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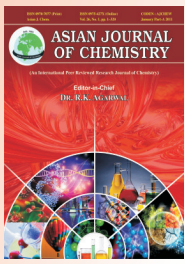

Simultaneous Determination of Three Active Ingredients in Wubishan Capsule by HPLC

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To establish an HPLC method for the simultaneous determination of 3 bioactive ingredients in Wubishan capsule. HPLC conditions included Kromasil-C_{18} column (250 mm × 4.6 mm, 5 μm) and the mobile phase was a mixture of MeOH-CH_3CN-H_2O (13:6:81). The flow rate was 1.0 mL/min, the column temperature was at 30 °C and the UV detection wavelength was 254 nm. The 3 active ingredients had good linear relationships: guanosine in the range of 0.0915-4.575 μg (R^2 = 0.999 3), loganin in the range of 0.116-5.801 μg (R^2 = 0.999 0) and schizandrin in the range of 0.0398-1.990 μg (R^2 = 0.999 3). Their average recoveries were 99.1 % (RSD = 1 %), 98.7 % (RSD = 2.4 %) and 97.1 % (RSD = 1.0 %), respectively. This method was steady with high precision and good repeatability and could be used for the determination of the 3 active ingredients and quality control of Wubishan capsule.

Keywords: Wubishan capsule, Guanosine, Loganin, Schizandrin, HPLC.

INTRODUCTION

Wubishan capsule, also known as 'Wubishuyu', is composed of 11 traditional Chinese medicines, including *Fructus Macrocarpii*, *Rhizoma Alismatis*, *Poria*, *Radix Achyranthis Bidentatae*, *Cortex Eucommiae*, *etc*. This complex preparation is very famous for its abundant bioactivities, such as strengthening spleen and invigorating kidney, regulating immune system, which would be extremely beneficial towards the paroxysmal nocturnal hemoglobinuria (PNH), therefore it's frequently used to treat consumption diseases[1-2], spermatorrhea[3], lumbodynia[4], consumptive micturition disorders[5-6], *etc*. Modern pharmacological researches have also confirmed such bioactivities of Wubishan Capsule as immunological enhancement, antitumor, cardiovascular enhancement, hypoxia endurance improvement, *etc*[7-10].

As the main medicinal ingredients of Wubishan capsule, guanosine, loganin and schizandrin exhibit various interesting activities, such as tumor-resistant, virus-resistant, kidney protection, reducing blood glucose and anti-shock. Currently, there is no comprehensive detection method to measure the above 3 active ingredients simultaneously, therefore, there is no effective way to control the quality of Wubishan capsule with easiness and convenience. In this study, a steady HPLC method, with high precision and good repeatability, was set up for the simultaneous determination of the above 3 active components inside Wubishan capsule, aiming to provide a rapid, simple and precise method for the quality control of Wubishan capsule.

EXPERIMENTAL

Agilent 1100 series HPLC system (Angilent Technologies, USA) was equipped with quaternary pump (G1311A), automatic sampler (G1313A), UV variable-wavelength detector (1314A-UV) and column oven (CTO-10ASVP); Pa2251 electronic analytical balance from Sartorius Group, Germany; Tw20 constant temperature bath box from Julabo Labortechnik GmbH Company, Germany.

Standards of guanosine, loganin and schizandrin were supplied by National Institute for Food and Drug Control (NIFDC, Beijing, China). Three batches of Wubishan Capsule were purchased from Hangzhou Laobaixing Pharmacy (Batch No: 20110203, 20110412 and 20111023). Methanol and acetonitrile were of HPLC grade and other reagents used were of analytical grade. Deionized water was prepared using a Millipore water purification system.

HPLC conditions: The separation of the 3 ingredients was carried out on a Kromasil-C_{18} (250 mm × 4.6 mm, 5 μm). The solvents used for HPLC separation were MeOH-CH_3CN-

H_2O (13:6:81) and the analysis was monitored at 254 nm with the column temperature of 30 °C and the injection volume was 20 µL.

Preparation of standard solution: Certain amounts of the 3 standard compounds were dissolved with methanol into volumetric flasks, respectively, then measured 1 mL guanosine, 1.5 mL loganin and 1.5 mL schizandrin standard solution, respectively, into a 20 mL volumetric flask and diluted to the desired concentrations.

Preparation of sample solution: Fifty capsules of Wubishan capsule were mixed finely, about 2 g sample was accurately weighted, then added into a 50 mL conical flask. About 30 mL MeOH was added into the flask and performed ultrasonic extraction in a constant temperature bath for 0.5 h. The extract solution was then cooled to room temperature and diluted to the volume. After centrifuged at 10 000 rpm for 10 min, the supernatant was passed through a 0.22 µm membrane filter and the filtrate was ready for the chromatographic analysis (Fig. 1).

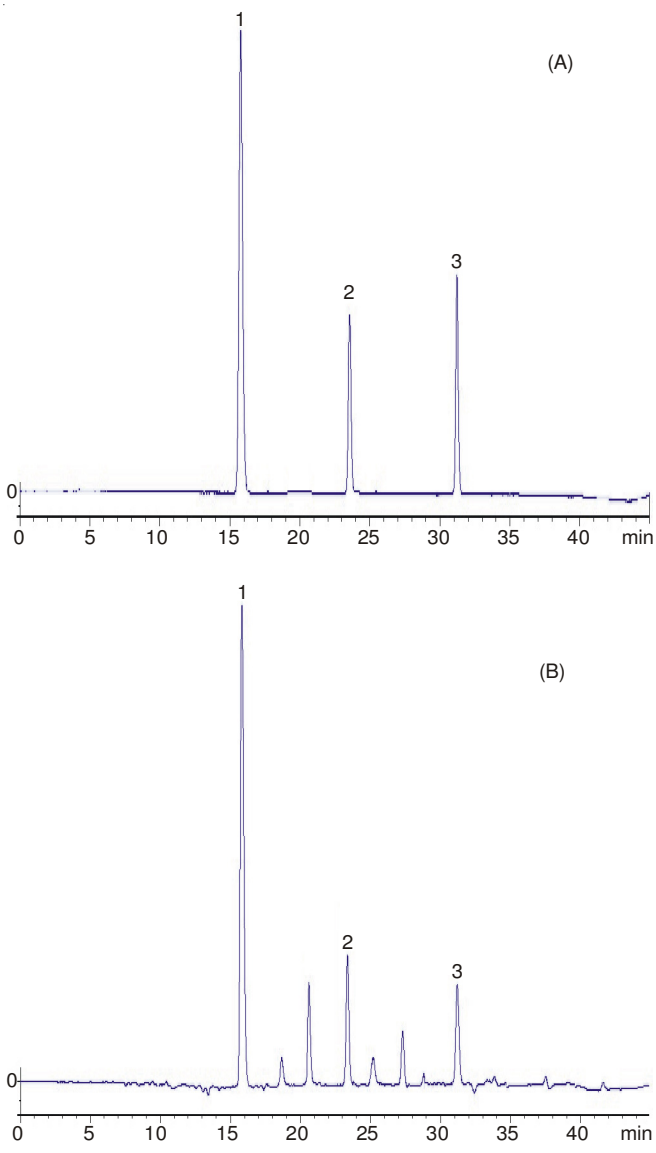

Fig.1. Typical HPLC chromatograms of the 3 ingredients mixture (A) and Wubishan Capsule (B) 1: guanosine; 2: loganin; 3: schizandrin

RESULTS AND DISCUSSION

Validation of the chromatographic method

Linear Range: Linear regression analysis for each component was performed by the external standard method. The above 3-compound solution was accurately injected 1, 5, 10, 20, 30 and 50 µL, respectively. The linearity of each compound was calculated by plotting the peak area (Y) *vs.* concentration (X) (Table-1). All the 3 components showed good linearities in wide concentration ranges.

TABLE-1
LINEAR REGRESSION EQUATION AND LINEAR RANGES

Components	Regression equation	R^2	Linear range (µg)
Guanosine	Y=23.05X+3.21	0.999 3	0.0915~4.575
Loganin	Y=3.12X+1.23	0.999 0	0.116~5.801
Schizandrin	Y=10.21X+0.99	0.999 3	0.0398~1.990

Precision: The standard mixture solution of the 3 components was injected into HPLC 6 times continuously and the area of each peak was used for the calculation of precision. The results showed that relative stand deviation (RSD) of peak area of each standard was 1.3, 2 and 1.8 %, respectively.

Stability: As for the stability test, the same sample solution was analyzed at designated time points in 24 h. The results showed that RSDs of peak areas were 0.8, 1.9 and 1.4 % and found to be stable for the experiment.

Repeatability: Repeatability was carried out using six samples solution after the same treatment procedure. The results showed that RSD of each peak area was 2, 1.8 and 2.2 %, respectively.

Recovery test: The sample with known targeted contents was spiked with certain amounts of the 3 standards. Then the spiked sample was processed in accordance with the established method for the HPLC detection. The average recoveries for guanosine, loganin and schizandrin determined were 97.1-99.1 % (Table- 2).

TABLE-2
RECOVERY RESULTS OF THE 3 ACTIVE INGREDIENTS

Compt.	Contents in samples (mg)	Added (mg)	Found (mg)	Recovery (%)	Mean (%)	RSD (%)
Guanosine	1.1450	1.1093	2.2454	99.2	99.1	1.0
	1.1253	1.1093	2.2091	97.7		
	1.1048	1.1093	2.2019	98.9		
	1.1506	1.1093	2.2587	99.9		
	1.1055	1.1093	2.2190	100.4		
	1.1520	1.1093	2.2458	98.6		
Loganin	0.3707	0.3447	0.7020	96.1	98.7	2.4
	0.3371	0.3447	0.6917	102.9		
	0.3577	0.3447	0.6963	98.3		
	0.3723	0.3447	0.7137	99.0		
	0.3540	0.3447	0.6957	99.1		
	0.3660	0.3447	0.7003	97.0		
Schizandrin	0.8488	0.7748	1.5938	96.2	97.1	1.0
	0.8019	0.7748	1.5685	98.9		
	0.8190	0.7748	1.5730	97.3		
	0.8533	0.7748	1.6010	96.5		
	0.7723	0.7748	1.5243	97.1		
	0.8344	0.7748	1.5848	96.8		

Application of the HPLC method for the quantitation studies: The experiment determined 3 samples of different batches with the above method. The contents of the 3 components were shown in Table-3.

TABLE-3
CONTENTS DETERMINATION OF THE 3 ACTIVE INGREDIENTS IN 3 BATCHES

Batch	Guanosine (mg/g)	Loganin (mg/g)	Schizandrin (mg/g)
20110203	1.53	0.55	0.80
20110412	1.95	0.33	0.55
20111023	2.23	0.35	0.85

Optimization of HPLC separation conditions: In order to get a better resolution separation of the determined ingredients and a shorter analytical time, 3 different column temperatures were compared: 25, 30 and 40 °C, then it was found that the higher temperature, the shorter retention time, while the resolutions were almost similar, so 30 °C was finally chosen for the isolation to protect the lifespan of the column.

Six chromatographic columns were compared in the pre-experiment: Kromasil-C_{18}, Zorbax SB-C_{18}, Symmetry Shield RP_{18}, Eclipse-C_{18}, Extend-C_{18} and Hypersil-C_{18}, according to the effect of separation, Kromasil-C_{18} (250 mm × 4.6 mm, 5 μm) was used for the further research of the methodology.

Besides, reflux extraction and ultrasonic extraction were investigated in the experiments. Considering the advantages of high efficiency, ultrasonic extraction was chosen as the way to prepare the sample solutions. In the mean time, the efficiency of different elution solvents was also compared, namely MeOH and CH_3CN, the results indicated that the 2 solvents almost had the same efficiency. Considering the toxicity and safety, MeOH was finally selected as the extract solvent.

Conclusion

From the content determination of 3 batches Wubishan capsule, it could be found that the batch prepared in October had higher contents of the 3 target compounds, which might because in the conventional harvest time. The bioactive components also accumulated to the highest contents, which also indicated that better bioactivities might be obtained. Therefore, further research would be carried out towards the bioactivity difference comparison among different products in different generation seasons.

In short, the above 3 active components were quantitatively determined in Wubishan Capsule. On the whole, this developed method was simple, accurate for the determination of guanosine, loganin and schizandrin and reliable for the quality control and further efficacy study of Wubishan capsule in clinic use and pharmacokinetic studies.

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