TLC Identification and Extraction Process of Rubiasin-1-methyl Ether from Yao Medicine Chuanlianzhu

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Abstract [**Objectives**] To establish a thin-layer chromatography (TLC) method for the determination of rubiadin-1-methyl ether in Yao Medicine Chuanlianzhu (*Damnacanthus giganteus*). [**Methods**] A silica gel G thin-layer plate was adopted for TLC. Petroleum ether (60 - 90 ℃)-chloroform-methanol-water (7 ∶ 15 ∶ 3 ∶ 1) was used as the developing solvent and inspected under ultraviolet lamp (365 nm). The content was determined by Inertsil ODS-3 C_{18} column (4.60 mm × 250 mm, 5 μm), mobile phase: acetonitrile-0.2% phosphoric acid gradient elution, detection wavelength 277 nm, flow rate 1.0 mL/min, column temperature 30 ℃, injection volume 10 μL. [**Results**] The spots of 10 Chuanlianzhu samples from different origins showed the same color at the same position as the control, and the spots were clear and specific. The injection volume of rubiadin-1-methyl ether showed a good linear relationship in the range of 2.90 - 145 μg (R = 0.999 6). The average recovery rate of rubiadin-1-methyl ether in the low, medium and high dose groups of Yao Medicine Chuanlianzhu was 98.72%, and RSD = 1.78%. [**Conclusions**] This method can effectively identify Yao Medicine Chuanlianzhu medicinal materials and accurately determine the content of rubiadin-1-methyl ether in the medicinal materials. It provides a scientific basis for the development and utilization of Yao Medicine Chuanlianzhu medicinal resources.

Key words Chuanlianzhu, Thin-layer chromatography (TLC), Extraction process, Rubiadin-1-methyl ether, Content determination

1 Introduction

Yao Medicine Chuanlianzhu (*Damnacanthus giganteus*) is also known as Jijinshen, Huangjipang, and Changyeshuzhugen. It mainly comes from the root of *Damnacanthus macrophyllus* Sieb. Cx Miq. var. giganteus (Makino) Koidz (DM). [D. indicus Gaertn. f. var. giganteus Makino]. According to the *Compilation of National Chinese Herbal Medicine*, Chuanlianzhu has the effects of replenishing qi and nourishing blood, astringent and stopping bleeding, and is mainly used for blood collapse and weak blood deficiency in women[1-2], and it is mainly distributed in Guangxi, Guangdong and Hunan provinces. Morinda officinalis is commonly used as a substitute for Morindae Officinalis Radix (Bajitian) to treat patients with weak blood deficiency, and relevant literature reports that the types and contents of amino acids and trace elements contained in the two are very similar[3-4]. The main effective components of Yao Medicine Chuanlianzhu are flavonoids, anthraquinones and iridoid glycosides[1,4]. In order to use Chuanlianzhu safely and effectively, it is very important to study the quality standard of Chuanlianzhu. In this study, we established a method of TLC identification and content determination of Yao Medicine Chuanlianzhu. It is expected to provide a reference for the quality evaluation of Chuanlianzhu and lay a foundation for the study of new methods for the quality control of Yao Medicine Chuanlianzhu.

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2 Instruments and reagents

2.1 Instruments 1260 Agilent High Performance Liquid Chromatograph; Simplicity Ultrapure Water System (Millipore China); Practum224-1CN Analytical Balance [Sartorius Scientific Instruments (Beijing) Co., Ltd.].

2.2 Reagents Rubiadin-1-methyl ether reference standard (China Institute for Food and Drug Control, batch No.: 112076-202101), methanol and acetonitrile were chromatographically pure (Fisher, 4 L), ethanol, phosphoric acid, acetic acid and other reagents were analytically pure, and the experimental water adopted ultrapure water.

Ten batches of Chuanlianzhu medicinal materials were collected from different regions of Guangxi, and were identified as root of by Zhong Wen, chief pharmacist of Guangxi International Zhuang Medicine Hospital affiliated to Guangxi University of Chinese Medicine, and the drying method was baking.

3 Methods and results

3.1 TLC identification

3.1.1 Preparation of sample solution. Took 2 g of coarse powder, added 50 mL of methanol, performed ultrasonic treatment (power 200 W, frequency 40 kHz) for 30 min, filtered, and evaporated the filtrate to dryness. The residue was dissolved by adding 20 mL of water, extracted twice with chloroform, 20 mL each time, combined with chloroform extract, evaporated, and dissolved by adding 2 mL of methanol to the residue as the test so-

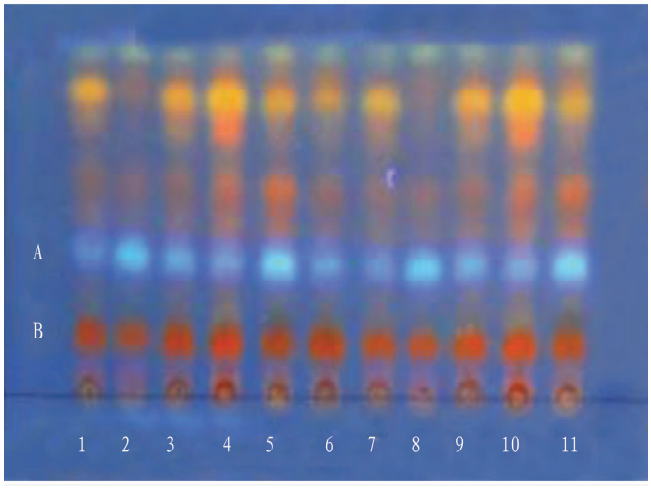
lution. In addition, 2 g of the control medicinal material of Chuanlianzhu was prepared into the control medicinal material solution in the same way.

Table 1 Sample information of Chuanlianzhu

Sample code	Source	Collection time
CLZ-1	Wuzhou City	November
CLZ-2	Cenxi City	November
CLZ-3	Quanzhou County	October
CLZ-4	Longsheng County	December
CLZ-5	Gongcheng County	November
CLZ-6	Pingle County	December
CLZ-7	Zhongshan County	December
CLZ-8	Fuchuan County	November
CLZ-9	Zhaoping County	September
CLZ-10	Yongfu County	December

NOTE The medicinal materials were collected from different areas of Guangxi in 2020.

3.1.2 TLC conditions and results[5]. The test was carried out in accordance with General Technical Requirements 0502 in Volume IV of *Chinese Pharmacopoeia*. Pipetted 3 μL of each of the above two solutions, separately spotted on the same silica gel GF_{254} thin layer plate, used petroleum ether (60 – 90 ℃)-chloroform-methanol-water (7 ∶ 15 ∶ 3 ∶ 1) as the developing solvent, developed, took out, dried, and placed under ultraviolet light (365 nm) for inspection. The test sample showed fluorescent spots of the same color at the corresponding position of the control medicinal material (Fig. 1).



NOTE 1. Chuanlianzhu control medicinal material; 2 – 11: 10 batches of Chuanlianzhu samples.

Fig. 1 Thin layer chromatography of 10 batches of Chuanlianzhu

3.2 Content determination

3.2.1 Chuanlianzhu sample preparation. We collected the whole plant of Chuanlianzhu, naturally dried, and pulverized to pass through No. 2 sieve for sample content determination.

3.2.2 Chromatographic conditions. Column: Inertsil ODS-3 C_{18} column (4.60 mm × 250 mm, 5 μm), mobile phase: acetonitrile-0.2% phosphoric acid, gradient elution, elution procedure is shown in Table 2, detection wavelength 277 nm[6], flow rate 1.0 mL/min, column temperature 30 ℃, injection volume 10 μL, and good resolution of each component under the above conditions.

Table 2 Gradient elution program

Time//min	Mobile phase	
	Acetonitrile//%	0.2% phosphoric acid aqueous solution//%
0	15	85
8	24	76
15	42	58
25	48	52
32	55	45
46	72	28
60	90	10

3.2.3 Preparation of reference solution. Accurately weighed 14.50 mg of rubiadin-1-methyl ether, put it into a 10 mL volumetric flask, added methanol to dissolve it and diluted it to the scale to obtain the reference stock solution with a concentration of 1.45 mg/mL; accurately weighed 0.5 mL of the above rubiadin-1-methyl ether reference stock solution, put it into a 10 mL volumetric flask, and diluted it with methanol solution to the scale to obtain the rubiadin-1-methyl ether reference solution with a concentration of 72.5 μg/mL.

3.2.4 Preparation of test solution. Accurately weighed 1.0 g of coarse powder of Chuanlianzhu, extract with 80% methanol under reflux for 1 h, centrifuged for 10 min at a radius of 0.65 cm and a 13 000 of r/min, and filtered the supernatant with a 0.45 μm microporous membrane to obtain the test solution.

3.2.5 Plotting of standard curve. Accurately pipetted 2, 5, 10, 15, 20 μL of rubiadin-1 methyl ether reference solution (concentration of 49.5 μg/mL) and injected it into the liquid chromatograph, with the peak volume and the injection volume (μg) as the ordinate and abscissa, respectively. The standard curve was drawn and the regression equation was calculated. Rubiadin-1-methyl ether $Y = 8.780 \times 10^6 X + 7.365 \times 10^4$ ($R^2 = 0.9996$). The results showed that the injection volume has a good linear relationship in the range of 2.90 – 145 μg.

3.2.6 Precision test. Accurately weighed a proper amount of reference solution (72.50 μg/mL), and continuously injected the sample for 6 times according to the conditions in Section **3.2.2**. The *RSD* of rubiadin-1-methyl ether peak area was 1.32%, which was lower than 3.0%, indicating that the instrument has good precision.

3.2.7 Stability test. Take 1.0 g of coarse powder of the same batch of Chuanlianzhu, accurately weighed it, prepared the test solution according to the method in Section **3.2.4**, and determined it according to the conditions in Section **3.2.2** at 0, 2, 4, 8, 12 and 24 h after the preparation of the test solution. The *RSD* of the peak area of Chuanlianzhu was 2.36%, less than 3.0%, indica-

ting good stability of the test solution within 24 h.

3.2.8 Repeatability test. Accurately weighed 1.0 g of coarse powder of the same batch of Chuanlianzhu medicinal material, weighed accurately, 6 portions in total, prepare the test solution according to the method in Section **3.2.4**, determined according to the conditions in Section **3.2.2**, calculated that the average content of Chuanlianzhu medicinal material is 0.318 mg/g, and the *RSD* was 2.47%, less than 3.0%, indicating the method has good repeatability.

3.2.9 Sample recovery test. Accurately weighed 0.5 g of coarse powder of Chuanlianzhu with known content (0.318 mg/g) from the same batch, weighed precisely, 9 portions in total, and divided into 3 groups, namely, low, medium and high sample groups (80%, 100% and 120% of the content of 0.5 g of Chuanlianzhu). The added samples were 0.127 2, 0.159 and 0.190 8 mg, respectively. The average recovery of rubiadin-1-methyl ether was 98.72% and *RSD* was 1.78% ($n=9$), which indicated that the method was accurate. The results are shown in Table 3.

Table 3 Recovery rate of rubiadin-1-methyl ether in Chuanlianzhu ($n=9$)

Component	Sample weight//g	Content in weighed sample//mg	Added weight//mg	Measured weight//mg	Recovery rate//%	Average recovery rate//%	*RSD*//%
Rubiadin-1-methyl ether	0.500 3	0.159 1	0.127 2	0.283 2	97.57	98.72	1.78
	0.500 6	0.159 2	0.127 2	0.281 9	96.47		
	0.500 2	0.159 1	0.127 2	0.284 8	98.85		
	0.501 0	0.159 3	0.159 0	0.321 6	102.06		
	0.501 2	0.159 4	0.159 0	0.316 7	98.94		
	0.501 6	0.159 5	0.159 0	0.317 0	99.05		
	0.500 8	0.159 3	0.190 8	0.345 9	97.82		
	0.500 7	0.159 2	0.190 8	0.352 1	101.09		
	0.500 3	0.159 1	0.190 8	0.345 8	97.85		

3.3 Extraction process of rubiadin-1-methyl ether from Chuanlianzhu medicinal materials

3.3.1 Single factor investigation. (i) Determination of the maximum absorption wavelength. Dissolved and diluted the rubiadin-1-methyl ether reference solution with methanol, took the methanol as a reference solution, and scanned with an ultraviolet-visible spectrophotometer in a wavelength range of 200 – 400 nm; the optimal absorption wavelength was determined to be 277 nm in accordance with the absorption curve of rubiadin-1-methyl ether.

(ii) Investigation of extraction methods. Took rubiadin-1-methyl ether as the evaluation index, took extraction time of 1 h and 25 mL of 80% methanol as the extraction conditions, the medicinal materials of Chuanlianzhu were extracted by rubiadin-1-methyl ether with reflux extraction, ultrasonic extraction and solvent impregnation, respectively. The results showed that the extraction rate of rubiadin-1-methyl ether by reflux extraction method was high, so the reflux extraction method was selected in this experiment.

(iii) Investigation of solvent concentration. The extraction time of rubiadin-1-methyl ether was fixed at 1 h and the volume of solvent was 25 mL, and the extraction rate of rubiadin-1-methyl ether in Chuanlianzhu was determined by single factor experiment with the concentration of ethanol and methanol. The designed concentrations of methanol and ethanol were 0%, 20%, 40%, 60%, 80% and 95%, respectively. The results showed that the extraction rate of 80% methanol reflux was higher.

(iv) Investigation of extraction volume. The extraction time of rubiadin-1-methyl ether was 1 h and 80% methanol, the extraction volume of solvent was 15, 25, and 35 mL, the extraction rate of rubiadin-1-methyl ether in Chuanlianzhu was determined by single factor test. The results showed that the extraction rate was the highest when the volume was 25 mL.

(v) Investigation of extraction time. With 25 mL of 25% methanol, the extraction rate of rubiadin-1-methyl ether in Chuanlianzhu was determined by single factor experiment with the extraction time of 0.5, 1.0, 1.5, 2.0 and 2.5 h. The results showed that the yield of rubiadin-1-methyl ether was higher when the extraction time was 1.0 h.

3.3.2 Orthogonal experimental design[7-8]. According to the relevant literature and the *Chinese Pharmacopoeia* (2020 edition), the extraction method with different methanol concentrations was finally adopted in this experiment, and the three conditions of solvent concentration (60% methanol, 80% methanol, 95% methanol), solvent volume (15, 25 and 35 mL) and extraction time (1, 1.5 and 2 h) were investigated. $L_9(3^4)$ orthogonal experimental design was selected according to the orthogonal design assistant software, as shown in Table 4.

Table 4 Factors and levels of orthogonal experiment

Level	Factor		
	A (Methanol concentration)//%	B (Solvent volume)//mL	C (Extraction time)//h
1	60	15	1.0
2	80	25	1.5
3	95	35	2.0

Took 1.0 g of Chuanlianzhu medicinal material powder, accurately weighed, extracted according to the above extraction method of rubiadin-1-methyl ether in Chuanlianzhu, then operated accord-

ing to the preparation method of the test solution, and carried out the experiment according to the $L_9(3^4)$ orthogonal design scheme. The content of rubiadin-1-methyl ether in Chuanlianzhu was determined by HPLC, as shown in Table 5.

3.3.3 Orthogonal experiment and result analysis. (i) Orthogonal experiment. The results of experimental variance analysis (Table 5-6) showed that the volume of extraction solvent had a significant effect on the extraction rate of rubiadin-1-methyl ether in Chuanlianzhu, followed by methanol concentration and extraction time. The results of variance analysis showed that the volume of extraction solvent had significant difference, while the methanol concentration and extraction time had no significant difference, and the strength of each factor was B > A > C. Therefore, the volume of extraction solvent was the factor with statistical significance for the extraction effect of rubiadin-1-methyl ether in Chuanlianzhu. Therefore, the most ideal extraction scheme in this experiment is $A_2B_2C_1$, that is, when the methanol concentration is 80%, the extraction solvent volume is 25 mL, and the reflux extraction time is 1 h, the extraction effect is optimal.

Table 5 Orthogonal experimental design and results

No.	Factor				Rubiadin-1-methyl ether//mg/g
	A	B	C	D (error)	
1	1	1	1	1	0.254 2
2	1	2	2	2	0.278 3
3	1	3	3	3	0.273 1
4	2	1	2	3	0.261 3
5	2	2	3	1	0.301 4
6	2	3	1	2	0.297 5
7	3	1	3	2	0.254 2
8	3	2	1	3	0.321 2
9	3	3	2	1	0.277 6
K_1	0.268 5	0.256 6	0.291 0	0.277 7	
K_2	0.286 7	0.300 3	0.272 4	0.276 7	
K_3	0.284 3	0.282 7	0.276 2	0.285 2	
R	0.018 6	0.043 7	0.018 2	0.008 5	

Table 6 Variance analysis results

Source of error	*SS*	*f*	*S*	*F*	*P*
A	0.000 586 640	2	0.000 29	4.522 82	>0.05
B	0.002 905 887	2	0.001 45	22.403 53	<0.05
C	0.000 576 487	2	0.000 29	4.444 54	>0.05
D (error)	0.000 129 707	2	0.000 06		

(ii) Verification experiment. Three batches of verification experiments were carried out according to the optimal process determined by orthogonal experiment. The results showed that the content of rubiadin-1-methyl ether in Chuanlianzhu was 0.320 5 mg/g, and the *RSD* was 1.93%. The results showed that the process had good reproducibility and could be used as the extraction process of rubiadin-1-methyl ether in Chuanlianzhu.

4 Discussion

We separately investigated developing agents such as Petroleum ether (60-90 ℃) -chloroform-methanol-water, petroleum ether (60-90 ℃) -dichloromethane-methanol-water, petroleum ether (60-90 ℃)-chloroform-methanol-formic acid, n-butanol-glacial acetic acid-water, toluene-ethyl acetate-formic acid-glacial acetic acid and cyclohexane-ethyl acetate-acetone, *etc.* The results showed that when petroleum ether (60-90 ℃)-chloroform-methanol-water (7∶15∶3∶1) was used as the developing agent, the spots were clear and the resolution was good. Besides, we also investigated different methanol-sour water systems and acetonitrile-sour water systems (0.05% phosphoric acid, 0.1% phosphoric acid, 0.2% phosphoric acid, 0.1% glacial acetic acid, and pure water). The results show that when acetonitrile-0.2% phosphoric acid solution is used as the mobile phase, the resolution was relatively good, the baseline was relatively stable, and the peak time was appropriate, thus the mobile phase was determined to be acetonitrile-0.2% phosphoric acid aqueous solution.

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