Quality control of tuirejieduling granules using high-performance liquid chromatography fingerprint method and simultaneous determination of four main active ingredients

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Submitted: 29-02-2012 Revised: 28-03-2012 Published: 30-04-2013

ABSTRACT

Background: The Tuirejieduling granule is a compound preparation made from four kinds of Chinese medicines. It is effective for anti-inflammation, antivirus, defervescence and anti-bacterium; however, its quality control standards have remained unknown. **Objective:** To establish a simple and accurate fingerprint method for quality control of the Tuirejieduling granule. **Materials and Methods:** The methanol extract of the Tuirejieduling granule was used for the fingerprint analysis and the four selected active ingredients (epigoitrin, phillyrin, saikosaponin A and glycyrrhetinic acid) in the extract were determined. The fingerprint method was performed on an Amethyst C18-P chromatography column by gradient elution with acetonitrile and aqueous phase (containing 0.5% H_3PO_4 (v/v), pH 3.0). **Results:** Under the optimal chromatographic condition, twenty peaks were chosen as fingerprint peaks of the Tuirejieduling granules extractions. The similarities of 10 batches of Tuirejieduling granule was more than 0.99. This indicates that the different batches of Tuirejieduling granules were under the consistent quality control. Good linear behaviors over the investigated concentration ranges were obtained with the values of R^2 higher than 0.99 for four studied active ingredients. The recoveries for spiked samples were in the range of 96.2-105.5%. The developed method was successfully applied to determine the contents of active constituents in different batches of Tuirejieduling granule. **Conclusion:** The HPLC fingerprint was proved to be a reliable method for the quality control of Tuirejieduling granule.

Key words: Fingerprint, high-performance liquid chromatography, quality control, Tuirejieduling granules

Access this article online

Website: www.phcog.com

DOI: 10.4103/0973-1296.111284

Quick Response Code:

INTRODUCTION

The Tuirejieduling granule is a compound preparation made from four kinds of Chinese medicines including Radix Isatidis (RI), Forsythia, Radix Bupleuri (RB) and Glycyrrhiza. It is effective for anti-inflammation, antivirus, defervescence and anti-bacterium. The preparation was improved based on the Tuirejieduling oral liquid for long-term clinical medication made by our institute [Approval No.: 2001, BZ01-036].[1] During the last decades, how to quality control of Chinese herbal compound was a hot spot of study. With the development of modern analytical technique, fingerprinting analysis of traditional Chinese medicine (TCM) was rapidly improved in recent years and widely used for quality control of Chinese herbal compound as a useful method.[2,3]

As a valuable cultural heritage of the Chinese nation, TCM has attracted increasing attention around the world. The active ingredients in TCM are the material basis of medical treatment. RI, Forsythia, RB and Glycyrrhiza are commonly used in TCM. Epigoitrin is one of the representative components in RI with the effective of against influenza virus[4] and is used as an important indicator for the quality

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control of RI in Chinese Pharmacopoeia (ChP).[5] As one of the main constituents in Forsythia, Phillyrin is used for its quality control in (ChP)[6] and was recently verified to have the effects of antioxidation and reducing blood-fat.[7,8] Saikoside (Ss), mainly existing as Ssa, Ssb, Ssc and Ssd, are the main constituents and active compounds in RB.[9] Among of them, Ssa is used for the quality control in ChP.[10] Some literatures also indicated that Ssa showed excellent anti-inflammatory[11,12] and anti-virus effect.[13,14] Glycyrrhetinic acid is considered as one of the active constituents in Glycyrrhiza due to its remarkable pharmacological effects on anti-inflammatory[15] and antitumor effect.[16] Therefore, the four active ingredients including epigoitrin, phillyrin, saikosaponin A and glycyrrhetinic acid were selected to control the quality of the Tuirejieduling granules.

In this study, an operational HPLC fingerprint method was proposed and could be effectively used for the quality control of the Tuirejieduling granules.

MATERIALS AND METHODS

Instrumentation

The HPLC (HITACHI, High-Technological Corporation, Tokyo, Japan) was equipped with a UV detector L-2400, Pump L-2130, 20 μL sample injection loop, and T2000P chromatography workstation. Chromatographic separations were carried out on an Amethyst C_{18}-P chromatography column with 5 μm particle size (250 mm × 4.6 mm i.d.; Sepax Technologies Inc.). UV-756 MC UV-visible optical depth meter (Shanghai Precision Scientific Instrument Co, Ltd, Shanghai, China) was used to scan the maximum absorption wavelength of the object. The Delta 320 acidometer (Mettler Toledo Instruments Co. Ltd., Shanghai, China) was used to control the acidity of the mobile phase.

HPLC procedure

A binary gradient elution system consisting of acetonitrile (A) and 0.5% (v/v) phosphoric acid aqueous solution (B) (pH 3.0) was used with the following gradient program: 0 ~ 100 min, 5 ~ 30% A; 100~ 120 min, 30 ~ 50% A; 120 ~ 140 min, 50 ~ 100% A. The flow rate was 1.0 mL min-1. The column temperature was 25°C. The detection wavelength was 210nm. The injection volume was 20 μL.

Standard solution and reagent

Epigoitrin (Approval No. 111753-200601, purity >98%), phillyrin (Approval No. 110821-200603, purity >98%), saikosaponin A (Approval No. 110777-200303, purity>98%) and glycyrrhetinic acid (Approval No.100001-200504, purity > 98%) were all provided by National Institute for the Control of Pharmaceutical and Biological Products. The Tuirejieduling granule samples were made by Wuhan Hospital of Combination of Traditional Chinese Medicine with Western Medicine. Acetonitrile (Tianjin Kermel Chemical Reagent Co. Ltd., Tianjin, China) and methanol (Tianjin Standard Science and Technology Co. Ltd, Tianjin, China) were chromatographic grade. Analytical grade phosphoric acid (Shanghai Reagent Co., Shanghai, China) and high purity deionized water obtained from a Labconco system (18.2 MX cm) were used for preparation of the mobile phase. The chemical structures of the reference compounds are shown in Figure 1.

Sample preparation

Tuirejieduling granule (1.00 g) was actually weighted. It was then transferred into a sealed flask (200 mL) containing 100 mL of methanol, extracted in an ultrasonic water bath for 15 min at room temperature. The resulting solution was filtered through a 0.45 μm polytetrafluoroethylene membrane filter (Tianjin Jinteng Instrument Factory, Tianjin, China). The extract was transferred into a 100 mL volumetric flask, and adjusted to volume with methanol. Also, the blank solution was prepared by the same procedure without adding the Tuirejieduling granules.

Standards preparation and calibration curves

Stock solutions were prepared from the above-mentioned standard chemicals by dissolving proper amounts of the solid in methanol, and then stored at 4°C. Working standards at the concentration of the calibration range were prepared by stepwise dilution of their stock solutions with methanol. The quantitative analysis was performed by the external standard method: the calibration curve for each species was obtained after subjecting a series of standard

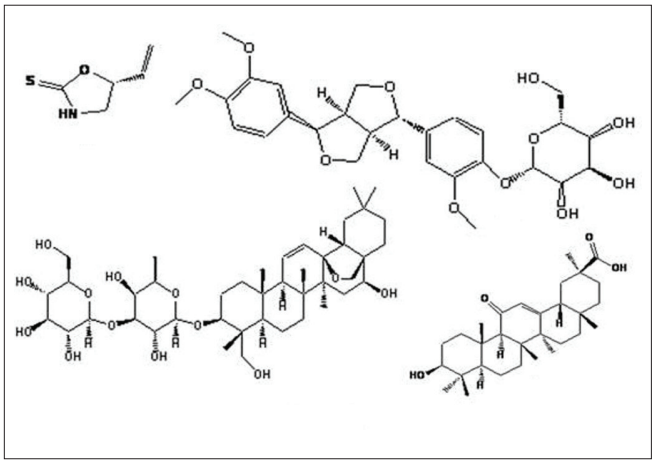

Figure 1: Chemical structures of the reference compounds: a. epigoitrin; b. phillyrin; c. saikosaponin A; d. glycyrrhetinic acid

solutions to the same analytical procedure. The calibration curve for each analyte was drawn by simple linear regression of each analytes concentration versus its peak area, and the calibration curve was used to calculate the concentration of analyte in the samples.

Calculation of the correlation coefficient

The correlation coefficients of entire chromatograms among samples were calculated and the correlation coefficient is expressed by the following formula:[17]

$$\gamma_{ij} = \sum_k (x_{ik} - m_i) \times (x_{ik} - m_j) / \sqrt{\sum_k (x_{ik} - m_i)^2} \times \sqrt{\sum_k (x_{ik} - m_j)^2} \quad k = 1, 2, ..., n$$

Where, x_{ik} and x_{ik} are the kth elements in two different fingerprints; say x_i and x_j, respectively, and n is the number of the elements in the fingerprints. m_i and m_j are the mean values of the n elements in fingerprints x_i and x_j, respectively, that is,

$$m_i = \sum_k x_{ik} / n, k = 1, 2, ...n$$

$$m_j = \sum_k x_{jk} / n, k = 1, 2, ...n$$

Here, γ_{ij} is more closed to 1, the two fingerprints are more similar.

RESULTS AND DISCUSSION

Selection of the detection wavelength

The experiment chose three wavelengths including 210, 225, and 245 nm to examine the separation ability of the chromatographic peaks and the peak areas of the chemicals in the samples [Figure 2]. The higher sensitivity to most of the chemicals as well as the highest sensitivity to the standards was obtained when using 210 nm. Therefore, the 210 nm was chosen as the final detection wavelength for the following study.

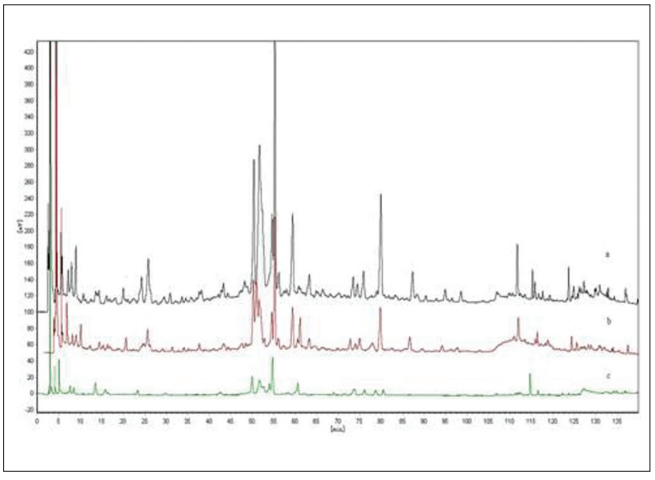

Figure 2: Chromatograms of the extraction sample at 210, 225, and 245 nm wavelengths. (a) 210 nm; (b) 225 nm; (c) 245 nm

Choosing the mobile phase

The chromatography showed large tailing peak when using acetonitrile and water system, while the tailing peak was greatly reduced after adding 0.5% phosphoric acid to the water phase. It was found that the peaks were unable to be completely separated when using different isocratic elution to modify the chromatography, while the separation of the peaks was well improved if the gradient elution was chosen. In this study, a binary gradient elution system consisting of acetonitrile (A) and 0.5% (v/v) phosphoric acid aqueous solution (B) (pH 3.0) was used (0 ~ 100 min, 5 ~ 30% A; 100 ~ 120 min, 30 ~ 50% A; 120 ~ 140 min, 50 ~ 100% A) to obtain relative good resolution [Figure 3].

Selection of the chromatography peak

The 20 peaks (Figure 3b) were chosen as fingerprint peaks of the Tuirejieduling granules extractions.[18]

Methodology validation

The analytical method of binary fingerprinting has been

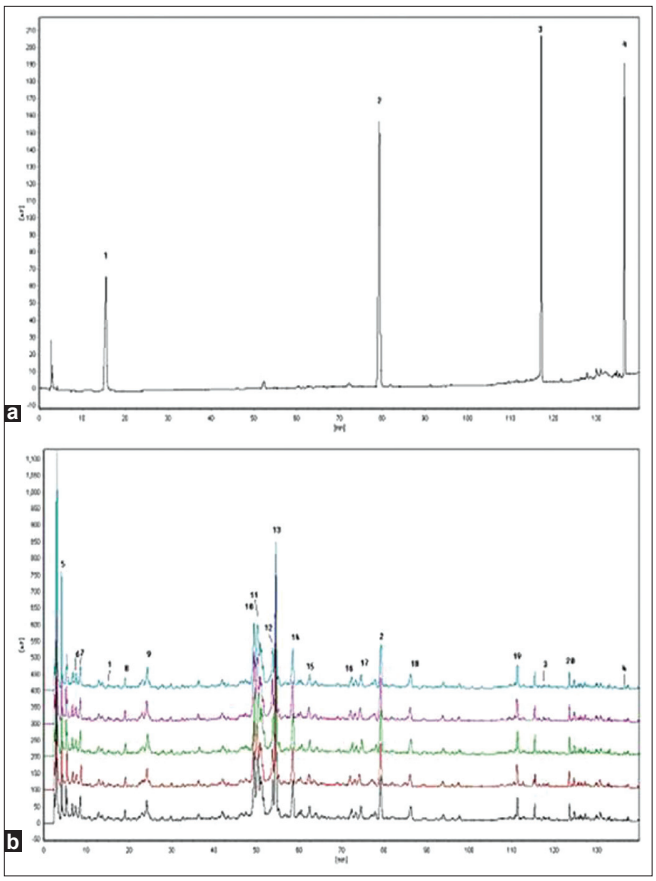

Figure 3: Chromatograms of the standard compounds (a) and the Tuirejieduling granules; (b) The compound name corresponding to Peak 1-4 are (1) epigoitrin; (2) phillyrin; (3) saikosaponin A; (4) glycyrrhetinic acid

validated based on the retention time and the peak area. The intra- and inter-day precisions of the proposed method were validated with the extraction of the same batch on 1 day ($n = 5$) and on five continuous days (one time a day), respectively. The results, which clearly demonstrated the reproducibility of the sample preparation, are listed in Table 1

The intra-day precisions were within the range of 0.03-0.98% ($n = 5$) for retention times and 0.70-2.86% ($n = 5$) for peak areas, while the inter-day precisions of the fingerprint were 0.09-1.00% ($n = 5$) for retention times and 1.34-3.63% ($n = 5$) for peak areas, respectively.

Linear, limits of detection and quantification

Five concentration gradients of standard compounds were injected in triplicate, and the calibration curves were obtained. Limit of detection (LOD) and quantification (LOQ) under the optimal chromatographic conditions were determined at signal-to-noise ratios (S/N) of 3 and 10, respectively. As can be seen in Table 2, good linear (coefficient of determination R^2>0.99) was achieved in the proportional bands for all target compounds.

Table 1: Analytical method validation results

Peak No.	RSD of retention time (%)		RSD of peak area (%)	
	Intra-day (n = 5)	Inter-day (n = 5)	Intra-day (n = 5)	Inter-day (n = 5)
1	0.40	0.46	1.73	3.36
2	0.38	0.60	0.91	3.63
3	0.64	0.90	2.45	3.21
4	0.42	0.86	2.44	3.21
5	0.98	1.00	1.18	3.61
6	0.58	0.99	2.10	2.65
7	0.53	0.83	2.72	2.89
8	0.45	0.73	1.64	1.34
9	0.55	0.64	1.52	1.60
10	0.56	0.63	2.46	2.61
11	0.53	0.52	2.53	3.42
12	0.48	0.52	2.86	3.02
13	0.38	0.79	1.98	3.54
14	0.35	0.88	1.78	2.54
15	0.29	0.45	1.08	1.71
16	0.37	0.40	1.35	2.60
17	0.14	0.16	2.76	3.05
18	0.11	0.12	0.70	2.39
19	0.08	0.10	1.57	2.58
20	0.03	0.09	1.68	3.37

Recovery Study

In order to evaluate the accuracy of the method, a known amount of the four standard chemicals were spiked into 1.00 g Tuirejieduling granules, and then subjected to the optimal extraction procedure as described above. Extraction solution was analyzed by the optimized HPLC conditions and the results are shown in Table 3

Calculation of the Similarity

In this study, the fingerprint of the batch of 081201 was chosen as the standard fingerprint (x_{jk}). The similarities of the other batches of Tuirejieduling granules; compared to the standard fingerprint were calculated according to the described section of calculation of the correlation coefficient. Table 4 shows the similarities of 10 batches of Tuirejieduling granules. The results indicate that the analyzed products show good similarities with correlation coefficients of the ten samples greater than 0.99, suggesting the different batches of Tuirejieduling granules with consistent quality control.

Table 3: Accuracy validation of ultrasonic-assisted extraction of epigoitrin, phillyrin, saikosaponin A and glycyrrhetinic acid in 1.00 g Tuirejieduling granules (means ± SD, n = 3)

Analyte	Addeda (mg)	Determinedb (mg)	RSD (%)	Recovery (%)
Epigoitrin	0	2.42 ± 0.01	0.2	
	2.00	4.41 ± 0.03	0.7	99.9
Phillyrin	0	25.93 ± 0.47	1.8	
	18.00	43.24 ± 0.67	1.5	96.2
Saikosaponin A	0	43.37 ± 0.47	1.1	
	44.00	88.99 ± 2.25	2.5	103.7
Glycyrrhetinic acid	0	3.37 ± 0.06	1.9	
	3.00	6.53 ± 0.20	3.1	105.5

[a]Amount added in 1.00 g Tuirejieduling granules; [b]Amount in 1.00 g Tuirejieduling granules

Table 4: The similarities of 10 batches of Tuirejieduling granules

Batch	Similarities	Batch	Similarities
081201	1.0000	090501	0.9977
090101	0.9996	090601	0.9954
090201	0.9981	090701	0.9978
090301	0.9968	090801	0.9979
090401	0.9982	090901	0.9982

Table 2: Analytical performance of the proposed method

Standard compounds	Calibration	R2	Linear range (mg L-1)	LOD (mg L-1)	LOQ (mg L-1)
Epigoitrin	$y = 7.88 \times 10^4\chi + 6.00 \times 10^3$	0.9979	0.39-25.00	0.08	0.23
Phillyrin	$y = 1.26 \times 10^5\chi - 1.22 \times 10^4$	0.9999	0.70-45.00	0.09	0.26
Saikosaponin A	$y = 4.69 \times 10^3\chi + 3.50 \times 10^3$	0.9998	6.88-440.00	0.53	1.75
Glycyrrhetinic acid	$y = 3.08 \times 10^4\chi - 0.04$	0.9999	0.95-61.00	0.06	0.19

Table 5: Content determination (means ± SD)

No.	Epigoitrin (%)	Phillyrin (%)	Saikosaponin A (%)	Glycyrrhetinic acid (%)
1	0.0232 ± 0.0002	0.2528 ± 0.0021	0.4328 ± 0.0065	0.0194 ± 0.0006
2	0.0257 ± 0.0006	0.2959 ± 0.0056	0.3940 ± 0.0010	0.0142 ± 0.0002
3	0.0297 ± 0.0012	0.2840 ± 0.0001	0.4251 ± 0.0020	0.0167 ± 0.0003
4	0.0239 ± 0.0001	0.2888 ± 0.0004	0.4366 ± 0.0010	0.0164 ± 0.0002
5	0.0265 ± 0.0003	0.3071 ± 0.0001	0.4371 ± 0.0037	0.0137 ± 0.0002
6	0.0282 ± 0.0002	0.3198 ± 0.0085	0.4322 ± 0.0014	0.0212 ± 0.0006
7	0.0226 ± 0.0003	0.2832 ± 0.0044	0.4256 ± 0.0044	0.0209 ± 0.0003
8	0.0238 ± 0.0002	0.2718 ± 0.0072	0.4218 ± 0.0022	0.0173 ± 0.0003
9	0.0241 ± 0.0000	0.3247 ± 0.0003	0.4245 ± 0.0020	0.0187 ± 0.0003
10	0.0258 ± 0.0001	0.3274 ± 0.0011	0.4335 ± 0.0030	0.0197 ± 0.0001

Content Determination

The contents of epigoitrin, phillyrin, saikosaponin A and glycyrrhetinic acid in the samples were calculated by the external standard method. The results are listed in Table 5.

CONCLUSIONS

In the present work, an impersonal, valid and rapid fingerprint analysis method was developed and applied for the quality control of the Tuirejieduling granule. The average fingerprint of 10 batches of samples from different time was obtained, 20 common peaks represents the major constituents of this traditional Chinese medicine prescription. The similarities of 10 batches of Tuirejieduling granule were more than 0.99, which indicates the preparations from different time were consistent. The results demonstrate that the method is feasible for comprehensive quality evaluation of Tuirejieduling granule.

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Cite this article as: Yang A, Wang X, Xiong C, Chen J, Ruan J. Quality control of tuirejieduling granules using high-performance liquid chromatography fingerprint method and simultaneous determination of four main active ingredients. Phcog Mag 2013;9:162-6.

Source of Support: Natural Science Foundation of Hubei Province of China (2008CDB225). **Conflict of Interest:** None declared.