

## **Simultaneous determination of chlorogenic and isochlorogenic acid contents in Xiao'er Yanbian granule by HPLC.**

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### **Abstract**

The contents of chlorogenic acid and isochlorogenic acid A in Xiao'er Yanbian Granule were determined simultaneously by Reversed-Phase HPLC with acetonitrile-methanol-0.1% phosphoric acid (20:6.5:73.5) as the mobile phase. Investigation of the injection amounts of the two types of chlorogenic acids by the methodology revealed that the injection amounts were in good linearity with the peak areas at a range of chlorogenic acid injection amount of 0.050~1.250 µg, and a isochlorogenic acid A injection amount range of 0.025~0.625 µg. The average recovery (n = 9) was 98.59% for chlorogenic acid, and 100.53% for isochlorogenic acid A, with RSDs of 0.42% and 1.17%, respectively. The contents of chlorogenic acid and isochlorogenic acid A in Xiao'er Yanbian Granule were determined to be 0.78 mg/g and 0.15 mg/g, respectively, this quantification method can be used for the comprehensive evaluation of the quality of Xiao'er Yanbian Granule.

**Keywords:** Xiao'er Yanbian Granule, chlorogenic acid, isochlorogenic acid A, HPLC

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### **Introduction**

Xiao'er Yanbian Granule is composed of eight TCM materials, namely Flos Lonicerae, Rhizoma Belamcandae, Radix Tinosporae, Radix Platycodi, Radix Scrophulariae, Radix Ophiopogonis, and Calculus Bovis Artificatus, which is usually used for swollen sore throat, oral erosion, pharyngitis, laryngitis, tonsillitis and other diseases [1]. The drug has been favored by the general public for its excellent efficacy in treatment of pediatric upper respiratory infections without any side effects. China now has nearly a hundred pharmaceutical companies; which manufacture Xiao'er Yanbian Granule, with a very good sale. To improve the quality of Xiao'er Yanbian Granule, enhance its efficacy and ensure its safety, the improvement of the quality control of Xiao'er Yanbian Granule is vitally important.

Flos Lonicerae is one of the most important medicinal materials in Xiao'er Yanbian Granule, which plays a leading role in terms of efficacy. Major active constituents in Flos Lonicerae include chlorogenic acids, isochlorogenic acids and flavonoids [2-6] of which; chlorogenic acid content is the highest that can reach up to 5.19% [2]; followed by isochlorogenic acid, which can be as high as 70% of that of the chlorogenic acid [7]. All the constituents have quite strong anti-bacterial, anti-inflammatory,

anti-oxidant, antipyretic and analgesic effects [8-10]. Therefore, it is reasonable to take Flos Lonicerae as the medicinal material used for the quality control of Xiao'er Yanbian Granule, in the 2010 Edition of Chinese Pharmacopoeia, where only the determination of chlorogenic acid content is listed under the article of quantitative determination of Xiao'er Yanbian Granule [11]. For a more scientific and comprehensive quality control of the preparation, multi-index evaluation method was adopted, in accordance with the "Measures for the Administration of Drug Registration of PRC" and relevant technical guidelines [12-13]. We established a HPLC method for the quantification of the active constituents in Flos Lonicerae [14-18]. This study is the first to simultaneously determine the chlorogenic acid and isochlorogenic acid A contents in Xiao'er Yanbian Granule using the ordinary isocratic elution and reversed-phase column with acetonitrile-methanol-0.1% phosphoric acid as the mobile phase. The results demonstrate that the method is simple, fast and practical.

### **Material**

#### **Materials**

Xiao'er Yanbian Granule samples (batch number: 140109, 140110, 140111, 140112, 140113, 140114) and the eight kinds of medicinal materials in the preparation were pro-

vided by Beijing Tong Ren Tang Pharmaceutical Co., Ltd.; chlorogenic acid reference substance (batch number: 1120136-20140130) and isochlorogenic acid A reference substance (batch number: 1120136-20140231) were purchased from Nanjing Tianqi Biotechnology Co., Ltd., the purities of reference substances were determined to be over 99.8% by normalization method.

#### Instruments

Agilent 1100 HPLC (G1379A degasser; G1311A quaternary pump; G1314A UVD detector; G132813 sample injector; Agilent 1100 chromatography workstation). Dikma diamonsil column (5  $\mu\text{m}$ , 4.6 mm $\times$ 150 mm).

#### Reagents

Reagents used in the mobile phase were chromatographically pure reagents; all the other reagents were of analytical grade.

### Methods and Results

#### Chromatographic conditions

Column: octadecylsilane bonded silica as the filler; mobile phase: acetonitrile-methanol-0.1% phosphoric acid

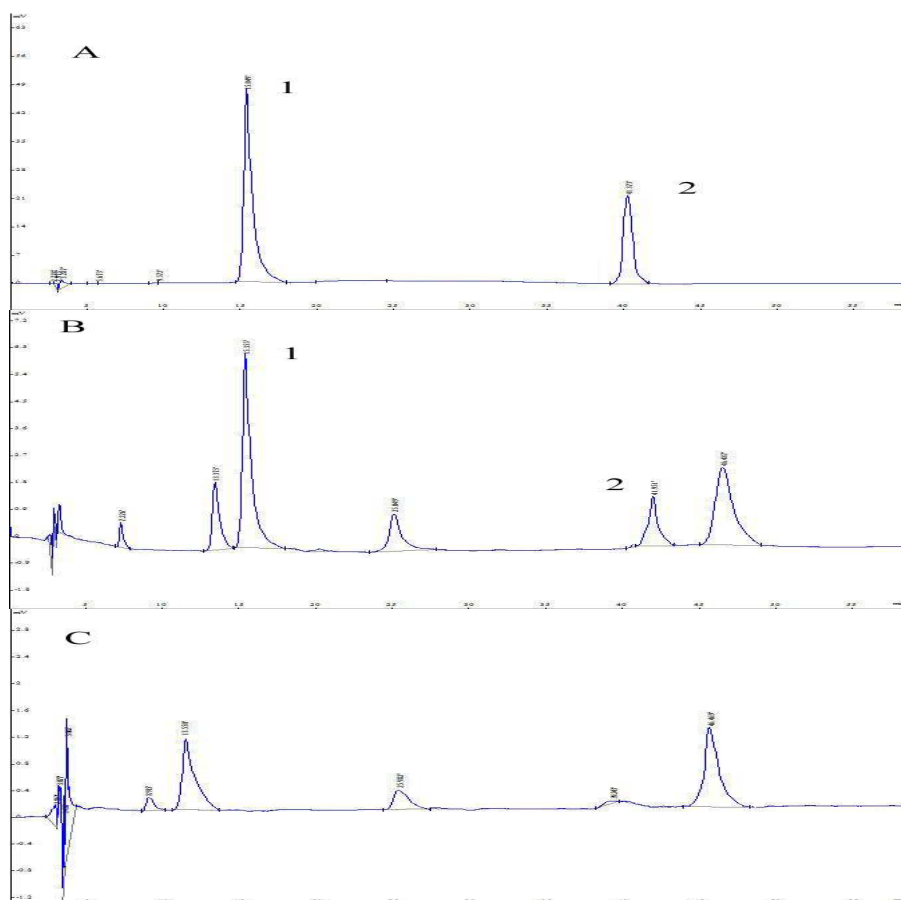
(20:6.5:73.5); detection wavelength: 326 nm; column temperature: 25°C; volume flow rate: 1.0 mL/min; number of theoretical plates: should not be less than 2,000 calculated based on chlorogenic acid peak.

#### Preparation of reference solutions

Appropriate amounts of chlorogenic acid and chlorogenic acid A reference substances were accurately weighed, placed in brown volumetric flasks separately, and added with methanol to prepare solutions containing 40  $\mu\text{g}$  of chlorogenic acid and 25  $\mu\text{g}$  of chlorogenic acid A per 1 mL A as the reference solutions.

#### Preparation of test solution

0.5 g of Xiao'er Yanbian Granule was accurately weighed, placed in a stoppered conical flask, added precisely with 25 mL of 70% methanol, stoppered tightly, and weighed, the Xiao'er Yanbian Granule was then ultrasonicated (power 250 W, frequency 33 kHz) for 30 min and 10 min, allowed to cool, and weighed again. After the lost mass was complemented with 70% methanol, the solution was shaken well, filtered by paper, the subsequent filtrate was filtered with 0.45  $\mu\text{m}$  microporous membrane, and the resulting filtrate was collected and used as the test solution.



**Figure 1.** HPLC chromatograms of four kinds of chlorogenic acids  
A. Mixed reference substances B. Xiao'er Yanbian Granule sample  
C. Negative sample 1. Chlorogenic acid 2. Isochlorogenic acid A

**Table 1.** Determination results of two types of chlorogenic acids in Xiao'er Yanbian Granule

Sample batch number	Chlorogenic acid mg/g)	RSD (%)	Isochlorogenic acid A (mg/g)	RSD (%)
140109	0.78	1.20	0.15	1.52
140110	0.79	1.21	0.12	1.57
140111	0.78	1.22	0.16	1.54
140112	0.76	1.19	0.21	2.01
140113	0.76	1.31	0.19	1.92
140114	0.79	1.29	0.11	1.60

**Preparation of blank sample**

Sample not containing Flos Lonicerae was taken, and prepared into the blank sample solution as per the methods under the "Preparation of test solution".

**Determination of samples**

Each 10 µL of the above reference, test and blank solutions were taken, injected into the HPLC, the results indicated that the blank sample was free of interference, the specificity of quantitative determination is shown in Fig. 1; mass fraction was calculated by one point external standard method, the sample determination results are shown in Table 1.

**Determination of detection wavelength**

Chlorogenic and isochlorogenic acids had the basic parent structure, which were both esters formed between caffeoyl and quinic acid in different locations with different number of caffeoyl<sup>19</sup>, so their spectral scan results were consistent, with maximum absorptions both at (326±2) nm, so the detection wavelength was set at 326 nm.

**Linearity range**

1, 5, 10, 20 and 25 µL of 0.050 mg/ml chlorogenic acid solutions and 0.025 mg/ml isochlorogenic acid A solutions were taken separately, and injected into the HPLC for determination of peak area. Standard curves were plotted with the peak area as the ordinate, and the injection volume as the abscissa. The results revealed that the injection volume was in a good linearity with the peak area at a chlorogenic acid concentration range of 0.05~1.25 µg/ml, and a isochlorogenic acid A concentration range of 0.02~0.625 µg/ml, respectively; and the regression equations were:  $Y = 3051758.7X - 3210$ ,  $r = 0.999$  for chlorogenic acid, and  $Y = 2881690.6X - 3820$ ,  $r = 0.998$  for isochlorogenic acid A.

**Precision test**

Five aliquots of 15 µL of test solutions were injected into the HPLC for determination of peak areas. The average peak area of chlorogenic acid and chlorogenic acid A were: 1260320 and 251691, respectively, and their RSDs were: 0.05% and 0.66%, indicating good precision.

**Stability test**

Test solutions under the "Precision test" were taken, and injected into the HPLC every 2, 4, 6, 8, 10, 12, 18 and 24 h for determination of peak area, as well as stability within 24 h, the RSD of peak area was 0.22% for chlorogenic acid and 0.56% for isochlorogenic acid A, indicating that the test samples were stable within the investigated 24 h.

**Reproducibility test**

Nine aliquots of samples with the same batch number (140109) were taken in high, medium and low doses for extraction, preparation and determination as per the methods under the preparation of test samples and chromatographic conditions. Average mass fractions of the two types of chlorogenic acids upon determination of same batch of samples nine times were: 0.78 mg/g for chlorogenic acid, and 0.15 mg/g for isochlorogenic acid A; and RSDs were: 1.02% and 2.04%, respectively, indicating good reproducibility of the method.

**Recovery test**

Sample recovery test was adopted. Nine aliquots of 0.5 g of same batch of samples (batch number: 140109) were taken in high, medium and low doses, plus a certain amount of reference substances, and extracted, prepared and determined as per the methods under the determination of test samples for calculation of recovery rates. Average recovery of the two types of chloro-

genic acids were 98.59% for chlorogenic acid, and 100.53% for isochlorogenic acid A, and RSDs (n = 9)

were 0.42% and 1.17%, respectively. The results are shown in Tables 2,3.

**Table 2.** Recovery test results of chlorogenic acid in Xiao'er Yanbian Granule

Sampling amount (g)	Measured sample amount (µg)	Chlorogenic acid addition amount (µg)	Total measured amount (µg)	Recovery (%)	Average recovery (RSD%)
0.5010	255.6	150.2	404.5	99.13	
0.5004	255.1	150.2	402.1	97.87	
0.5001	255.0	150.2	402.2	98.00	
0.5009	255.3	250.4	502.3	98.64	
0.5012	255.4	250.4	502.6	98.72	98.59
0.5016	255.4	250.4	502.1	98.52	(0.42)
0.5003	255.0	350.6	601.9	98.94	
0.5002	255.1	350.6	601.5	98.80	
0.4992	255.0	350.6	601.0	98.69	

**Table 3.** Recovery test results of isochlorogenic acid A in Xiao'er Yanbian Granule

Sampling amount (g)	Measured sample amount (µg)	Isochlorogenic acid A addition amount (µg)	Total measured amount (µg)	Recovery (%)	Average recovery% (RSD%)
0.5010	75.1	45.2	121.1	101.77	
0.5004	75.0	45.2	119.8	99.12	
0.5001	75.0	45.2	121.3	102.43	
0.5009	75.1	75.2	150.9	100.80	
0.5012	75.2	75.2	151.1	100.93	100.53 (1.17)
0.5016	75.2	75.2	151.2	101.06	
0.5003	75.0	105.3	180.2	99.91	
0.5002	75.0	105.3	180.1	99.81	
0.4992	75.0	105.3	179.2	98.96	

**Table 4.** Comparison of peak area between present method and method in literature

Method	Sample batch number	Injection volume (µL)	Chlorogenic acid	Isochlorogenic acid A
Present method	140109	15	1 275 485	254 709
Method in literature	140109	15	1 275 496	251 908

It can be seen from the data of gradient elution conditions reported in the literature in Tab. 6 that the isochlorogenic acid A contains the impurity peak, affecting the precision of the quantitative determination. Peak resolution chroma

tograms showed that the resolution of present method was superior to the conditions reported in the literature, with the resolutions of both chlorogenic and isochlorogenic acids reaching 3 or above.

### Comparison of present method with reported gradient determination method

Peak was determined by chromatographic methods used herein and reported in the literature [13], respectively, using the same column, the same sample solution and the same inject volume, the peak area data are shown in Tab. 4.

### Discussion

Flos Lonicerae is one of the most important medicinal materials in Xiao'er Yanbian Granule. The major chemical constituents of the herb are also the major active ingredients of the granule. Flos Lonicerae ; which mainly contains phenolic acids dominated by chlorogenic acids, as well as anthocyanins has good antibacterial activity [6,10]. Therefore, to ensure the safety and efficacy for better quality control of Xiao'er Yanbian Granule, the determination of the chlorogenic acid content was done [16-17], i.e. the simultaneous determination of chlorogenic acid and isochlorogenic acid A contents in Xiao'er Yanbian Granule.

This study was the first to simultaneously determine the chlorogenic acid and isochlorogenic acid A contents in medicinal herb Flos Lonicerae and its preparation Xiao'er Yanbian Granule using the ordinary isocratic elution and reversed-phase column with acetonitrile-methanol-0.1% phosphoric acid (20:6.5:73.5) as the mobile phase. By adjusting the proportion of the mobile phase, the quantitative chromatograms of the two constituents completed peak appearance in 20~30 min on three different columns, each peak had a good resolution, thus achieving a simple, fast, scientific, standardized and multi-index control of the quality of Xiao'er Yanbian Granule.

Compared with the reported gradient elution method, the present method reduces the performance requirements on instrument in terms of the contents of chlorogenic acid and chlorogenic acid A, where HPLC with gradient elution capability is no longer needed, and the quantitative determination of the two types of constituents is possible as long as there is an ordinary UV detector HPLC, thus substantially reducing the cost of experiments.

This method solves the problems of higher costs and cumbersome steps in testing of chlorogenic acid and isochlorogenic acid A in Xiao'er Yanbian Granule, thus achieving simple, fast and low-cost determination of drug quality. Meanwhile, it also provides the methodological basis and reference of multi-index determination for other Flos Lonicerae-containing preparations.

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