Determination of Chlorogenic Acid, Geniposide, Total Flavonoids and Total Triterpenes in Wulan-13

Xiaohua WU[1], Burenbatu[2]*

1. College of Clinical Medicine, Inner Mongolia Minzu University, Tongliao 028000, China; 2. Affiliated Hospital of Inner Mongolia Minzu University, Tongliao 028000, China

Abstract [**Objectives**] This study was conducted to establish a method for the determination of chlorogenic acid, geniposide, total flavonoids and total triterpenes in Wulan-13. [**Methods**] The contents of chlorogenic acid and geniposide were determined by HPLC, and the contents of total flavonoids and total triterpenes were determined by an ultraviolet spectrophotometer. [**Results**] There was a good linear relation between the mass of chlorogenic acid reference substance and the peak area in the range of 0.05 - 0.45 μg, and the regression equation was $Y = 2\,524.1X + 3.194\,3$, ($r = 0.999\,8$). A good linear relationship was found between the mass of gardenoside reference substance and the peak area in the range of 0.776 - 6.984 μg, and the regression equation was $Y = 1\,670.5X + 64.804$, ($r = 0.999\,8$). There was also a good linear relation between the mass of rutin reference substance and its absorbance in the range of 0.008 08 - 0.048 48 mg, and the regression equation was $Y = 12.916X + 0.014$, ($r = 0.999$). The mass of oleanolic acid reference substance had a good linear relation with its absorbance in the range of 0.004 18 - 0.020 9 mg, and the regression equation was $Y = 51.89X - 0.083\,9$, ($r = 0.999\,1$). [**Conclusions**] The content determination method is simple, reliable and reproducible, and suitable for controlling the contents of chlorogenic acid, geniposide, total flavonoids and total triterpenes in Wulan-13.

Key words Wulan-13, HPLC, Ultraviolet spectrophotometer, Content determination

1 Introduction

The Mongolian medicine Wulan 13 decoction is contained in "Wulan-13 (Mariqinzhusumu or Baimaihong Decoction)" included in the fifth chapter of *Guanzhezhixi*. In 1984, it was included in *Standard of Inner Mongolia Mongolian Medicine* with the name of "Yihe-Wulan-13 Decoction"[1]. In 1998, it was promoted to the national standard, and was recorded in the *Drug Standard of Ministry of Public Health of the People's Republic of China* (*Mongolian Medicine Volume*), and its name was changed to Wulan Shisanwei Tangsan or Wulan-13. It is a kind of reddish-brown powder consists of thirteen kinds of medicinal materials, including Inula helenium, Sophorae Flavescentis Radix, Ramulus Rubi, Kaempferia galanga, Chebulae Fructus, Gardeniae Fructus, Toosendan Fructus, Rubiae Radix, Eriobotryae Folium, drug shellac, acorn, Arnebiae Radix and Trollii Flos. The powder is slightly fragrant, and bitter and astringent in flavor, and has the effect of removing pathogenic heat from blood[2]. It is mainly used for treating blood heat, headache, red eyes and hypertension. There has been no other literature report on the simultaneous determination of geniposide and chlorogenic acid in Gardeniae Fructus, which is the main drug in the prescription. In order to reasonably and effectively control the internal quality of the preparation, the content of geniposide, and chlorogenic acid, total flavonoids and total triterpenes existing in most Chinese medicinal materials and plants were determined by HPLC and ultraviolet spectrophotometry. Methodological investigation proved that the method was simple and reproducible, and the chemical properties of chlorogenic acid, geniposide, rutin and oleanolic acid were stable, which could be used as quality control indicators of the prescription.

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* Corresponding author. E-mail: dr_burenbatu@126.com

2 Materials

2.1 Instruments Ultra high performance liquid chromatography (Agilent1260); Precisa electronic balance (ha225sm); freeze drying machine (FD-1A-50 +) (Biocool (Beijing) Instrument Co., Ltd.); KH-500DE CNC ultrasonic cleaner (Kunshan Hechuang Ultrasonic Instrument Co., Ltd.); double-beam ultraviolet spectrophotometer (genesys50); low speed centrifuge (LC-4012).

2.2 Reagents and test drugs Chlorogenic acid reference substance (batch No.: D0015642, purity 96.9%), purchased from Beijing Manhage Biotechnology Co., Ltd.; geniposide reference substance (batch No.: ST0333WL0120, purity 97.1%), purchased from Zhongke Huajian (Beijing) Technology Co., Ltd.; rutin reference substance (batch No.: BL40842, purity ≥ 98%), purchased from Henan Reference Material R&D Center; oleanolic acid reference substance (batch No.: C23PC501A, purity ≥ 98%), purchased from Zhongke Huajian (Beijing) Technology Co., Ltd.; Wulan-13 from Affiliated Hospital of Inner Mongolia Minzu University (batch No.: 20201135).

Merck chromatographic methanol, ultrapure water and sodium nitrite solution (batch No.: 20200018), Tianjin Tianli Chemical Reagent Co., Ltd.; aluminum nitrate (batch No.: 20201209), Sinopharm Chemical Reagent Co., Ltd.; sodium hydroxide (batch No.: C22PB951A, purity ≥ 96%, AR), Sinopharm Chemical Reagent Co., Ltd.; vanillin (batch No.: C21PB3122A, purity ≥ 99%), Tianjin Tianli Chemical Reagent

Co., Ltd.; glacial acetic acid (batch No.: C22PC209D, purity ≥ 99.9%); other reagents, all analytically pure.

3 Methods and results

3.1 HPLC (high performance liquid chromatography) determination of chlorogenic acid and geniposide content

3.1.1 Chromatographic conditions. The chromatography column used was Mars120 C_{18} chromatography column (250 mm × 4.6 mm, 5 μm). Mobile phase: Methanol was used as mobile phase A, and 0.1% phosphoric acid water was used as mobile phase B. The ratio and gradient changes are shown in Table 1. HPLC was performed with a flow rate at 1 mL/min and a column temperature at 30 ℃. The detection wavelength were 327 nm (chlorogenic acid) for 0 – 31 min, and 238 nm (geniposide) for 31 – 60 min. The injection volume was 5 μL.

Table 1 Gradient elution program for Wulan-13

Time//min	Mobile phase A//%	Mobile phase B//%
0	5	95
60	35	65

3.1.2 Preparation of test solution. First, 5 g of Wulan-13 raw material powder was accurately weighed, and added in a round bottom flask. Next, 17 times of water was added to form a suspension. After soaking for 30 min, the suspension was boiled with an electric heating sleeve for 45 min, and then taken out and cooled to room temperature. Centrifugation was performed at 2 000 r/min for 10 min to obtain a supernatant, which was poured into an evaporating dish and freeze-dried for 48 h. Next, 0.4 g was precisely weighed, added in a 25 mL brown volumetric flask, and dissolved in methanol to the mark. The obtained solution was filtered through a 0.22 μm microporous filter membrane[3].

3.1.3 Preparation of mixed reference solution. Appropriate amounts of geniposide and chlorogenic acid reference materials were accurately weighed, and added in a brown volumetric flask. Next, methanol was added to the mark, and the reference substances were dissolved through 3 – 5 s of ultrasonic treatment. After shaking well for full dissolution, a mixed reference solution of 0.776 mL/mg geniposide and 0.05 mL/mg chlorogenic acid was obtained.

3.1.4 Investigation of linear relation. First, 1, 3, 5, 7 and 9 μL of the mixed reference solution in Section **3.1.3** were injected according to conditions in Section **3.1.1**, respectively, and the chromatographic peak areas were recorded. A standard curve was drawn with the mass (g) of corresponding reference substance as the abscissa *X* and the peak area of the sample as the ordinate *Y*. The regression equation, correlation coefficient and linear range of each component are shown in Table 2. It showed that there was a good linear relation between each indicator component and chromatographic peak area within its range.

Table 2 Linear regression equations, linear ranges and correlation coefficients of two components to be measured

To-be-measured component	Linear equation	R^2	Linear range//μg
Geniposide	$Y = 1\,670.5.1X + 64.804$	0.999 8	0.776 – 6.984
Chlorogenic acid	$Y = 2\,524.1X + 3.194\,2$	0.999 8	0.050 – 0.450

3.1.5 Precision test. The mixed reference solution in Section **3.1.3** was injected 6 times according to conditions in Section **3.1.1**, and the peak areas of various components and their corresponding *RSD* values were recorded. The results showed that the *RSD* values of geniposide and chlorogenic acid were 1.06% and 1.14%, respectively, indicating good precision of the equipment.

3.1.6 Stability test. From the test sample prepared according to the method described in Section **3.1.2**, 0.4 g was weighed, and stood at room temperature. The test sample was injected for analysis according to the chromatographic conditions described in Section **3.1.1** at 0, 3, 6, 12, 18 and 24 h after preparation. The peak area of each indicator component was recorded, and their *RSD* values were calculated. The *RSD* values of geniposide and chlorogenic acid were 1.85% and 1.68%, respectively, indicating good stability of the samples within 24 h.

3.1.7 Repeatability test. Six parts of samples were prepared in parallel using the preparation method for test substances in Section **3.1.2**, and about 0.4 g was weighed. Next, the samples were analyzed separately according to the chromatographic conditions in Section **3.1.1**. The peak area of each component was recorded, and the *RSD* value of corresponding peak areas was calculated. The *RSD* values of geniposide and chlorogenic acid were 1.49% and 1.73%, respectively, indicating good repeatability of the method.

3.1.8 Recovery test. Approximately 0.2 g of Wulan-13 Decoction dry powder with known contents was weighed, totaling six parts. Corresponding contents of geniposide and chlorogenic acid reference materials were accurately added. Test solutions were prepared using the method described in Section **3.1.2**, and injected according to conditions in Section **3.1.1** for measurement. The recovery values and *RSD*% of the six samples at different concentration points were calculated. The results are shown in Table 3 and Table 4.

Table 3 Results of chlorogenic acid recovery test

Sample No.	Original amount//mg	Added amount//mg	Measured value//mg	Recovery//%	Average//%	*RSD*//%
1	0.497 2	0.487	0.965	96.06	95.99	1.88
2	0.497 2	0.487	0.958	94.52		
3	0.497 2	0.487	0.972	97.39		
4	0.497 2	0.487	0.964	95.85		
5	0.497 2	0.487	0.953	93.59		
6	0.497 2	0.487	0.977	98.52		

Table 4 Results of geniposide recovery test

Sample No.	Original amount // mg	Added amount // mg	Measured value // mg	Recovery // %	Average // %	*RSD* // %
1	7.518 4	7.432	15.112	102.17	103.70	1.19
2	7.518 4	7.432	15.111	102.16		
3	7.518 4	7.432	15.268	104.27		
4	7.518 4	7.432	15.306	104.78		
5	7.518 4	7.432	15.313	104.88		
6	7.518 4	7.432	15.244	103.95		

3.2 Determination of total flavonoid content using an ultraviolet spectrophotometer

3.2.1 Preparation of reference solution. Into a 25.00 mL volumetric flask, 5 mg of rutin reference substance was accurately weighed, and methanol was added to dissolve it. Then, methanol was added to the mark to obtain a reference solution with a concentration of 0.200 0 mg/mL.

3.2.2 Preparation of test solution of Wulan-13. First, 5 g of original medicinal powder of Wulan-13 was accurately weighed and added into a round bottom flask. Next, 17 times of water was added to form a suspension. After soaking for 30 min, the suspension was boiled in an electric heating sleeve for 45 min, and then taken out and cooled to room temperature. Next, centrifugation was performed at 2 000 r/min and 10 min to obtain a supernatant, which was poured into an evaporating dish and freeze-dried for 48 h. Next, 0.4 g of the residue was accurately weighed and added in a 25 mL brown volumetric flask, and 1 mL was pipetted and added in a 25 mL volumetric flask. After dissolving in methanol, the solution was diluted to constant volume.

3.2.3 Determination. First, 1 mL of solution to be detected was precisely pipetted into a 25 mL volumetric flask, and 6 mL of methanol was added, followed by 1 mL of 5% $NANO_2$ (sodium nitrite solution). The solution was shaken well and stood for 6 min. Next, 1 mL of 10% $Al(NO_3)_3$ (aluminum nitrate solution) was added, and the solution was mixed well and stood for 6 min. Next, 10 mL of 4% NAOH (sodium hydroxide solution) was added, and the obtained solution was diluted with ultrapure water to constant volume, and shaken well and stood for 15 min. The absorbance was determined at 510 nm with number 0 as control. Finally, a standard curve was drawn with absorbance Y as the ordinate and mass concentration X (mg/mL) as the abscissa[4].

3.2.4 Investigation of linear relation. Into a 25 mL volumetric flask, 0.00, 1.00, 2.00, 3.00, 4.00, 5.00 and 6.00 mL of reference solution were accurately pipetted, and methanol was added to make up the volume to 6 mL. The obtained solution was determined by the method in Section **3.2.3**. A standard curve was drawn with absorbance Y as the ordinate and mass X (mg) of rutin as the abscissa, and the regression equation was calculated.

The equation of the standard curve was $Y = 12.916X + 0.014$ ($r = 0.999$), indicating that rutin had a good linear relation with absorbance A in the range of 0.008 08 – 0.048 48 mg.

3.2.5 Precision test. Six copies of reference solution were prepared in accordance with the method in Section **3.2.1**, and determined for 6 times according to the conditions in Section **3.2.3**. The absorbance and *RSD* of each component were recorded. The results showed that the *RSD* of total flavonoids was 0.21%, indicating that the precision of the equipment was good.

3.2.6 Stability test. According to the method described in Section **3.2.2**, 0.4 g of test sample was prepared. The prepared test solution was determined for 6 times according to the conditions described in Section **3.2.3** at 0, 10, 20, 30, 40, and 60 min after preparation. The absorbance and *RSD* values of each component were recorded. The results showed that the *RSD* of total flavonoids was 0.44%, which indicated that the sample was stable within 60 min.

3.2.7 Repeatability test. According to the method described in Section **3.2.2**, six parts of test sample were prepared in parallel. Next, 0.4 g of sample was weighed and determined for 6 times according to conditions in Section **3.2.3**. The absorbance and *RSD* values of each component were recorded. The results showed that the *RSD* of total flavonoids was 0.32%, which indicated that the method had good repeatability.

3.3 Determination of total triterpenes using an ultraviolet spectrophotometer

3.3.1 Preparation of reference solution. Into a 10.00 mL volumetric flask, 2 mg of oleanolic acid reference substance was accurately weighed, and methanol was added to dissolve it. Next, methanol was added to constant volume to obtain a reference substance solution with a concentration of 200 μg/mL.

3.3.2 Preparation of test solution of Wulan-13. First, 5 g of the original powder of Wulan-13 was accurately weighed, and added into in a round-bottomed flask, and 17 times of water was added to make a suspension, in which the original powder was soaked for 30 min. Next, the suspension was heated in an electric heating sleeve for 45 min, and then taken out and cooled to room temperature. Next, centrifugation was performed at 2 000 r/min for 10 min to obtain a supernatant, which was poured into an evaporating dish and freeze-dried for 48 h. Finally, 0.4 g of the residue was accurately weighed and added in a 25 mL brown volumetric flask, and 1 mL was pipetted and added in a 25 mL volumetric flask, and dissolved in methanol and diluted to constant volume.

3.3.3 Determination. First, 0.3 mL of the liquid to be measured was accurately pipetted and added in a 10 mL volumetric flask. The solvent was evaporated in a 70 ℃ water bath. Next, 0.2 mL of newly prepared 5% vanillin-glacial acetic acid solution and 0.8 mL of perchloric acid were added in turn. After mixing well and plugged tightly, the obtained solution was heated in a 70 ℃ water bath for 15 min, and then taken out and rapidly cooled in water, and 4 mL of ethyl acetate was added 5 min later. After mixing well and standing for 15 min, the absorbance was determined at 540 nm[5].

3.3.4 Investigation of linear relation. First, 0, 0.1, 0.2, 0.3,

0.4, 0.5 and 0.6 mL of oleanolic acid reference solution were accurately pipetted and added in 10 mL stoppered test tubes, respectively. Next, the absorbance was determined according to the method in Section **3.3.3**. A standard curve was drawn with absorbance Y as the ordinate and mass concentration X (mg) of oleanolic acid as the abscissa to calculate a regression equation.

The equation of the standard curve was $Y = 51.89X - 0.0839$ ($r = 0.9991$), indicating that oleanolic acid had a good linear relation with absorbance A in the range of 0.004 18 – 0.020 9 mg.

3.3.5 Precision test. Six copies of the reference solution in Section **3.3.1** were prepared, and determined for 6 times according to the conditions in Section **3.3.3**, and the absorbance and *RSD* value of each component were recorded. The results showed that the *RSD* of total triterpenes was 1.47%, which indicated that the precision of the equipment was good.

3.3.6 Stability test. From the test drug sample prepared by the method in Section **3.3.2**, 0.4 g was taken and determined according to the conditions in Section **3.3.3** for 6 times at 0, 10, 20, 30, 40 and 60 min after preparation, and the absorbance and *RSD* of each component were recorded. The results showed that the *RSD* of total triterpenes was 0.13%, which indicated that the sample was stable within 60 min.

3.3.7 Repeatability test. Six samples were prepared in parallel according to the preparation method in Section **3.3.2**, and about 0.4 g was weighed and determined for 6 times according to the conditions in Section **3.3.3**, and the absorbance and *RSD* value of each component were recorded. The results showed that the *RSD* of total triterpenes was 0.08%, which indicated that the method had good repeatability.

4 Discussion

4.1 Thoughts on the selection of detection wavelength The selection of wavelength is very important when HPLC and ultraviolet spectrophotometry are applied for detection, as it has a direct impact on the content of components. After purchasing the reference substances, all the reference substances were scanned by an ultraviolet spectrophotometer in the range of 200 – 600 nm. Combined with *Pharmacopoeia of the People's Republic of China* (PRC) and literature records, the maximum absorption wavelengths of the four reference substances were finally determined as chlorogenic acid 329 nm, geniposide 238 nm, rutin 510 nm and oleanolic acid 540 nm, respectively.

4.2 Thoughts on the selection of components for determination In this study, the contents of the main components in Wulan-13 were preliminarily determined, including chlorogenic acid, geniposide, total flavonoids, and total triterpenes. The most active ingredients in Wulan-13 are flavonoids, which have pharmacological effects such as protecting nerves, preventing myocardial ischemia, lowering blood pressure, improving memory loss, promoting gastric ulcer healing, protecting reproductive tissues, resisting inflammation and tumors, and lowering blood sugar. They mainly exist in various single herbs of Wulan-13 formula, such as Sophorae Flavescentis Radix, Inulae Radix, Ramulus Rubi, Rhizoma Kaempferiae, Toosendan Fructus, Eriobotryae Folium, and Trollii Flos. Secondary active ingredients are triterpenes, which are widely present in following herbs of Wulan-13 formula: Sophorae Flavescentis Radix, Inulae Radix, Ramulus Rubi, Chebulae Fructus, Gardeniae Fructus, Toosendan Fructus, and Eriobotryae Folium, and have blood sugar-lowering, anti-tumor, antioxidant and liver-protecting effects. However, the proportion of Gardeniae Fructus in this prescription is relatively high, and it is the main medicinal material for clearing blood heat. According to the pharmacopoeia, the most effective components of Fructus Gardeniae are iridoids, and geniposide is one of the most active substances in Gardeniae Fructus. Many studies have shown that geniposide has pharmacological effects such as lowering blood pressure, lowering lipid, antithrombotic, neuroprotective, antiplatelet aggregation, improving myocardial ischemia reperfusion injury, protecting vascular endothelial cells and anti-atherosclerosis, and is closely related to cardiovascular diseases such as hypertension and hyperlipidemia. Chlorogenic acid is a common active substance found in most Chinese medicinal materials and plants, exerting various pharmacological activity such as removing pathogenic heat from the blood and toxic materials from the body, soothing liver and gallbladder, anti-inflammatory and antibacterial. Meanwhile, its content can also be used as an important indicator for quality control of medicinal materials. Studies have also shown that chlorogenic acid can effectively reduce the blood pressure of patients with hypertension. The main indications of Wulan-13 are excessive blood heat, headache, red eyes and hypertension. Therefore, the chemical components with the effects of lowering blood pressure and clearing blood heat can be selected for content determination, so as to provide some ideas for the reform of technical dosage forms and lay a certain foundation.

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