

# Determination and Pharmacokinetics of 6,7-Dimethoxycoumarin in Rat Plasma after Intragastric Administration of Different Decoctions of Yinchenhao Tang

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## Abstract

A simple and sensitive reversed-phase high-performance liquid chromatography method with UV detection is developed and validated for the determination of 6,7-dimethoxycoumarin in rat plasma and comparative analysis of its pharmacokinetics after intragastric administration of 6,7-dimethoxycoumarin and three different decoctions of Yinchenhao Tang. The plasma samples are deproteinated with acetonitrile. The components are separated on a Kromasil C<sub>18</sub> column (250 × 4.6 mm, 5 μm) with methanol–1% acetic acid solution–tetrahydrofuran (30:63:7, v/v/v) as the mobile phase, and the UV detector is set at 340 nm. Coumarin is used as an internal standard. The linear calibration curve is obtained in the concentration range of 25–2500 ng/mL. The lower limit of quantitation of the method is 25 ng/mL. The intra- and inter-day precision are less than 12%, and the accuracy determined with relative error ranges from –2.9% to 1.7%. The data obtained from rat plasma are analyzed with Topfit 2.0 Pharmacokinetic Software. With pharmacokinetic analysis, the main parameters after intragastric administration of 6,7-dimethoxycoumarin, Herba Artemisiae Scopariae decoction, Artemisiae Scopariae decoction plus Radix et Rhizoma Rhei and Fructus Gardeniae decoction, Yinchenhao Tang are as follows: T<sub>1/2</sub> is 0.29, 1.30, 1.07, and 1.75 h, AUC<sub>0→∞</sub> is 919.1, 1215.0, 2035.3, and 2537.9 ng·h/mL, AUC<sub>0→t</sub> is 928.5, 1325.9, 2094.4, and 2612.6 ng·h/mL, respectively.

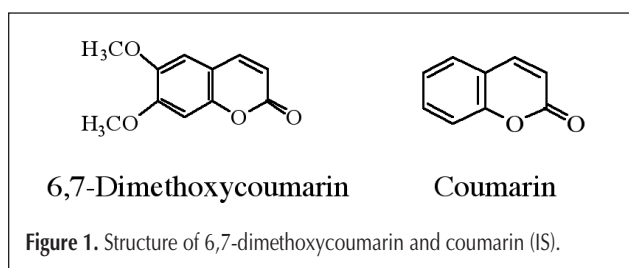
## Introduction

Traditional Chinese medicinal (TCM) prescriptions have been used for over 1,000 years. Most of them are composed of many herbs, which contain complicate chemical constituents. Because of a difference of formulation, the activation is different, thus the pharmacokinetic characteristics of active con-

stituents are different.

Yinchenhao Tang is a famous prescription in a classical Chinese medical work “Shang han lun,” which consists of Herba Artemisiae Scopariae, Radix et Rhizoma Rhei, and Fructus Gardeniae, and it is used clinically in China and Japan for the treatment of hepatitis. In this prescription, Herba Artemisiae Scopariae is the most important crude drug. 6,7-Dimethoxycoumarin (Figure 1) was extracted from the leaf and stem of Artemisia Scoparia by Waldst et Kit, and it was reported that 6,7-dimethoxycoumarin was an important chemical substance with activities to cure hepatic injury in Herba Artemisiae Scopariae (1), while it possesses vasodilator and hypertensive action (1), immune suppressive activity (2–3), anti-angina effect on the heart, and antiatherogenic effect in hyperlipidaemic diabetic rabbits (4–5). Tsai determined the concentration of 6,7-dimethoxycoumarin in rat plasma after i.v. administration by high-performance liquid chromatography (HPLC) (6).

This work developed an RP-HPLC method with UV detection to determine the concentration of 6,7-dimethoxycoumarin in rat plasma and compare the pharmacokinetics of the 6,7-dimethoxycoumarin after intragastric administration of 6,7-dimethoxycoumarin, Herba Artemisiae Scopariae decoction, Herba Artemisiae Scopariae decoction plus Radix et Rhizoma Rhei and Fructus Gardeniae decoction, Yinchenhao Tang, respectively.



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## Experimental

### Materials and reagents

6,7-Dimethoxycoumarin (99.6%) and coumarin (99.7%) used as the standard substance and internal standard (IS) were isolated in our laboratory (Department of Pharmaceutical analysis, Shenyang Pharmaceutical University, Shenyang, China). Acetonitrile, methanol, and tetrahydrofuran (THF) (HPLC grade) were purchased from Concord Tech Reagent Company (Tianjin, China). Acetic acid and other analytical reagents (AR) were purchased from Shenyang Chemical Reagent Factory (Shenyang, China). Herba Artemisiae Scopariae, Radix et Rhizoma Rhei, and Fructus Gardeniae were collected in Dongling district of Shenyang, China, and they were authenticated by Qishi Sun, Professor of Pharmacognosy (Shenyang Pharmaceutical University, Shenyang, China).

### Chromatographic system

The HPLC system was composed of a Shimadzu LC-10ATvp series binary pump, a Shimadzu SPD-10Avp UV detection, and an HT-130 column heater. An ANASTAR Chromatography Data System was used for data acquisition and integration. Separations were carried out with a Kromasil C<sub>18</sub> column (Sciencelab, 250 × 4.6 mm, 5 μm) and a mobile phase of methanol–1% acetic acid solution–THF (30:63:7, v/v/v). Chromatography was performed at a flow-rate of 0.8 mL/min with a wavelength of 340 nm and operated at 30°C.

### Preparation of standard solutions and quality control samples

6,7-Dimethoxycoumarin and coumarin stock standard solutions (0.1 mg/mL) were prepared by dissolving 10 mg of 6,7-dimethoxycoumarin and coumarin in 100 mL of methanol and kept in a refrigerator (4°C), respectively. The working solutions were prepared by appropriate dilution of the stock solution with methanol.

The linearity of the assay was assessed by preparing six different concentrations of standard samples with blank plasma and working solution. The standard samples were prepared under the same conditions as those of the test samples. Calibration was performed by least-squares linear regression of the peak-area ratio of the component to the IS versus the respective plasma concentration with a weight of  $1/x^2$  (reciprocal of square of concentration) factor. Quantitation was based on the ratio of the peak area of the analyte against that of the IS.

Quality control (QC) samples were prepared at low, medium, and high concentrations in the same way as the plasma samples for calibration. These QC samples were used to determine the accuracy, precision, and recovery of the HPLC method, and stability of the plasma sample. All working solutions were stored at 4°C before use.

### Preparation of administrations

For the 6,7-dimethoxycoumarin solution, dried 6,7-dimethoxycoumarin was dissolved in right volume of 1% solution of Polysorbate 80 (4.0 mg/mL, calculated by the quantity

of 6,7-dimethoxycoumarin).

For the Herba Artemisiae Scopariae decoction, dried Herba Artemisiae Scopariae (8 g) was extracted twice, each with 80 mL of boiling water for 1 h. The extracts were mixed with 95% alcohol of 3 times the volume. They were stored in a refrigerator (4°C) for 18 h, and then the supernatant was filtered and concentrated to be as dry as possible. The residue was dissolved in right volume of 1% solution of Polysorbate 80 (4.0 mg/mL, calculated by the quantity of 6,7-dimethoxycoumarin).

For the Herba Artemisiae Scopariae decoction plus Fructus Gardeniae and Radix et Rhizoma Rhei decoction, dried Fructus Gardeniae (3 g) and Radix et Rhizoma Rhei (2 g) were extracted twice, each with 50 mL of boiling water for 1 h. The extracts were mixed with 95% alcohol of 3 times the volume. After being stored in a refrigerator (4°C) for 18 h, the supernatant was filtered and concentrated to be as dry as possible. Dried Herba Artemisiae Scopariae (8 g) was extracted and treated following the previous process. The two parts of the powder were mixed uniformly and then dissolved in right volume of 1% solution of Polysorbate 80 (4.0 mg/mL, calculated by the quantity of 6,7-dimethoxycoumarin).

For the Yinchenhao Tang, dried Herba Artemisiae Scopariae (8 g), Fructus Gardeniae (3 g), and Radix et Rhizoma Rhei (2 g) were prepared together with Yinchenhao Tang corresponding to the previous method. The residue was dissolved in right volume of 1% solution of Polysorbate 80 (4.0 mg/mL, calculated by the quantity of 6,7-dimethoxycoumarin).

### Animals, drug administration, and blood sampling

Male Wistar rats (200–220 g) were obtained from the Laboratory Animal Center of Shenyang Pharmaceutical University (Shenyang, China). These animals were pathogen-free and kept in this laboratory for at least 3 days before experiments. Standard Laboratory food and water was available continuously, except when food was withdrawn 12 h prior to experiments.

Twenty rats were divided into 4 groups at random. 6,7-Dimethoxycoumarin solution and three decoctions (Herba Artemisiae Scopariae decoction, Herba Artemisiae Scopariae decoction plus Fructus Gardeniae and Radix et Rhizoma Rhei decoction, and Yinchenhao Tang) were given to each group by oral administration, respectively.

Blood samples (0.5 mL) were collected into heparinized tubes from each rat from the suborbital vein at 0, 0.08, 0.17, 0.25, 0.5, 1.0, 1.5, 2.0, 3.0, 5.0, 7.0, and 9.0 h after intragastric administration. Blood was immediately processed for plasma by centrifugation at 3,000 rpm for 10 min. Plasma samples were frozen and maintained at –20°C until analysis.

### Preparation of plasma sample

An aliquot of plasma (200 μL), mixed with 100 μL IS solution (10 μg/mL), 100 μL methanol, and 400 μL acetonitrile, was vortex-mixed for 30 s and centrifuged at 3,000 rpm for 10 min. The supernatant was evaporated to dryness under nitrogen stream at 40°C. The residue was reconstituted with 100 μL mobile phase, and a 20 μL portion of the sample solution was injected into the HPLC system.

## Results and Discussion

### Specificity

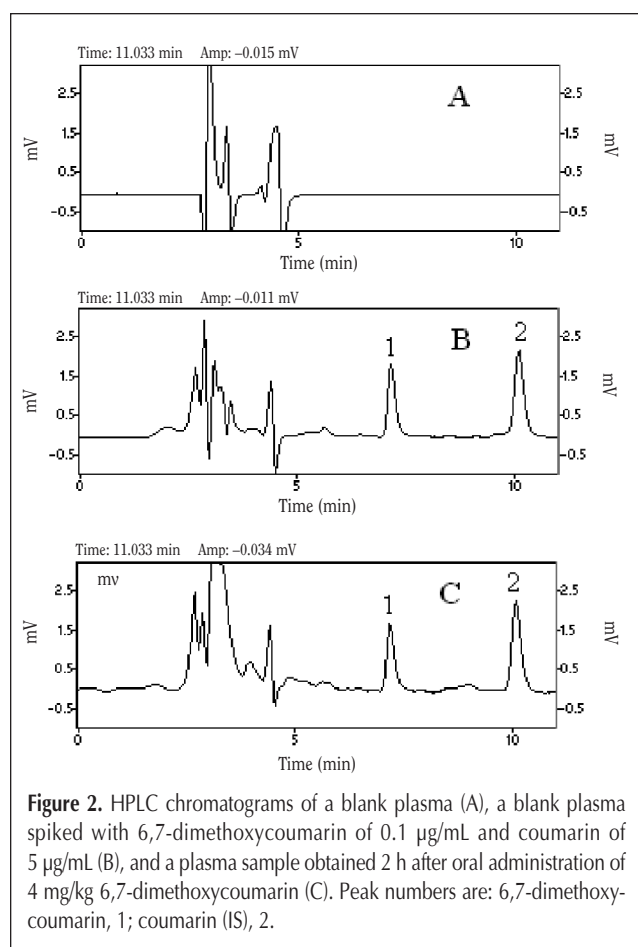
Typical chromatograms of the blank plasma sample and blank plasma spiked with the analyte and IS are given in Figures 2A and 2B, in which the retention time was 7.6 min for 6,7-dimethoxycoumarin and 10.5 min for coumarin. There is no coeluting disturbing peak from endogenous substance in the vicinity of the two peaks on the chromatogram of the blank plasma. A chromatogram of plasma sample of rat obtained 2 h after intragastric administration of 6,7-dimethoxycoumarin is given in Figure 2C.

### Calibration curve and lower limit of quantitation

Samples of plasma were spiked with standard solution and IS solution, then extracted and analyzed to serve as calibration standards. The calibration curves for the determination of 6,7-dimethoxycoumarin in rat plasma are linear over the ranges of 25 × 2500 ng/mL. All of the results were calculated using a  $1/x^2$  weighted regression. The linear equation was  $Y = 3.70 \times 10^{-4}x + 2.989 \times 10^{-3}$  with a correlation coefficient ( $r^2$ ) of 0.9973 ( $n = 6$ ), where  $x$  is the analyte concentration and  $y$  is the peak area ratio of analyte to IS and the LLOQ was 25 ng/mL.

### Precision and accuracy

The precision and accuracy of the assay were estimated by



analyzing QC samples of low (50 ng/mL), medium (250 ng/mL), and high (1000 ng/mL) concentrations. The concentrations of QC samples were calculated with the calibration curve obtained on the same day. The results are shown in Table I. The intra- and inter-day precisions were satisfactory with relative standard deviation (RSD) less than 12%. The accuracy determined with a relative error (RE) ranged from -2.9% to 1.7% ( $n = 18$ ).

### Extraction recovery

The extraction recovery was calculated from the plasma samples spiked with the component at three different concentrations (50, 250, and 1000 ng/mL for 6,7-dimethoxycoumarin). The extraction recovery of the constituent was determined by comparing the peak area of the analyte in plasma samples that had been spiked with the analyte prior to extraction with those of the samples to which the analyte had been added post-extraction. The data obtained from different concentrations of 6,7-dimethoxycoumarin is summarized in Table II.

### Stability

Sample stability was tested as follows: frozen stability, freeze-and-thaw stability, extracted solution stability under ambient temperature, and solution stability at 4°C.

The QC samples prepared in rat plasma, after undergoing three freeze-thaw cycles, showed no significant degradation. Also, these were stable in plasma at -20°C for up to 1 month. In extracts, the components were stable at ambient temperature for up to 24 h without any significant degradation. Stock solutions of these components in methanol were stable at 4°C for up to 1 month. Experiments to assess stability for greater periods are in progress. Some of the results are shown in Table III.

**Table I. Accuracy and Precision of the HPLC Method for Determination of 6,7-Dimethoxycoumarin in Rat Plasma**

Concentration (ng/mL)		Precision RSD (%)		Accuracy RE %
Added	Found	Intra-day	Inter-day	
50	50.8	2.9	6.1	1.7
250	242.6	1.9	11.6	-2.9
1000	1003.4	2.2	7.2	0.3

**Table II. Recovery of 6,7-Dimethoxycoumarin from Spiked Rat Plasma ( $n = 3$ , mean ± sd)**

Concentration (ng/mL)		Mean recovery (%)	RSD (%)
Added	Measured		
50	49.3 ± 0.9	98.6	1.8
250	248.7 ± 3.8	99.5	1.5
1000	992.2 ± 4.4	99.2	0.4

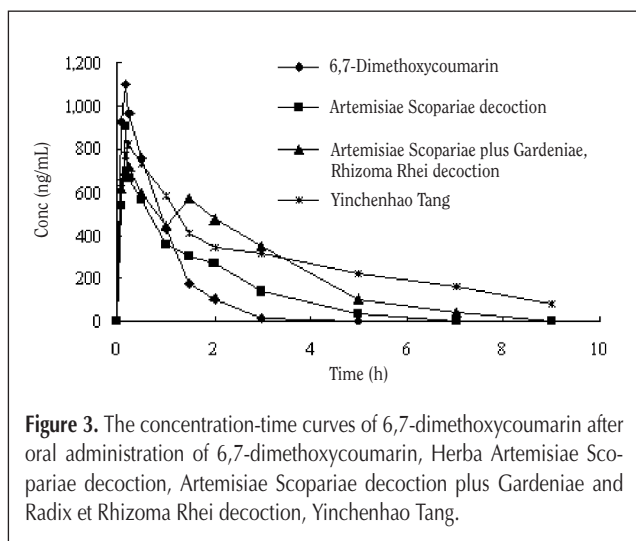
**Table III. Summary of the Stability of 6,7-Dimethoxycoumarin in Rat Plasma**

Concentration (ng/mL)	Storage in freezer (-20°C)			Freeze-thaw cycles		
	Before (ng/mL)	After (ng/mL)	RE (%)	Before (ng/mL)	After (ng/mL)	RE (%)
50	48.9	45.1	-7.8	48.3	51.1	5.8
250	252.6	262	3.7	262.4	257.5	-1.9
1000	1000.8	995.2	-0.6	1002.5	1023.8	2.1

**Table IV. Pharmacokinetic Parameters of 6,7-Dimethoxycoumarin in Rat Plasma After Intra-gastric Administration of Different Decoctions**

No.*	C <sub>max</sub> (ng/mL)	t <sub>max</sub> (h)	t <sub>1/2</sub> (h)	K <sub>e</sub> (1/h)	AUC <sub>0→t</sub> (ng·h/mL)	AUC <sub>0→∞</sub> (ng·h/mL)
1	1104.9 ± 195.9	0.17 ± 0.00	0.29 ± 0.02	2.38 ± 0.19	919.1 ± 195.8	928.5 ± 197.4
2	905.6 ± 180.3	0.24 ± 0.04	1.30 ± 0.37	0.60 ± 0.19	1215.0 ± 194.9	1325.9 ± 283.5
3	747.8 ± 272.1	0.22 ± 0.04	1.07 ± 0.37	0.71 ± 0.24	2035.3 ± 375.1	2094.3 ± 378.6
4	833.0 ± 203.4	0.22 ± 0.04	1.75 ± 0.33	0.41 ± 0.09	2537.9 ± 316.2	2612.6 ± 319.7

\* 6,7-dimethoxycoumarin (1), Artemisiae Scopariae decoction (2), Artemisiae Scopariae decoction plus Gardeniae and Rhizoma Rhei decoction (3), and Yinchenhao Tang (4).



**Figure 3.** The concentration-time curves of 6,7-dimethoxycoumarin after oral administration of 6,7-dimethoxycoumarin, Herba Artemisiae Scopariae decoction, Artemisiae Scopariae decoction plus Gardeniae and Radix et Rhizoma Rhei decoction, Yinchenhao Tang.

### Application of the analytical method in pharmacokinetic studies

The method described here was successfully employed to quantitate 6,7-dimethoxycoumarin in rat plasma and was applied to the pharmacokinetic comparative study of 6,7-dimethoxycoumarin after intra-gastric administration of different decoctions. The data were processed with Topfit 2.0 Pharmacokinetic Software, and the pharmacokinetic parameters of them after intra-gastric administration to rats are listed in Table IV. The plots of mean plasma concentration versus time are shown in Figure 3.

After intra-gastric administration of 6,7-dimethoxycoumarin solution, it was absorbed fast and the plasma concentration-time profile fitted with two compartment models. After intra-gastric administration of three different decoctions, the eliminative speeds are slower than after intra-gastric admini-

stration of 6,7-dimethoxycoumarin solution. The AUC (area under curve) values increased significantly and T<sub>max</sub> had no change after intra-gastric administration of Herba Artemisiae Scopariae decoction plus Fructus Gardeniae and Radix et Rhizoma Rhei decoction and Yinchenhao Tang compared with intra-gastric administration of Artemisiae Scopariae decoction. The abnormal points of Artemisiae Scopariae plus Fructus Gardeniae, and Rhizoma Rhei decoction at 1.5, 2, and 3 h may be the indications of second absorption in intestinal or enterohepatic circulation. Maybe the components fundamentally changed because of interactions of components during the boiling progress, so that there are evident differences of co-existing components effecting the pharmacokinetics of 6,7-dimethoxycoumarin between Yinchenhao Tang and Herba Artemisiae Scopariae decoction plus Fructus Gardeniae and Radix et Rhizoma Rhei decoction. These showed that Radix et Rhizoma Rhei and Fructus Gardeniae could reduce the eliminative speed and enhance bioavailability of 6,7-dimethoxycoumarin without increasing absorptive speed. Further studies about the effects of co-existing components are performing.

### Conclusion

A rapid, simple, specific, and sensitive HPLC-UV method for the quantitation of 6,7-dimethoxycoumarin in rat plasma has been developed and validated. The method described has been successfully used to quantitate 6,7-dimethoxycoumarin in rat plasma for the pharmacokinetic comparative study of 6,7-dimethoxycoumarin after intra-gastric administration of different decoctions.

These feasible pharmacokinetic parameters are very useful

for the further studies of Yinchenhao Tang in vivo. Because of the complexity of chemical constituents in TCM formulas, pharmacokinetic study of active constituents in TCM is essential to illustrate their action mechanism and to investigate the impact of combination. Pharmacokinetic study of 6,7-dimethoxycoumarin will play an important role in the further development of TCM formulas.

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