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Three bioactive components determination by TLC and HPLC for Compound Shanzha Granules

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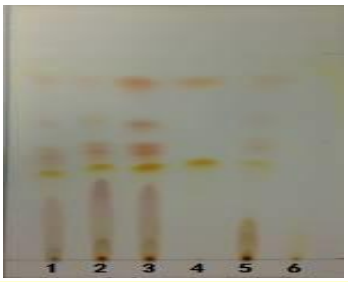
Abstract: **To establish the quality standard of compound Shanzha Granules. The chief components of the preparation, Crataegi Fructus and Cassiae Semen were identified by TLC. The contents of Autrantio-obtusin and Chrysophanol in Cassiae Semen were determined by HPLC. The separation was performed on Agilent SB-C18 (4.6×250mm, 5μ m) with acetonitrile-0.1% phosphoric acid as mobile phase for gradient elution. The relevant spots on TLC plates were clear by the blank reference. The contents showed good linear, it is in the range of 0.0357 ~ 0.3570μg (r=0.9999) for Autrantio-obtusin and 0.0520 ~ 0.5200μg (r=0.9999) for Chrysophanol. The average recovery rate was 99.64% for Autrantio-obtusin and 100.18% for Chrysophanol. The established qualitative and quantitative methods are simple, accurate and reproducible, which can be used for the quality control of compound Shanzha Granules.**



Keywords: Compound Shanzha Granules; Quality Control; Chlorogenic acid; Autrantio-obtusin; Chrysophanol; TLC; HPLC

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1. Introduction

Compound Shanzha Granule, a compound preparation of Chinese herbal medicine recorded by Chinese People's Liberation Army medical preparation standard (2002), is composed of two kinds of Chinese herbal medicines, Crataegi Fructus and Cassiae Semen. It is mainly used in the treatment of Hyperlipidemia [1]. The original quality standard was established in 2002, which can not reflect the quality of the traditional Chinese medicine preparation for the technique limitation of the time. For a better control of the quality of compound Shanzha Granules, the qualitative identifications of Crataegi Fructus and Cassiae Semen were revised and an effective HPLC method for the quantitative determination of two active components in Cassiae Semen was presented. This newly established quality standard was successfully applied for the analysis of compound Shanzha Granules.

2. Apparatus and Materials

2.1 Apparatus

JM-B2102 Electronic balance (Jiming Weighing Inspection Equipment Co. Ltd, Yuyao, China); FA1604 Electronic analytical balance (Hengping instrument factory, Shanghai, China); KH-100B Ultrasonic extraction device (Kechuang ultrasound Instrument Co. Ltd, Kunshan, China); polyamide thin layer (Sijia Chemical Plastic Factory, Taizhou, China); silica gel H plates (No.401 Hospital of PLA, Qingdao, China); Agilent 1260 high performance liquid chromatography(HPLC).

2.2 Materials

Compound Shanzha Granules (Batch No: 20130802, Jinan Military Region General Hospital; Batch No: 20130625, the 159th Hospital of PLA; Batch No: 20131009, Shenyang Military Region General Hospital); Negative control samples were self-made; Reference substances of Chlorogenic acid (Batch No:110753-201314), Autrantio-obtusin (Batch No: 111900-201303), Chrysophanol (Batch No: 110796-201319) and reference herbal medicine Cassiae Semen (Batch No: 121011-201005) were purchased from the National Institutes for Foodand Drug Control, China; Reference herbal medicine Crataegi Fructus was purchased from the National Institute for the Control of Pharmaceutical and Biological Products, China; Acetonitrile (HPLC grade) were purchased from Siyou Meticulous Chemical Products Co. Ltd (Tianjin, China); Other chemicals and solvents are all analytical grade.

3. Identification

3.1 Crataegi Fructus

2g of Granules were dissolved in 10 ml of methanol for ultrasonic extraction for 15 min, and filtered used for sample solution. Chlorogenic acid was dissolved into 1mg/ml of methanol, which was used as reference solution，which was used as reference solution. 1.5g of

the reference of Crataegi Fructus and 2g of Granules with Crataegi Fructus removed were samely prepared as the control medicinal solution and the negative control sample solution separately. According to the guideline of thin-layer chromatography (TLC) described in Chinese Pharmacopoeia 2010, Vol I, Appendix VI B, the qualitative experiment was carried out with polyamide thin layer as the coating substance and with a mixture of ethyl acetate, methanol and formic acid (10:4:1) as the mobile phase. After separately developing 5μL of the sample solution, reference solution, control medicinal solution of and negative control sample solution on the plate, the solutions were dried in air. Sprayed with 0.1% Bromocresol green (Bromocresol green 0.1g, dissolved in 100mL ethanol, adding 1% NaOH to blue) and blew, the spots of the chromatograms were visualized. Results in the sample solution chromatograms, the same colored speckles were appeared at the corresponding positions of the reference solution and control medicinal solution. No other spots interfered with the identification of Chlorogenic acid in compound Shanzha granules.

3.2 Cassiae Semen

20g of Granules (sugar-free:10g) were dissolved in 60mL of ethanol for ultrasonic extraction for 60 min, and filtered. Then the filtrate was evaporated to dryness in water bath, and redissolved with 30 ml of water and extracted twice with ethyl acetate 30mL each time. After that, the ethyl acetate layer was collected and washed with 30mL water for two times. At last, ethyl acetate solution was collected and evaporated for dryness, and the residue was dissolved in 1 ml of ethyl acetate and used for sample solution. Autrantio-obtusin and Chrysophanol were dissolved in the ethyl acetate to prepare the solution with a concentration of 1mg/ml, which was used as reference solution. 1.0g of the reference of Cassiae Semen, were dissolved in 10mL of ethanol for ultrasonic extraction for 60min and filtered. Then the filtrate was evaporated to dryness in water bath, dissolved in 10 ml of water and extracted with ethyl acetate 20mL for two times. The ethyl acetate layer was collected and evaporated for dryness, and then the residue was dissolved in 1mL of ethyl acetate and used as the control solution of Cassiae Semen. By using 20g of the Granules with Cassiae Semen removed, the negative control sample solution was prepared with the same method for the sample solution. The experiment was carried out by following the guideline of thin-layer chromatography (TLC) described in Chinese Pharmacopoeia 2010, Vol I, Appendix VI B, with silica gel H as the coating substance and with a mixture of petroleum ether and acetone (2:1) as the mobile phase. After separately developing 5μL of the sample solution, reference solution and negative control sample solution on the plate, the solutions were dried in air. Sprayed with 10% sulphuric acid alcohol solutio and heated at 105℃, the spots of the chromatograms were visualized. Results in the sample solution chromatograms, the same colored speckles were appeared at the corresponding positions of the reference solution and control medicinal solution. No other components interfered with the identification of Autrantio-obtusin and Chrysophanol in compound Shanzha Granules.

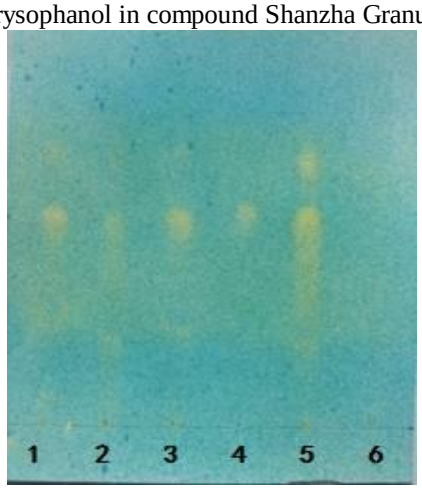


Figure 1. TLC of Crataegi Fructus in Compound Shanzha Granules: 1-3) samples; 4) Chlorogenic acid; 5) reference Crataegi Fructus; 6) negative control sample.

Figure 2. TLC of Cassiae Semen in Compound ShanzhaGranules: 1-3) samples; 4) Autrantio-obtusin and Chrysophanol; 5) reference Cassiae Semen; 6) negative control sample.

4. Assay

4.1 Preparation of solutions

Standard stock solutions of Autrantio-obtusin and Chrysophanol were prepared with methanol. By diluting and mixing the stock solutions, working standard solution contained each of the two compounds were prepared to reached the final concentrations of 14.28μg•mL^{-1} of Autrantio-obtusin, 20.8μg•mL^{-1} of Chrysophanol, and then filtered through 0.45μm membrane filter.

The granules were ground into fine powder 0.5g of the powder was accurately weighed into a 50mL flask with addition of 25mL 70% ethanol and weighed again,

and then it was refluxed for 2h. The solution was cooled down and made up the loss. Then the solution was shaken thoroughly and filtered through a filter paper. The filtrate was filtered through the 0.45μm membrane filter.

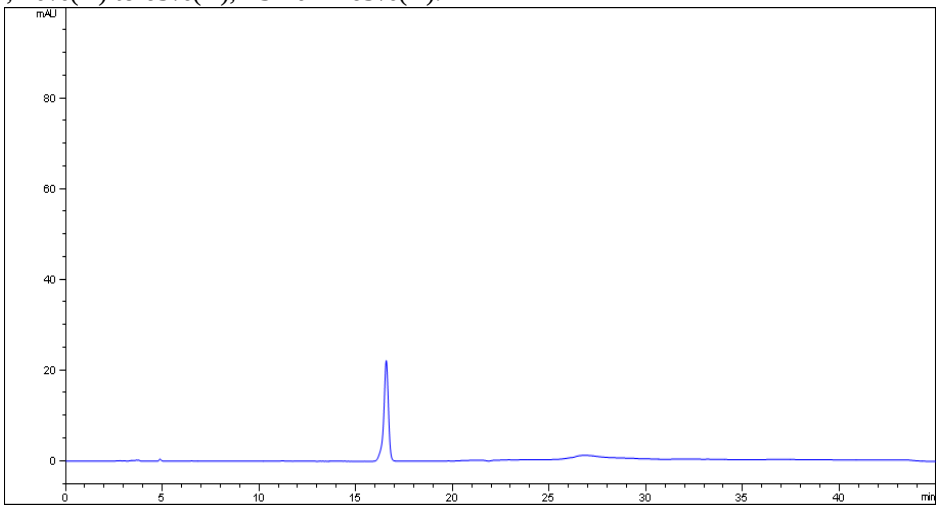
The negative control sample solution was prepared with the same method for sample solutions which was described in "3.1.2".

4.2 Chromatographic conditions

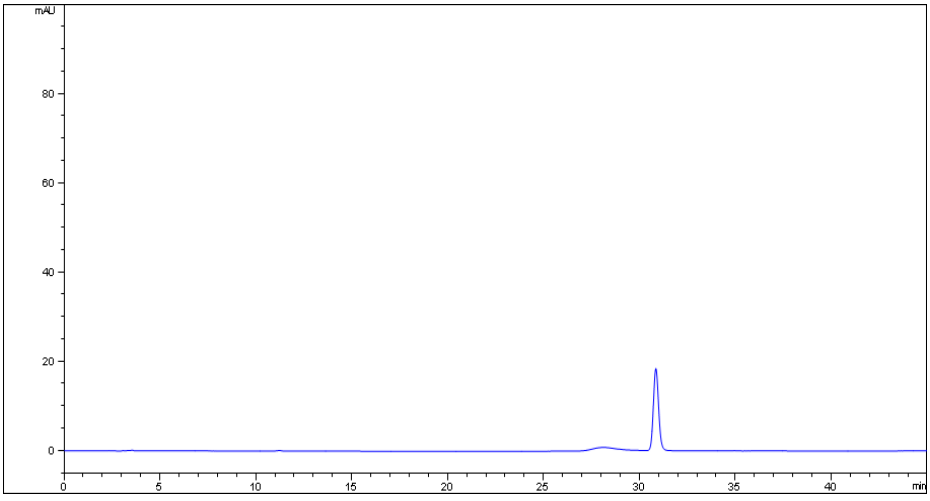
The samples were chromatographed under the following chromatographic conditions. Chromatographic column: Agilent SB-C18 (4.6×250mm, 5μm); Mobile phase: acetonitrile (solvent A) and 0.1% acetate acid (solvent B) [2], the gradient elution program: 0–10 min, 0% (A) to 40% (A); 10-25min, 40%(A) to 65%(A); 25-40min65%(A). Flow rate: 1.1 ml•min^{-1}. Column temperature: 30℃. Detection wavelength: 280nm. Injection volume: 10μL.

4.3 Specificity

Reference substance solution， sample solution and negative control sample solution as described above were analyzed. The injection volume of each solution was 10μL. The sample solution has a corresponding peak in the corresponding position of the peak of reference solution with the same retention time, 16.5min for Autrantio-obtusin and 30.8min for Chrysophanol, respectively. Moreover, there was no negative interference. As showed in Figure 3.



A



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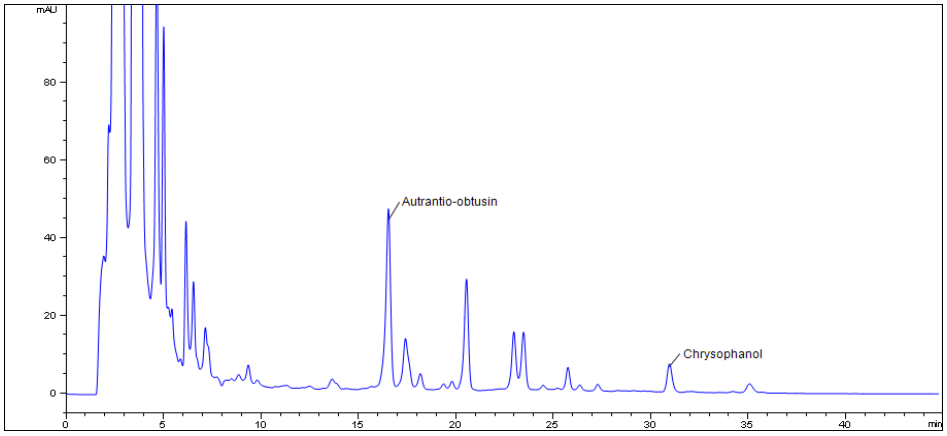

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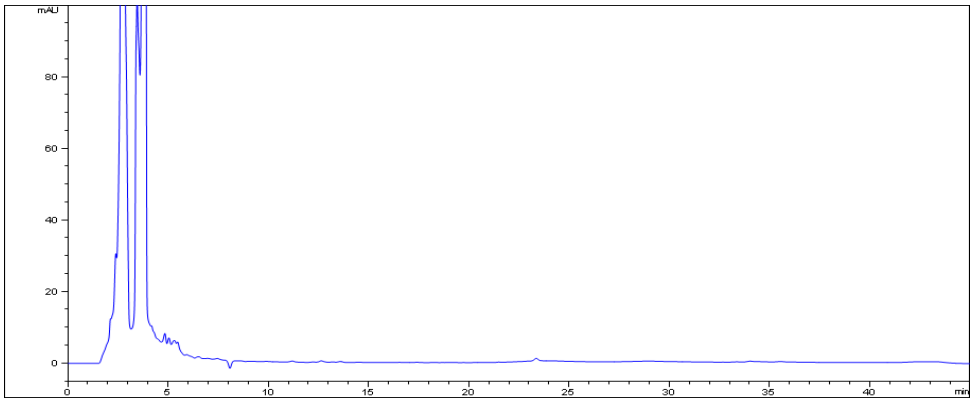

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Figure 3. HPLC of A) Autrantio-obtusin, B) Chrysophanol, C) Compound Shanzha Granules and D) negative sample (lack of Cassiae Semen).

Table 1 Recovery of Autrantio-obtusin

Sampling Quantity（g）	Sample Content（mg）	Addition（mg）	Determination（mg）	Recovery（%）	Average（%）	RSD (%)
2.5010	0.1901	0.133	0.319	96.92		
2.5010	0.1901	0.133	0.328	103.68		
2.5012	0.1901	0.133	0.323	99.92		
2.5002	0.1900	0.166	0.357	100.60		
2.5004	0.1900	0.166	0.360	102.41	99.64	2.83
2.5005	0.1900	0.166	0.360	102.41		
2.5006	0.1900	0.229	0.410	96.07		
2.5004	0.1900	0.229	0.414	97.82		
2.5001	0.1900	0.229	0.412	96.94		

4.4 Linearity of calibration curves

All calibration curves were plotted based on linear regression analysis of the integrated peak areas (Y) versus sample size (μg, X) of the two active components in the standard solutions at six concentrations. The corresponding regression equations were computed and found to be: Y=5210.2X+0.1468 (r=0.9999) for Autrantio-obtusin and Y=617.64X-0.0392 (r=0.9999) for Chrysophanol. Good linear relationships were obtained in the range of 0.0357～0.3570μg for Autrantio-obtusin and 0.0520～0.5200μg for Chrysophanol.

4.5 Precision, reproducibility and stability

The precision was determined by repeated analysis of the standard solution at the same concentration. The RSD% of peak areas were 1.03% for Autrantio-obtusin, and 0.93% for Chrysophanol.

Six batches of compound Shanzha Granules were extracted and analyzed with the proposed procedure to test the reproducibility. Their peak areas were measured and compared with standard solutions under the same conditions described above. The RSD% was 1.18% for Autrantio-obtusin, and 2.71% for Chrysophanol.

For stability test, the sample test solution (Batch No:20131009) was analyzed at hour 0h，2h，4h，6h，8h and 12h, respectively. The RSD% of peak areas was 1.16% for Autrantio-obtusin, 2.17% for Chrysophanol. The experimental results indicated that samples were stable in 12h.

Table 2 Recovery of Chrysophanol

Sampling Quantit(g)	Sample Content（mg）	Addition （mg）	Determination （mg）	Recovery （%）	Average （%）	RSD(%)
2.5001	0.26501	0.208	0.472	99.51		
2.5001	0.26501	0.208	0.475	100.96		
2.5002	0.26502	0.208	0.471	99.03		
2.5001	0.26501	0.270	0.541	102.22		
2.5003	0.26503	0.270	0.533	99.25	100.18	1.09
2.5002	0.26502	0.270	0.533	99.25		
2.5001	0.26501	0.354	0.618	99.71		
2.5003	0.26503	0.354	0.621	100.56		
2.5004	0.26504	0.354	0.623	101.12		

4.6 Recoveries

Recovery studies were carried out at three different concentration levels. The known amount of reference standard was added into the samples, and the recovery studies subjected to the proposed HPLC method. The mean recoveries were 99.64% for Autrantio-obtusin and 100.18% for Chrysophanol (Table 1, Table 2).

4.7 Sample Analysis

According to the method for the sample solution preparation, two active components of three batches of compound Shanzha Granules were determined. The results are listed in Table

Table 3 Analysis results of the marker constituents in Compound Shanzha Granules

Batch No	Autrantio-obtusin (mg g^{-1})		Chrysophanol (mg g^{-1})	
	$\overline{X} \pm S$	RSD (%)	$\overline{X} \pm S$	RSD (%)
20131009	0.076±0.002	2.0	0.106±0.002	1.97
20130625	0.042±0.001	2.4	0.022±0.001	2.60
20130802	0.160±0.001	0.4	0.103±0.001	0.06

4. Discussion

Compared with the original quality standard of compound Shanzha Granules, this study has made some progress in the identification of Crataegi Fructus and Cassiae Semen. In the identification of Crataegi Fructus, the Chemical identification was replaced by TLC, which improved specificity of identification; This study tended to identify flavonoids of Crataegi Fructus, but the chief active components of Cassiae Semen is ingredients [3], ingredients' polarity is smiliar with flavonoids, so that the two components cannot be easily separated in TLC, result showed that there was negative interference in the corresponding position of reference substance; Tried to use the ursolic acid, chlorogenic acid, citric acid as control sample, only chlorogenic acid was found that it has the same yellow spot in the corresponding place, so we choose chlorogenic acid as the reference.

In the TLC of the old quality standard for Cassiae Semen, the sample was extracted with methanol, then the methanol solution was extracted with ether. In our study, methanol replaced by ethanol and ether replaced by ethyl acetate. A more simple，rapid and environmental result was obtained. The proccess of adding hydrochloric acid and reflux was deleted for more clear and reproduciblespots, and the method is also simpler than the old one. In our study, we choose aurantio obtusin and chrysophanol as references of TLC, which is same with Chinese Pharmacopoeia (2010 Ed) about TLC of Cassiae Semen. Aurantio obtusin is exclusive component in Cassiae Semen [4-5], which improves the specificity.

The quality standard of compound Shanzha granules has no determination of the main ingredients of content. In our study, one of the important steps is the selection of proper factors. These procedure-related factors, classified into extraction conditions and chromatographic conditions were examined. For example, in terms of preparation of sample solutions，we choose 70% ethanol as the extract solvents acooding to references [6-7] and the effects of different consumption (25, 50, 75, 100mL) and extracting time (1.0, 1.2, or 2.0h) on the separation were compared. We also compared different gradient elution of mobile phase. It is concluded that with 20mL 70% ethanol for 2h can be used as the most optimal methol, gradient elution program was designed as follow: 0–10 min, 0% (A) to 40% (A); 10-25min, 40% (A) to 65% (A); 25-40min65% (A). The results show that the developed methods are simple, precise, rapid and reproducible for quantitation of compound Shanzha granules.

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