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# Simultaneous Determination of Five Constituents in "Tzyy-Yun-Gau" Medicine by High Performance Liquid Chromatography

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## ABSTRACT

An HPLC method was applied for the simultaneous determination of five marker substances in the traditional Chinese medicine preparation "Tzyy-Yun-Gau". These substances included shikonin, deoxyshikonin,  $\beta,\beta$ -dimethylacrylshikonin, and acetylshikonin in *Arnebiae* Radix; and ferulic acid in *Angelicae* Radix.

Tzyy-Yun-Gau was partitioned in a mixture of n-hexane and methanol, and a fraction taken into the methanol layer was analyzed. The samples were run through an HPLC column (Inertsil ODS-2, 4.6 mm I.D.  $\times$  250 mm) at 30°C with a mobile phase consisting of methanol, acetonitrile and 2% acetic acid with linear gradient elution at a flow-rate of 1.0 mL/min. A UV detector was used for detection. The detection wavelength varied with time, which was 325 nm during 0~25 min, 525 nm during 25~58.5 min, 440 nm during 58.5~62 min, and 525 nm during 62~80 min. This could be a successful separation method for the simultaneous determination of five marker substances in "Tzyy-Yun-Gau".

Key words: Tzyy-Yun-Gau, *Arnebiae* Radix, *Angelicae* Radix, shikonin, deoxyshikonin,  $\beta,\beta$ -dimethylacrylshikonin, acetylshikonin, ferulic acid

## INTRODUCTION

Quality control is an important factor in obtaining the Good Manufacturing Practice (GMP) standard for Chinese medicinal preparations. In recent years, a number of analytical methods for Chinese medicinal preparations have been established in our laboratories<sup>(1,2)</sup>. Our aim is to develop simple and expedient analytical methods for routine quality control. In this study, we selected a traditional preparation, Tzyy-Yun-Gau, which contains *Angelicae* Radix, *Arnebiae* Radix, sesame oil, lard oil, and yellow wax, and is a kind of topical medicine used extensively in the treatment of frostbite, burns, rub and so on. Although the compositions and the analytical methods of *Angelicae* Radix<sup>(3-23)</sup> and *Arnebiae* Radix<sup>(24-31)</sup> have been well established, we found that in existing literature, only shikonin<sup>(32)</sup> in Tzyy-Yun-Gau has been studied and analyzed. Therefore this study employed HPLC to simultaneously determine the content of five marker substances. These substances included shikonin, deoxyshikonin,  $\beta,\beta$ -dimethylacrylshikonin, and acetylshikonin in *Arnebiae* Radix; and ferulic acid in *Angelicae* Radix.

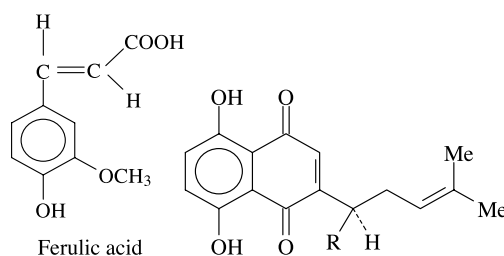
## MATERIALS AND METHODS

### I. Materials

According to Ref. 33, the materials for Tzyy-Yun-Gau preparation are *Angelicae* Radix, *Arnebiae* Radix, sesame oil, lard oil, and yellow wax. Each material was obtained from the herbal market, and *Angelicae sinensis* Radix and *Arnebiae* Radix were pulverized through a #8 mesh sieve (2.36 mm).

### II. Chemicals and Reagents

The structures of the marker substances are shown in



R	
Deoxyshikonin	-H
Shikonin	-OH
Acetylshikonin	-O-CO-CH <sub>3</sub>
$\beta,\beta$ -Dimethylacrylshikonin	-O-CO-CH=C(CH <sub>3</sub> ) <sub>2</sub>

Figure 1. Structures of marker constituents.

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Figure 1. Ferulic acid was purchased from Sigma. Shikonin, deoxyshikonin,  $\beta,\beta$ -dimethylacrylshikonin and acetylshikonin were purified and identified in our laboratory, and the internal standard emodin was obtained from Extrasynthese (France). Acetonitrile, methanol, n-hexane (HPLC grade), and acetic acid (analytical-reagent grade) were obtained from Riedel-de Haen (Germany). Ultra-pure distilled water with a resistivity greater than 18M $\Omega$  was used.

### III. Liquid Chromatography

HPLC was conducted with an HP model 1100 system equipped with an HP G1315A Photodiode Array Detector, HP G1314A Variable Wavelength Detector, HP G1311A QuatPump, HP G1322A Degasser, HP G1313A Auto-sampler, and HP G1316A Column Oven. Peak areas were calculated with an HP Vectra VE PC 5/100 integrator. An Inertsil ODS-2 reversed-phase column (4.6 mm I.D.  $\times$  250 mm) was used.

The mobile phase was a mixture of methanol, acetonitrile and 2% acetic acid solution with linear gradient elution (0 min, 5: 5: 90; 70 min, 40: 40: 20), filtered through a 0.45  $\mu$ m membrane filter (Millipore) and degassed prior to use. The analysis was carried out at a flow-rate of 1.0 mL/min. A UV detector was used for the detection of the marker substances. The detection wavelength varied with time, which was 325 nm during 0–25 min, 525 nm during 25–58.5 min, 440 nm during 58.5–62 min, and 525 nm during 68–80 min.

### IV. Calibration Method

The standard solutions of each marker substance were diluted with 70 % methanol to afford sequential concentrations. Each diluted solution contained the internal standard, emodin, of 108  $\mu$ g/mL. After filtering through a 0.45  $\mu$ m filter, 20  $\mu$ L of each concentration was injected into the HPLC column for analysis. The calibration graphs were plotted after linear regression of the peak area ratio with concentration.

### V. Reproducibility Test

The standard solutions with various concentrations of ferulic acid (8.3, 33.2, 132.6  $\mu$ g/mL), shikonin (11.5, 40.6, 184.5  $\mu$ g/mL), deoxyshikonin (10.4, 65.3, 408.0  $\mu$ g/mL),  $\beta,\beta$ -dimethylacrylshikonin (25.1, 100.2, 501.0  $\mu$ g/mL), and acetylshikonin (24.1, 96.5, 482.5  $\mu$ g/mL) were used for a reproducibility test. An intra-day test (injecting each concentration three times during 24 hr), and inter-day test (injecting each concentration four times during 7 days with each injection separated by at least 24 hr) were used to check reproducibility.

### VI. Preparation of Tzzy-Yun-Gau

*Angelicae Radix* 18.75 g and *Arnebiae Radix* 37.5 g were weighed and pulverized, and sesame oil 150 g and lard oil 75 g were then added. The mixture was boiled for 10 min

and filtered while hot. Finally yellow wax 18.75 g was added to the filtrate.

### VII. Preparation of Sample Solutions

Samples of 300 mg each of the above-mentioned Tzzy-Yun-Gau or commercial Tzzy-Yun-Gau was partitioned with a mixture of n-hexane 10 mL and methanol 10 mL in a separator (2,500 rpm, 30 min). Then the methanol layer was then separated and a suitable amount of internal standard emodin was added to the solution to give a concentration of 108  $\mu$ g/mL.

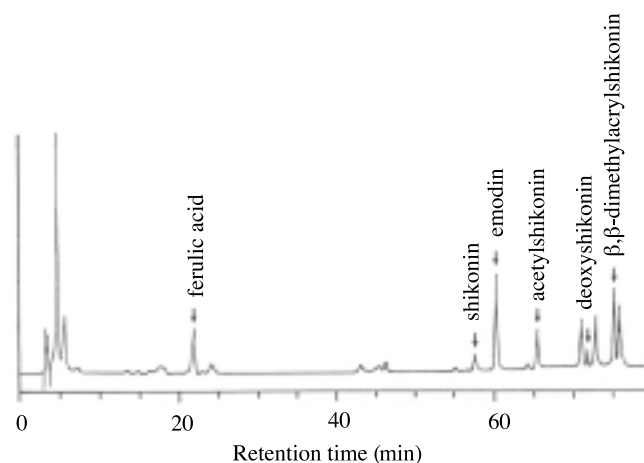
### VIII. Recovery Test

The methanol layer from Tzzy-Yun-Gau was divided into four portions, one portion used as a control group and the rest being spiked with different concentrations of standard solutions to afford various concentrations of ferulic acid (8.3, 33.2, 132.6  $\mu$ g/mL), shikonin (11.5, 40.6, 184.5  $\mu$ g/mL), deoxyshikonin (10.4, 65.3, 408.0  $\mu$ g/mL),  $\beta,\beta$ -dimethylacrylshikonin (25.1, 100.2, 501.0  $\mu$ g/mL), and acetylshikonin (24.1, 96.5, 482.5  $\mu$ g/mL). Internal standard emodin was added to each solution to a concentration of 108  $\mu$ g/mL. All samples were filtered through a 0.45  $\mu$ m filter, injected into the HPLC column for analysis and the recovery calculated.

## RESULTS AND DISCUSSION

### I. Separation Result by HPLC

A methanol extract of Tzzy-Yun-Gau was analyzed with an HPLC instrument. Ferulic acid, shikonin, deoxyshikonin,  $\beta,\beta$ -dimethylacrylshikonin, acetylshikonin and the internal standard were completely separated as shown in Figure 2. The retention times of the marker substances and internal standard were 21.7, 57.4, 75.6, 66.1 and 60.1 min, respectively. On the inspection of three-dimensional chro-



**Figure 2.** Chromatogram of ferulic acid, shikonin, deoxyshikonin,  $\beta,\beta$ -dimethylacrylshikonin, acetylshikonin and internal standard in Tzzy-Yun-Gau.

**Table 1.** Reproducibilities of intra-day and inter-day analysis of Tzyy-Yun-Gau

Compound	Concentration ( $\mu\text{g/mL}$ )	CV%	
		intra-day (n=3)	inter-day (n=4)
Ferulic acid	132.60	2.41	3.24
	33.20	1.08	2.95
	8.30	2.59	4.65
Shikonin	184.00	0.54	1.01
	46.00	0.57	3.50
	11.50	3.81	5.30
Deoxyshikonin	408.00	0.23	0.98
	65.30	0.31	1.15
	10.40	0.76	2.37
$\beta,\beta$ -Dimethylacrylshikonin	501.00	0.95	1.28
	100.20	2.92	1.73
	25.10	4.01	2.72
Acetylshikonin	482.50	4.91	2.84
	96.50	3.21	1.86
	17.40	4.25	3.63

**Table 2.** Recovery of each marker substance from Tzyy-Yun-Gau

Compound	Added <sup>a</sup> ( $\mu\text{g/mL}$ )	Found <sup>a</sup> ( $\mu\text{g/mL}$ )	Recovery <sup>b</sup> (%)
Ferulic acid	132.60	134.00	101.06 $\pm$ 4.57
	33.20	34.21	103.04 $\pm$ 4.57
	8.30	8.89	107.11 $\pm$ 4.12
Shikonin	184.00	184.22	100.12 $\pm$ 0.68
	46.00	46.85	101.85 $\pm$ 1.77
	11.50	11.86	103.11 $\pm$ 4.09
Deoxyshikonin	408.00	406.29	99.58 $\pm$ 1.16
	65.30	65.23	99.90 $\pm$ 3.36
	10.40	10.59	101.86 $\pm$ 4.89
$\beta,\beta$ -Dimethylacrylshikonin	501.00	569.94	113.76 $\pm$ 3.79
	100.20	126.69	126.44 $\pm$ 2.20
	25.10	30.54	121.69 $\pm$ 4.60
Acetylshikonin	482.50	462.86	95.93 $\pm$ 4.06
	96.50	98.60	102.18 $\pm$ 1.29
	17.40	17.17	98.70 $\pm$ 2.60

<sup>a</sup> Mean, n=7. <sup>b</sup> Mean  $\pm$  S.D., n=7.

matograms, these five constituents all showed high purities. The commercial preparation also showed satisfactory separation.

## II. Calibration Line

The regression equations and correlation coefficients of calibration lines for those marker substances were as follows, where  $y$  is the peak area ratio of the marker to the internal standard and  $x$  is the concentration of the marker.

Ferulic acid in the concentration range of 8.3~132.6  $\mu\text{g/mL}$ ;  $y = -0.1963 + 0.7553x$ ,  $r = 0.9998$  ( $n = 5$ ).

Shikonin in the concentration range of 11.5~184.0  $\mu\text{g/mL}$ ;  $y = -0.0440 + 0.1048x$ ,  $r = 0.9993$  ( $n = 5$ ).

Deoxyshikonin in the concentration range of 10.4~408.0  $\mu\text{g/mL}$ ;  $y = -0.0050 + 0.0226x$ ,  $r = 0.9999$  ( $n = 5$ ).

$\beta,\beta$ -Dimethylacrylshikonin in the concentration range of 25.1~501.0  $\mu\text{g/mL}$ ;  $y = -0.0111 + 0.0034x$ ,  $r = 0.9999$  ( $n = 5$ ).

Acetylshikonin in the concentration range of 25.1~501.0  $\mu\text{g/mL}$ ;  $y = -0.0059 - 0.0323x$ ,  $r = 0.9994$  ( $n =$

**Table 3.** Contents of each marker substance in two different preparations of Tzyy-Yun-Gau

Compound	Our preparation ( $\mu\text{g/g}$ )	Commercial preparation ( $\mu\text{g/g}$ )
Ferulic acid	36.01 $\pm$ 0.07	2.03 $\pm$ 0.03
Shikonin	22.13 $\pm$ 0.54	2.79 $\pm$ 0.05
Deoxyshikonin	23.05 $\pm$ 1.41	3.52 $\pm$ 1.18
$\beta,\beta$ -Dimethylacrylshikonin	54.89 $\pm$ 0.16	11.99 $\pm$ 0.81
Acetylshikonin	30.60 $\pm$ 0.84	5.91 $\pm$ 0.51

Data represented as mean  $\pm$  S.D.,  $n = 7$ .

5).

These results showed good linear relationships between the peak-area ratio and concentration.

## III. Reproducibility Test

The coefficients of variation (C.V. %) were calculated from the results as shown in Table 1. The intra-day relative standard deviations were 1.08~2.59%, 0.54~3.81%, 0.23~0.76%, 0.95~4.01%, and 3.21~4.91%, respectively. The inter-day relative standard deviations were 2.95~4.65%, 1.01~5.30%, 0.98~2.37%, 1.28~2.72%, and 1.86~3.63%, respectively. Results showed very good reproducibility.

## IV. Recovery Test

Recovery of the analysis were 101.06  $\pm$  4.57~107.11  $\pm$  4.12% for ferulic acid, 100.12  $\pm$  0.68~103.11  $\pm$  4.09% for shikonin, 99.58  $\pm$  1.16~101.86  $\pm$  4.89% for deoxyshikonin, 113.76  $\pm$  3.79~126.44  $\pm$  2.20% for  $\beta,\beta$ -dimethylacrylshikonin, and 95.93  $\pm$  4.06~102.18  $\pm$  1.29% for acetylshikonin, respectively (Table 2).

## V. Analysis of the Commercial Preparations

The contents of marker substances in commercial preparation are markedly different from the product prepared in our laboratory, as shown in Table 3. This is probably due to the different sources of the crude drugs and the different manufacturing process.

## CONCLUSIONS

A multi-component HPLC method was developed for the simultaneous quantification of five marker substances in Tzyy-Yun-Gau. A multi-solvent of methanol, acetonitrile and 2% acetic acid solution was used as the mobile phase with a gradient elution program, with an Inertsil ODS-2 reversed-phase column as the stationary phase. Four detection wavelengths were varied depending on the retention times of the respective components. The internal standard, emodin, used to determine the calibration line resulted in a precise and reliable quantification analysis. The method can be applied for analyzing commercial preparations and has the advantage of simplicity in sample preparation. It could be applicable for the quality control of Tzyy-Yun-Gau in the future.

## ACKNOWLEDGMENTS

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## 紫雲膏製劑中多成分同時分析研究

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### 摘 要

使用 HPLC 進行傳統中藥軟膏製劑紫雲膏之成分分析，開發出包含紫草中 shikonin, deoxyshikonin,  $\beta,\beta$ -dimethylacrylshikonin, acetylshikonin 及當歸中 ferulic acid 等五種指標成分之多成分同時定量分析方法。

紫雲膏經採用 n-hexane 及 methanol 二種溶媒，進行分配並取 methanol 層為 HPLC 分析之對象。將製備之試料通過保持在 30℃ 恆溫之 HPLC 層析管 (Inertsil ODS-2, 4.6 mm I.D.  $\times$  250 mm)，移動相採用 methanol, acetonitrile 及 2% acetic acid 之混合溶液，進行梯度沖提法，以 1.0 mL / 分之流速沖提。五種指標成分之檢測使用 UV 檢出器，檢測波長設定 0-25 分為 325 nm；25-58.5 分為 525 nm；58.5-62 分為 440 nm；最後 62-80 分為 525 nm。這個分離法對於紫雲膏中五種指標成分是精確且值得使用之定量法。

關鍵詞：紫雲膏，紫草，當歸，shikonin，deoxyshikonin， $\beta,\beta$ -dimethylacrylshikonin，acetylshikonin，ferulic acid