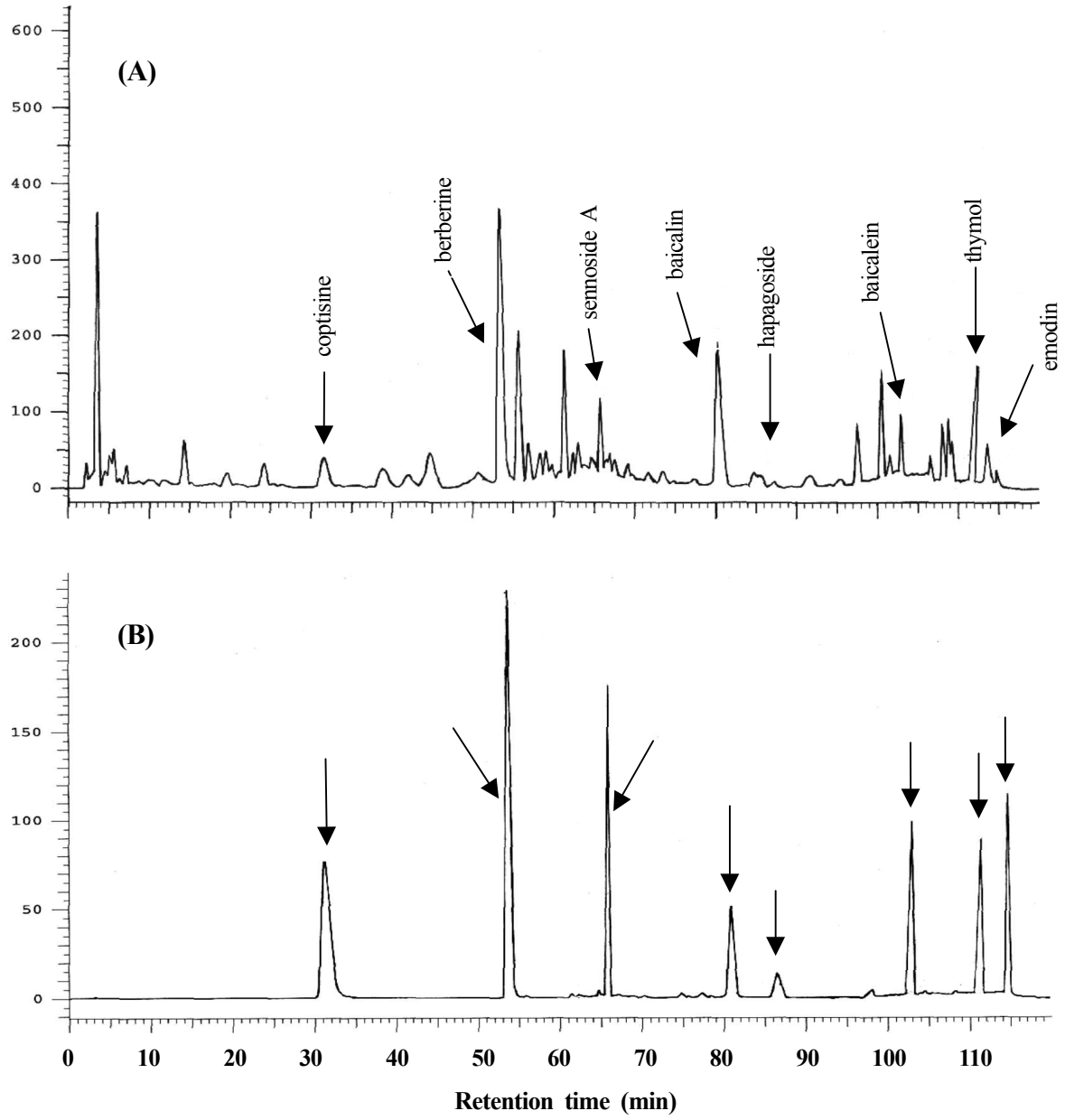
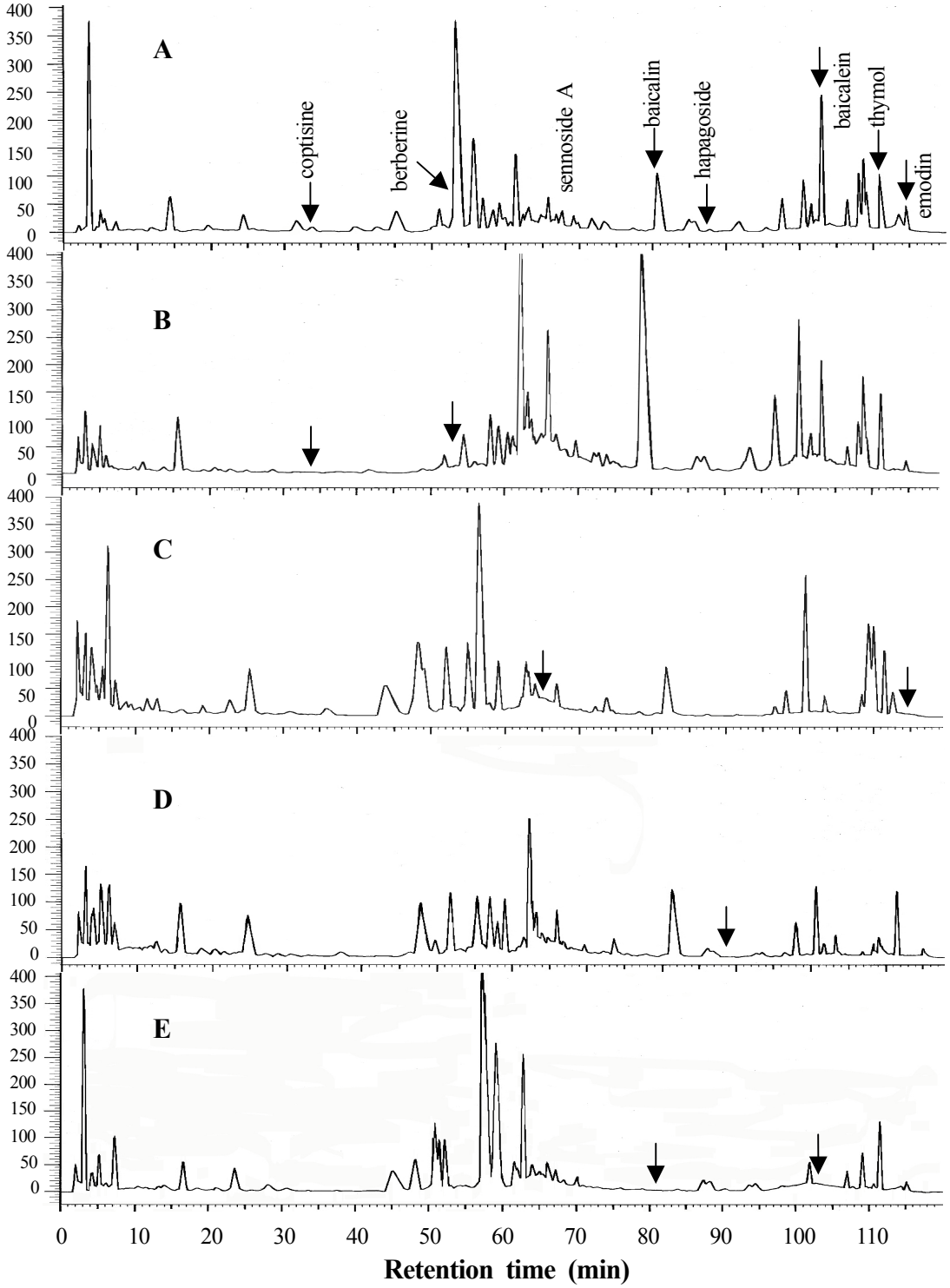


Fig. 2. HPLC Chromatograms of (A) marker substances in ethanol extractions of Rui-Yun-Gau, (B) marker substances.

marker substances were listed in Table 2. All calibration curves were in good linearity with correlation coefficient of 0.9987~0.9997.

Extraction Methods

The HPLC chromatograms and the contents of all marker substances extracted with four extraction methods are shown in Fig. 4 and Table 3. The results indicated that the method C (addition of 450 mL of ethanol, and then reflux at 80 °C for 3 hrs) or B (addition of 450 mL of 50% ethanol, and then reflux at 90 °C for 3 hrs) afforded higher yield of the seven marker substances.

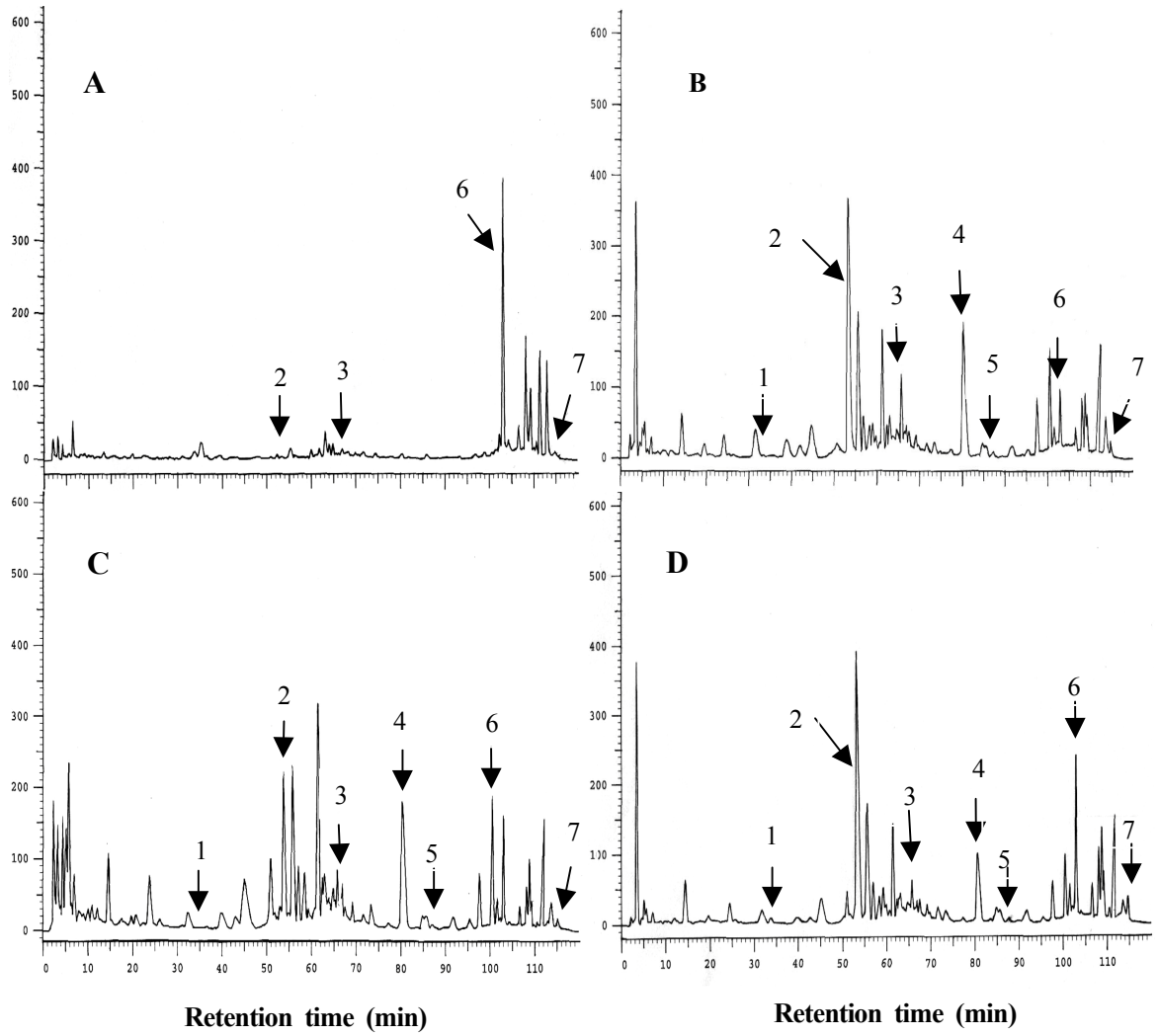


A: Ethanol extract of Rui-Yun-Gau containing internal standard, thymol.
B: Ethanol extract of Rui-Yun-Gau without Coptidis Rhizoma and Phellodendri Cortex.
C: Ethanol extract of Rui-Yun-Gau without Rhei Rhizoma.
D: Ethanol extract of Rui-Yun-Gau without Scrophulariae Radix.
E: Ethanol extract of Rui-Yun-Gau without Scutellariae Radix.

Fig. 3. Chromatograms of marker substances in ethanol extracts of Rui-Yun-Gau made from incomplete materials.

Table 2. Calibration curves of marker substances

Compound	Concentration range μg/mL	Regression equation	r
coptisine	2.50~80.00	y = 0.0406 x - 0.4600	0.9997
berberine	7.50~240.00	y = 0.2790 x + 0.0296	0.9992
sennoside A	3.75~120.00	y = 0.0078 x + 0.0059	0.9996
baicalin	10.94~350.00	y = 0.0210 x + 0.0364	0.9991
harpagoside	1.09~35.00	y = 0.0028 x - 0.0018	0.9994
baicalein	10.94~350.00	y = 0.0237 x + 0.0075	0.9993
emodin	1.25~40.00	y = 0.0181 x + 0.0449	0.9987



A: Addition of 450 mL of sesame oil and stored the mixture either at room temperature (25 °C) for one day, and then refluxed at 150 °C for 3 hrs.
B: Addition of 450 mL of 50% ethanol, and then refluxed at 90 °C for 3 hrs.
C: Addition of 450 mL of ethanol, and then refluxed at 80 °C for 3 hrs.
D: Addition of 450 mL of water, and then refluxed at boiling temperature for 3 hrs.
1: coptisine; 2: berberine; 3: sennoside A; 4: baicalin; 5: harpagoside; 6: baicalein; 7: emodin

Fig. 4. HPLC chromatograms of four kinds of extraction conditions in Rui-Yun-Gau.

Table 3. The relative extraction ratio of seven maker substances of Rui-Yun-Gau

Compound	A	B	C	D
coptisine	--	5.26 ± 0.71(89.6)	5.87 ± 0.31(100.0)	0.16 ± 0.51(2.7)
berberine	0.07 ± 0.43(0.2)	25.45 ± 0.25(89.2)	29.17 ± 0.05(100.0)	0.68 ± 0.08(2.3)
sennoside A	0.10 ± 1.48(0.2)	39.33 ± 0.21(87.1)	45.13 ± 0.18(100.0)	0.05 ± 1.19(0.1)
baicalin	--	64.65 ± 0.18(88.2)	73.32 ± 0.11(100.0)	0.36 ± 0.53(0.5)
harpagoside	--	4.72 ± 1.85(100.0)	3.87 ± 1.08(82.0)	0.01 ± 0.43(0.2)
baicalein	1.47 ± 0.14(5.9)	23.01 ± 0.32(91.7)	25.08 ± 0.08(100.0)	0.61 ± 2.80(2.4)
emodin	0.01 ± 1.52(0.6)	1.23 ± 2.28(69.1)	1.78 ± 1.74(100.0)	0.10 ± 0.77(5.6)

Data represented as mean (mg/one dose) ± C.V. value (%)

A: Addition of 450 mL of sesame oil and stored the mixture either at room temperature (25 °C) for one day, and then refluxed at 150 °C for 3 hrs.

B: Addition of 450 mL of 50% ethanol, and then refluxed at 90 °C for 3 hrs.

C: Addition of 450 mL of ethanol, and then refluxed at 80 °C for 3 hrs.

D: Addition of 450 mL of water, and then refluxed at boiling temperature for 3 hrs.

--: Not detected.

Precision and Accuracy

The relative standard deviations of the intra-day and inter-day analysis were 0.02~1.30% and 0.15~4.79%, suggesting that the method had very good reproducibility (Table 4).

Table 4. Reproducibilities of intra-day and inter-day analysis of Rui-Yun-Gau

Compound	Concentration (μg/mL)	Mean ± S.D. (R.S.D %)	
		intra-day (n = 3)	inter-day (n = 4)
coptisine	80.00	80.64 ± 0.62(0.77)	81.12 ± 0.53(0.65)
	20.00	19.34 ± 0.11(0.57)	19.52 ± 0.94(4.79)
	5.00	4.49 ± 0.04(0.78)	5.37 ± 0.04(0.79)
berberine	240.00	239.12 ± 1.29(0.54)	242.48 ± 3.59(1.48)
	60.00	60.57 ± 0.07(0.12)	61.13 ± 0.50(0.82)
	15.00	14.32 ± 0.01(0.10)	15.83 ± 0.05(0.32)
sennoside A	120.00	120.34 ± 1.50(1.25)	120.67 ± 3.08(2.55)
	30.00	29.57 ± 0.06(0.21)	29.35 ± 0.10(0.35)
	7.50	7.62 ± 0.02(0.25)	7.39 ± 0.01(0.15)
baicalin	350.00	348.77 ± 3.07(0.88)	349.21 ± 1.08(0.31)
	87.50	87.15 ± 0.09(0.10)	87.79 ± 0.25(0.29)
	21.88	22.45 ± 0.02(0.09)	21.56 ± 0.20(0.93)
harpagoside	35.00	35.39 ± 0.01(0.02)	35.89 ± 0.10(0.27)
	8.75	8.94 ± 0.12(1.30)	8.53 ± 0.07(0.84)
	2.19	2.07 ± 0.02(1.17)	2.32 ± 0.03(1.18)
baicalein	350.00	351.26 ± 0.74(0.21)	350.86 ± 0.81(0.23)
	87.50	87.89 ± 0.22(0.25)	88.13 ± 0.92(1.04)
	21.88	21.56 ± 0.01(0.03)	22.16 ± 0.25(1.11)
emodin	40.00	39.55 ± 0.26(0.65)	40.56 ± 0.07(0.18)
	10.00	9.78 ± 0.01(0.11)	9.39 ± 0.05(0.58)
	2.50	2.38 ± 0.01(0.30)	2.24 ± 0.06(2.64)

Table 5. Recovery of seven marker substances from Rui-Yun-Gau

Compound	Concentration (μg/mL)	Recovery (%) Mean ± S.D. (R.S.D %)
coptisine	80.00	101.26 ± 0.14 (0.14)
	20.00	90.65 ± 0.77 (0.85)
	5.00	106.80 ± 0.36 (0.34)
berberine	240.00	99.67 ± 0.21 (0.21)
	60.00	99.51 ± 0.18 (0.18)
	15.00	106.20 ± 0.54 (0.51)
sennoside A	120.00	95.56 ± 0.52 (0.54)
	30.00	96.83 ± 0.76 (0.79)
	7.50	103.33 ± 1.79 (1.73)
baicalin	350.00	100.86 ± 0.23 (0.23)
	87.50	111.83 ± 0.41 (0.37)
	21.88	95.93 ± 0.39 (0.41)
harpagoside	35.00	97.71 ± 0.31 (0.32)
	8.75	112.22 ± 2.24 (2.00)
	2.19	101.37 ± 0.80 (0.79)
baicalein	350.00	93.79 ± 0.19 (0.20)
	87.50	91.90 ± 1.95 (2.12)
	21.88	100.57 ± 0.19 (0.19)
emodin	40.00	94.22 ± 0.92 (0.98)
	10.00	107.40 ± 2.52 (2.35)
	2.50	101.20 ± 0.93 (0.92)

Recoveries of the analysis were shown in Table 5. All of the recoveries are greater than 90.65%.

LOD of these marker substances under above conditions was 5 ng/mL.

Quantitative Analysis of Marker Substances in Water-Based and Oil-Based Patch Preparations of Rui-Yun-Gau

The HPLC chromatograms and the contents of marker substances in water-based and oil-based patch preparations, as shown in Fig. 5 and Table 6, were quite different from each other. This is probably due to the different sources of the pharmaceutical excipients, the different manufacturing process, and the component release.

In this report, we established a precise and reliable quantification method for the simultaneous determination of seven marker substances in Rui-Yun-Gau. The method can be used for quality control of manufacturing process of the patches of Rui-Yun-Gau in the future.

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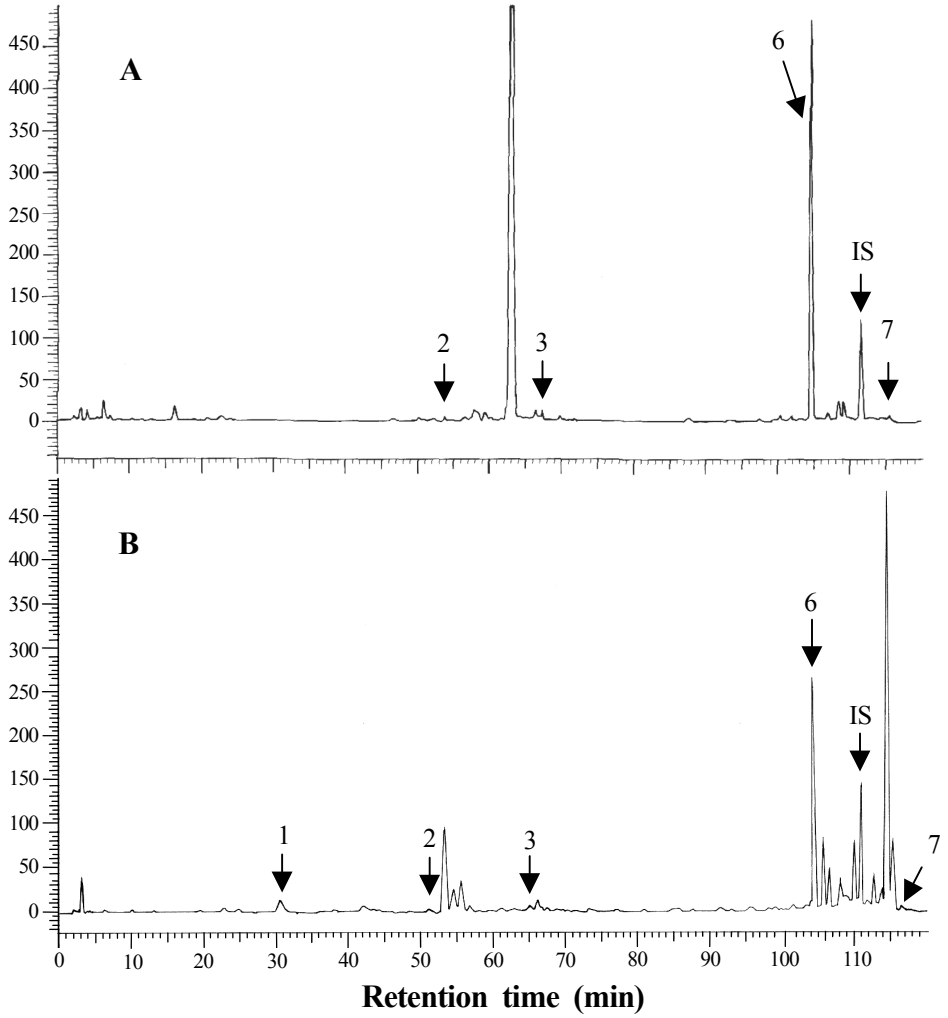

A: water based patch; B: oil based patch.
1: coptisine; 2: berberine; 3 :sennoside A; 4 : baicalin; 5:harpagoside; 6: baicalein; 7: emodin.

Fig. 5. HPLC chromatograms of water based and oil based patch preparations of Rui-Yun-Gau.

Table 6. Contents of marker substances in water-based and oil-based patches of Rui-Yun-Gau

Compound	Water-based Patch	Oil-based Patch
coptisine	--	3.0 ± 0.67
berberine	53.0 ± 0.37	17.0 ± 0.88
sennoside A	61.0 ± 2.59	6.0 ± 1.24
baicalin	--	--
harpagoside	--	--
baicalein	43.0 ± 0.37	24.0 ± 1.54
emodin	9.0 ± 1.37	4.0 ± 0.70

Data represented as mean (μg/one piece) ± C.V. value (%)

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液相層析法對綠云膏貼劑中七種成分之同時分析研究

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使用逆相 HPLC 模式進行綠云膏貼劑之成分分析，開發出包含黃連中 berberine、coptisine；黃柏中 berberine；大黃中 emodin、sennoside A；玄參中 harpagoside；黃芩中 baicalin、baicalein 等七種指標成分之多成分同時定量分析方法。同時以水性基劑及油性基劑等不同製程，探討對綠云膏成分之影響。

綠云膏之試料通過保持在 30 °C 恒溫之 HPLC 層析管（Inertsil ODS-2, 4.6 mm i.d. × 250 mm），移動相採用 10% 及 60% acetonitrile 之混合溶液（各以磷酸調整 pH 值為 2.8），進行梯度沖提法，以 1.0 mL/分之流速沖提。使用 UV 偵測器，偵測波長設定 240 nm。

本法之回收率，同日間及異日間的變異係數均在 5% 以下，回收率在 90.65~112.22% 之間。這個分析法對於綠云膏製劑中七種指標成分是安定且值得信賴之定量法。

關鍵詞：綠云膏，高效液相層析法。

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