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Simultaneous determination of six active constituents of *Inula* flowers dispensing granule by HPLC-DAD.

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Abstract

Objective: To develop an HPLC-DAD method for simultaneous determination of chlorogenic acid, caffeic acid, 1, 5-dicaffeoylquinic acid, rutin, spinacetin and quercetin in *Inula* flower dispensing granule.

Methods: Chromatographic separation was performed on a thermo hypersil BDS C18 column (4.6 mm × 250 mm, 3 μm) with a 0.5% citric acid-acetonitrile gradient at the flow rate of 1 ml/min. The column temperature was maintained at 35°C, and detection wavelength was set at 360 nm.

Results: Chlorogenic acid, caffeic acid, 1, 5-dicaffeoylquinic acid, rutin, spinacetin and quercetin were linear in the ranges of 0.101-2.020 μg (r=0.9998), 0.052-1.040 μg (r=0.9997), 0.210-4.200 μg (r=0.9997), 0.142-2.840 μg (r=0.9998), 0.045-0.900 μg (r=0.9996) and 0.050-1.000 (r=0.9997), respectively. The average recoveries were 98.37%, 98.57%, 99.11%, 98.93%, 98.81% and 99.26%, respectively, and the RSDs were 0.82%, 0.91%, 0.86%, 1.19%, 0.93% and 0.86%, respectively.

Conclusion: The HPLC-DAD method was accurate, convenient and reproducible for quality control of the multi-component in *Inula* flower dispensing granule.

Keywords: *Inula* flowers dispensing granule, Composition, HPLC-DAD, Chlorogenic acid, Caffeic acid, Quercetin.

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Introduction

Inula, a traditional Chinese herbal medicine, refers to the dry capitulum of *Inula japonica* Thunb or *Inula britannica* L. It is used for treating phlegm accumulation, cold wind cough, asthma and cough with profuse sputum, and chest congestion [1]. The main chemical components of this herb are flavonoids, phenolic acids, sesquiterpene lactones, and sterols [2-4]. Thus, *Inula* possesses antibacterial, anti-tumor, and hepato-protective properties [5]. Phenolic acids and flavonoids are the major effective antioxidant and antibacterial components of *Inula* flowers.

Chinese dispensing granule refers to single granule extracted and concentrated from prepared Chinese Materia Medica by modern pharmaceutical technologies. In effect, it is a product of modernization and industrialization of Traditional Chinese Medicine (TCM). The granule, which can be taken mixed with boiled water, has some advantages in that it is usually taken in small doses and the dose can be modified in line with the symptoms of disease. At the present, the quality control of TCM dispensing granules has not been formally issued. Moreover, quality researches on *Inula* granules are rarely reported [6]. Previous studies have analysed *Inula* with respect to chlorogenic acid content [7], phytochemical constituents [8] and flavonoid composition [9]. The chlorogenic acid content of *Inula* granule has been determined by a combination of thin Layer chromatography (TLC) and HPLC [9]. In another study, the chlorogenic acid and caffeic acid contents of *Inula* were simultaneously measured by HPLC [10]. However, there are no studies involving measurement of two or more components of the granules as indices of quality control. The present study was carried out to determine the chlorogenic acid, caffeic acid, 1, 5-dicaffeoylquinic acid, rutin, spinacetin, and quercetin levels in seven batches of *Inula* granule from various companies by a new method of HPLC-DAD, so as to provide some references for quality control and clinical practice.

Instruments and Materials

Agilent 1260 high performance liquid chromatograph (type G1315B DAD detector, Agilent Technologies); Type CPA225D 1/100000 balance (Satorius); Type MSE 1/100000 balance (Satorius and Type AS3120 ultrasonic generator (Aote Baoensi Instruments Co. Ltd) were used.

Seven different batches of *Inula* granules were provided by Jiangyin Tianjiang Pharmaceutical Co., Ltd (batch numbers: 0907043, 0912235, 0927182, 1509021 S, 1203010 S, 1308003 S and 1305012 S). Standard chlorogenic acid (0703-202131),

caffeic acid (0153-200130), 1, 5-dicaffeoylquinic acid (10226-201304), rutin (1502-300103), spinacetin (100132-330623), and quercetin (1021-205032) were provided by Chinese National Institute for Food and Drug Control. Chromatographically pure carbinol and acetonitrile were products of Tianjing Concord Technology, while citric acid (analytically pure) was purchased from Sinopharm Chemical Reagent Co., Ltd). The water used was pure.

Methods

Trials for ascertaining chromatographic conditions and system suitability: Using a Thermo Hypersil BDS C18 (4.6 mm × 250 mm, 3 μm) chromatographic column, and mobile phase of 0.5% citric acid aqueous solution (A)-acetonitrile (B), a gradient elution procedure was carried out at a flow rate of 1.0 ml/min, column temperature of 35°C and detection wavelength of 360 nm. The injection volume of the mixture of standards was 10 μL.

Preparation of standard solutions: The following amounts of standards were accurately and separately weighed: 10.122 mg chlorogenic acid, 5.201 mg caffeic acid, 10.503 mg 1, 5-diclofenac quinacrine, 10.140 mg rutin, 4.510 mg spinach protein and 5.021 mg dermatoside. Each standard was put in a separate 10 ml volumetric flask and dissolved in 70% methanol to achieve standard stock solutions containing 1.012 mg/ml chlorogenic acid, 0.520 mg/ml caffeic acid, 1.050 mg/ml 1, 5-diclofenac quinacrine, 1.014 mg/ml rutin, 0.451 mg/ml spinacetin, and 0.502 mg/ml quercetin. A mixture of the stock solutions was obtained by mixing 1 ml of stock solution of chlorogenic acid, 1.0 ml caffeic acid, 2.0 ml 1, 5-dicaffeoylquinic acid, 1.4 ml rutin, 1.0 ml spinacetin, and 1.0 ml quercetin in a 10 ml volumetric flask, and making up the volume to mark with 70% methanol. This resulted in final concentrations of 0.101 mg/ml chlorogenic acid, 0.052 mg/ml caffeic acid, 0.210 mg/ml 1, 5-dicaffeoylquinic acid, 0.142 mg/ml rutin, 0.045 mg/ml spinacetin, and 0.050 mg/ml quercetin.

Preparation of the test sample solution: The *Inula* granule samples were ground, and 0.5 g of each sample was accurately weighed and put into a stoppered conical flask to which 30 ml of 70% methanol was added. The flask was then weighed, and the sample was extracted for 60 min by ultrasound (power 150 W, frequency 40 kHz), after which the flask was re-weighed on cooling. After making up for losses in 70% methanol, the flask was shaken and the contents were filtered through a 0.22 μm microporous membrane. The filtrate was used as the test sample solution.

Investigation of linear relationship: Under the outlined chromatographic conditions, 1, 2, 4, 8, 16, and 20 μL of the mixed standard solution were accurately withdrawn for analysis. A standard curve was drawn with an abscissa of amount of standard compound (μg), and an ordinate of the range of peaks, so as to get regression equations and correlation coefficients.

Test of precision: Using the chromatographic conditions stated earlier, a volume of mixed control solution was successively injected 6 times into the column to record the area of peaks.

Test of repeatability: Six test samples from the same (batch number 0907043) were prepared and injected for analysis under the chromatographic conditions as stated earlier, and the RSD values of the standards were determined.

Stability test: Samples from the same batch (batch number 0907043), placed at room temperature were injected into the column and analysed at 0, 2, 4, 6, 8, 12, and 24 h under the same chromatographic conditions as stated earlier.

Test of loading recovery: Six (6) samples of *Inula* granule of known concentrations (chlorogenic acid, caffeic acid, 1, 5-dicaffeoylquinic acid, rutin, spinacetin, and quercetin were 4.51, 3.03, 6.42, 5.61, 2.54, and 2.82 mg/g, respectively) were used. Each sample (0.5 g) was accurately weighed and placed in a stoppered conical flask, and then mixed with known amounts of the control stock solutions of chlorogenic acid, caffeic acid, 1, 5-dicaffeoylquinic acid, rutin, spinacetin, and quercetin. The mixtures were processed into the loading test sample solutions according to rules of "2.3" determined under the stipulated chromatographic conditions, so as to calculate the mean recovery and RSD of each standard compound.

Determination of constituents of *Inula* granule: About 0.5 g powder of *Inula* granule sample, precisely weighed, was processed as outlined earlier, and injected under the same chromatographical conditions as described previously. The area of each chromatographic peak was recorded, and the contents of the six components in the samples were determined from a standard calibration curve.

Results

Chromatographic conditions and system suitability

The gradient elution procedure is summarized in Table 1.

Under the above chromatographic conditions, the resolutions between chromatographic peaks of chlorogenic acid, caffeic acid, 1, 5-dicaffeoylquinic acid, rutin, spinach, quercetin and their adjacent chromatographic peaks were all greater than 1.5, and the number of theoretical plates was above 9000. The DAD spectra showed that the peaks of chlorogenic acid, caffeic acid, 1, 5-dicyclareoylquinic acid, rutin, spinach and quercetin in the sample spectrograms were all uni-component peaks.

Table 1. *Gradient elution conditions.*

T (min)	A (%)	B (%)
0	88	12
10	76	24
35	65	35
45	35	65

55	20	80

Table 2. Regression equations, correlation coefficients and linear range of six components (n=6).

Compounds	Linear equation	R	Linear range/μg
Chlorogenic acid	y=43121x+1105.2	0.9998	0.101-2.020
Caffeic acid	y=30712x+2822.3	0.9997	0.052-1.040
1, 5-dicaffeoylquinic acid	y=50520x+1731.1	0.9997	0.210-4.200
Rutin	y=20832x+2033.2	0.9998	0.142-2.840
Spinacetin	y=23543x+1221.5	0.9996	0.045-0.900
Quercetin	y=42038x+983.6	0.9997	0.050-1.000

Table 3. Contents of six active constituents of Inula flower dispensing granule.

Batch no.	Concentration ($mg \cdot g^{-1}$)					
	Chlorogenic acid	Caffeic acid	1, 5-dicaffeoylquinic acid	Rutin	Spinacetin	Quercetin
0907043	4.51	3.03	6.42	5.61	2.54	2.82
0912235	4.46	3.07	6.35	5.51	2.63	2.89
0927182	4.43	2.99	6.30	5.49	2.48	2.72
1509021 S	4.27	2.78	6.13	5.23	2.18	2.24
1203010 S	4.19	2.65	6.02	5.12	2.10	2.01
1308003 S	4.20	2.72	6.05	5.15	2.09	2.13
1305012 S	4.15	2.60	6.16	5.21	2.13	2.08

Linearity test

The regression equations and correlation coefficients obtained in the linearity tests are shown in Table 2. The regression equations and correlation coefficients indicate that the HPLC-DAD method has a very good degree of linearity.

Precision test

The RSD values of chlorogenic acid, caffeic acid, 1, 5-dicaffeoylquinic acid, rutin, spinacetin, and quercetin were 1.21%, 1.03%, 0.93%, 1.06%, 1.34%, and 0.85%, respectively. This demonstrates that the HPLC-DAD method used has very high precision.

Repeatability test

In the repeatability test, the RSD values of chlorogenic acid, caffeic acid, 1, 5-dicaffeoylquinic acid, rutin, spinacetin, and quercetin were 1.32%, 1.06%, 1.21%, 1.36%, 0.91%, and 1.40%, respectively. These RSD values indicate that the HPLC-DAD method has very high degree of repeatability.

Stability test

In the 24 h stability test, the RSD values of chlorogenic acid, caffeic acid, 1, 5-dicaffeoylquinic acid, rutin, spinacetin, and quercetin were 1.30%, 0.78%, 1.19%, 1.05%, 1.28%, and 0.94%, respectively. These results show that the tested solutions remained stable within 24 h at room temperature.

Load recovery

The mean recovery of the six components ranged from 97.22 to 99.48%, and all RSD values were less than 2.0%, indicating that the method had a good accuracy.

Composition of Inula granule

The concentrations of the six components of *Inula* granule are shown in Table 3.

Discussion

In the selection of wavelength for use in the analysis, the six components were scanned at 200-400 nm with a Diode Array Detector (DAD), and it was found that chlorogenic acid, caffeic acid and 1, 5-dichenoylacetic acid absorbed maximally at 327 nm, and rutin absorbed maximally at 254 nm. Spinacetin absorbed maximally at 210 nm and 360 nm, while quercetin had maximum absorption at 260 and 370 nm. In view of factors like chromatographic peak area and baseline stability, 360 nm was chosen as the detection wavelength. During the preparation of samples, various solvents were first separately investigated for their suitability e.g. methanol, water, and methanol (60%, 70%, and 80%). The results revealed that 70% methanol had the best effect on the extraction of the six components. In addition, preliminary trials showed that 60 min of ultrasound resulted in complete extraction of sample. Hence the ultrasonic duration was set at 60 min. In addition, trials with gradient elution using various combinations of solvents revealed that with acetonitrile -0.05% phosphoric acid, all the six components in the test samples were separated at baseline, each with a well-defined and sharp chromatographic peak.

The results of analysis of the constituents of *Inula* granule indicated that chlorogenic acid, caffeic acid, 1, 5-dicaffeoylquinic acid, rutin, spinacetin and quercetin were present in all the batches studied. There were only minor differences in composition between the batches from different companies, which are an indication of stability in quality in the various batches. The order of abundance of the components was 1, 5-dicaffeoyl quinic acid>rutin>chlorogenic acid>caffeic acid>spinach quercetin.

Inula dispensing granule is a common Chinese patent medicine used in clinics, but its quality control has so remained sketchy. In this study, HPLC-DAD was developed and used successfully for the first time to determine the contents of six active components of the dispensing granule. This technique, which has very high accuracy, is easy to operate, and is considered a reference method for quality control.

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