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Full Length Research Paper

Validation of a high performance liquid chromatography (HPLC) method for the quality control of the Traditional Chinese Medicine (TCM) formulation, Traditional Chinese formula Huanglianjiedutang (HLJDT)

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The aim of this study was to optimize a high performance liquid chromatography (HPLC) method for the simultaneous quantification of the four main compounds geniposide, berberine, baicalin and crocin present in the Traditional Chinese formula Huanglianjiedutang (HLJDT). This formula contains Coptidis Rhizoma, Radix Scutellariae, Cortex Phellodendri and Fructus Gardeniae in a ratio of 1.5: 1: 1: 1.5. The decoction of the formula was prepared in the traditional Chinese way. The most suitable boiling time for the decoction turned out to be 60 min. Validation of the method was performed to demonstrate its reproducibility, precision, stability, repeatability, and recovery rate. Excellent behaviours over the investigated concentrations were observed with the values of RSD ≤ 5%. For the estimation of the recovery rate, half of the herbs were boiled together with the pure compounds. As crocin turned out to be an unstable compound its recovery rate could not be determined. The optimized HPLC method provides an accurate and reproducible analysis for the simultaneous determination of geniposide, berberine, baicalin, and crocin in the formulation HLJDT which is a very important tool for the quality control of this Traditional Chinese Medicine (TCM) formula.

Key words: High performance liquid chromatography (HPLC), quantification, validation, huanglianjiedutang (HLJDT), geniposide, berberine, crocin, baicalin.

INTRODUCTION

WHO introduced the use of chromatographic methods for the standardization of medicinal plants and herbal medicinal products. It is also accepted as a strategy for identification and evaluation of the quality of herbal medicinal products. Nowadays the high performance liquid chromatography (HPLC) is the most common analytical method to determine either the quality or the quantity of a compound in the phytochemical evaluation (Anonymous, 1992; Farnsworth et al., 1985). The Traditional Chinese Medicine (TCM) formula Huanglianjiedutang (HLJDT) contains Coptidis Rhizoma, Radix Scutellariae, Cortex Phellodendri, and Fructus Gardeniae in the ratio 1.5: 1: 1: 1.5. The single herbs of this formula are used to treat cold, high fever, gastro-intestinale dysfunctions, jaundice, obstipation, eczema, and epistaxis (Körfers and Sun, 2009).Pharmacological studies demonstrated that the main compounds berberin, baicalin, crocin, and geniposide show several activities on animals such as rabbits, guinea pigs, rats or mice. Baicalin is the major antibacterial active compound of *Scutellaria baicalensis*. It has antiallergic and

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antiinflammatory effects as well as a liver protective activity.

Geniposide and crocin, both isolated from the fruit of *Gardenia jasminoides,* have positive effects on the digestive system of rats and rabbits, for example, liver protection or increased bile secretion. Berberine, being one of the main compounds of *Phellodendron chinense* and *Coptis chinensis*, has strong inhibitory effects *in vitro* on several bacteria such as *Staphyllococcus aureus* or *Diplococcus pneumoniae* (Zhu, 1998). The Pharmacopoeia of the People´s Republic of China provides some information on HPLC methods for Cortex Phellodendri, Fructus Gardeniae, and Radix Scutellariae (Anonymous, 2005). But these HPLC data for the investigations of the single herbs cannot be transferred directly to the analysis of a complex herbal mixture like HLJDT. Literature reports to some extent of analytical parameters on HLJDT. Some analytical parameters of HLJDT are reported in literature to some extent (Shen et al., 2003; Cao and Shen, 2003). In the described methods the peak separation turned out not to be satisfactory. Additionally some of the main compounds of the decoction could not be detected properly. So crocin could not be identified at 240 nm as described in literature as it has its absorption maximum at 420 nm.

An UV-visible diode-array detection (UV-DAD) was needed in order to detect the compounds simultaneously due to the fact that the main compounds have their absorption maxima at different wavelengths. Therefore, it was important to optimize the HPLC method in order to provide a satisfactory peak separation for the quality control. It was also essential to optimize the extraction time of the decoction in order to dissolve a high concentration of active compounds in the aqueous extract. In the complex system of a TCM formula not all of the compounds are stable and soluble in the same ratio.

For the optimization of the extraction procedure the herbal mixture was boiled under different conditions and the results were evaluated. In this study, a chromatographic HPLC fingerprint of HLJDT was developed as a method for the quality control of a complex system. The present study reports of an optimized HPLC method for the simultaneous determination and validation of geniposide, berberine, baicalin and crocin which are the main ingredients of the TCM formulation HLJDT.

MATERIALS AND METHODS

Chemicals and reagents

Methanol, acetonitrile (both of HPLC grade) and phosphoric acid 85% (analytical grade) were purchased from Beijing Chemical Reagents Company. The reference standards were provided as follows: geniposide and baicalin (both purity 91%) were isolated and authenticated by scientists of the Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing. Berberine hydrochloride (purity 98.1%) and crocin-I (purity 98%) were purchased from SFDA Beijing.

Plant material

The bark of *Phellodendron chinensis* SCHNEID. (Cortex Phellodendri; Fam. Rutaceae) as well as the rhizomes of *Coptis chinensis* FRANCH. (Rhizoma Coptidis; Fam. Ranunculaceae) were collected from Sichuan Province, the fruits of *Gardenia jasminoides* ELLIS (Fructus Gardeniae; Fam. Rubiaceae) from Jiang Xi Province, and the roots of *Scutellaria baicalensis* GEORGI (Radix Scutellariae; Fam. Labiatae) from Inner Mongolia autonomous region. All plant material was collected during winter 2008, and authenticated by scientists of the Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing.

Sample preparation

Optimization of the extraction method

The determination of the most suitable boiling time is important because not all of the compounds are stable and not all are dissolved at the same time in an equal ratio into the solvent. Therefore 5 g of the herbal mixture were boiled for 30, 60 and 90 min, respectively, and the main ingredients geniposide, berberine, baicalin, and crocin were quantified by HPLC by means of calibration curves. For the calibration curves the following concentrations were used: geniposide 37.5 to 240 µg/ml, berberine 37.5 to 240 µg/ml, baicalin 5 to 32 µg/ml, and crocin 5 to 32 µg/ml.

Preparation of the decoction

1.5 g Rhizomae Coptidis, 1 g Radix Scutellariae, 1 g Cortex Phellodendri, and 1.5 g Fructus Gardeniae were air-dried, powdered, mixed and decocted with 50 ml water for 60 min without stirring. The extracted solution was cooled and filtered twice through the same cotton. 2 ml of this decoction were filled up with MeOH in a 25 ml volumetric flask. The samples were filtered and used for HPLC analysis.

Preparation of the solution of the analytes

3.21 mg berberine, 3.47 mg crocin, 3.72 mg geniposide, and 3.61 mg baicalin were dissolved in MeOH and transferred into a 10 ml volumetric flask. The solution was made up to volume with MeOH, filtered and used for HPLC analysis.

HPLC analysis

HPLC analysis was carried out on Agilent Technologies 1200 series HPLC instrument with Zorbax C18-RP column (4.6×250 mm; 5 µm). The spectrophotometric detection was performed at 240, 265 and 420 nm, respectively. The sample injection volume was 10 µl. The flow rate was 1.0 ml/min. The mobile phase was H_3PO_4 (0.001 mol) for solvent A and CH_3CN for solvent B.

The binary gradient program consisted of an 8 min gradient system of B, increasing of 5 to 15%, followed by B increasing to 18% from 8 to 15 min. The gradient was changed by increasing B to 20% from 15 to 20 min, and increasing B again to 25% from 20 to 30 min. Then B was maintained at 25% from 30 to 50 min. Finally, B was returned back to 5% at 50.1 min and maintained from

50.1 to 60 min for analysis of the next sample. Peak areas and concentrations were used for the evaluation along with the retention times (Rt).

METHOD VALIDATION

Precision - Injection repeatability

The precision is the ability of a measurement to be reproduced consistently under unchanged conditions. The precision, also called injection repeatability, is one of the parameters in a validation process. It presents the results of repeated measurements of one sample under unchanged conditions by injecting the sample several times (Kazakevich et al., 2007). A decoction of HLJDT was prepared and measured five times during the same day, and under the same conditions at a temperature of 18 ± 5°C. During the whole measurement the sample was kept in the HPLC appliance. The standard deviation of the results from the average was calculated. The percentage RSD should be ≤ 5%.

Repeatability - Analysis repeatability

The repeatability is the variation in measurements taken by a single person or instrument on the same item and under the same conditions. A measurement may be said to be repeatable when the variation is smaller than some agreed limit. For this reason multiple samples have to be prepared and measured by the same analytical method (Kazakevich et al., 2007). Five decoctions of HLJDT were prepared under the same conditions and the main ingredients determined in five separate runs on two consecutive days for estimating the inter-day variation. Each run was performed twice at a temperature of 18 ± 5°C. The repeatability was determined by calculating the percentage deviation observed during the analysis and expressed as percentage RSD.

Stability of the decoction

The stability is defined as a quality of being free from change or variation. The post-preparative stability was investigated to check the stability of the decoction over a certain period of time at room temperature. Therefore the decoction was analysed immediately after preparation, then after 2, 4, 8, 16 and 24 h twice each time. During the whole time, the sample was stored in the HPLC appliance at room temperature (18 ± 5°C). The standard deviation of the four main compounds of the decoction was calculated. The percentage RSD should be ≤ 5%.

Recovery rate

The recovery rate is used to compare the degree between the determined results and the real value or recognized value of the reference by using a certain measuring method. According to guidelines in the TCM the recovery rate for the content determination should be 95 to 105%. If the amount of a compound is difficult to determine the rate may require less than 90% (Wangsheng, 2003).

RESULTS AND DISCUSSION

Optimization of the HPLC method

Chromatographic fingerprints play an important role in the quality control of complex herbal medicines like TCM formulations. Therefore an HPLC system for the separation of the main ingredients of HLJDT as well as for the pure compounds was developed. A satisfactory peak separation was achieved with a mobile phase consisting of acetonitrile and H_3PO_4 (0.001 mol) in a gradient elution. Consequently this HPLC system was selected for the validation procedure. The four main compounds of the decoction were determined by comparison of their retention times (min) with a chromatogram of pure compounds. Furthermore, three different wavelengths (240, 265 and 420 nm) were needed in order to detect the four compounds properly. The retention times (Rt) of the four main compounds were 14.6 min for geniposide, 30.3 min for berberine, 35.3 min for crocin, and 35.6 min for baicalin, respectively. Figure 1 shows an HPLC chromatogram of the pure compounds at different wavelengths, whereas the HPLC chromatogram of the decoction is presented in Figure 2.

Optimization of the boiling time

The most suitable boiling time concerning the extraction of the highest amount of main compounds turned out to be 60 min. After this time about 100 µg/ml geniposide, 80 µg/ml berberine, and 8 µg/ml baicalin could be proved in the aqueous extract. Crocin was not a stable compound. Referring to these results the decoction was boiled for 60 min in the further experiments.

Precision – Injection repeatability

One sample of the decoction was injected five times within one day. The calculated percentage RSD values depicted in Table 1 show that a satisfactory injection repeatability of this RP-HPLC method for geniposide, berberine, baicalin, and crocin was achieved and that the proposed method provided also an acceptable intra-day variation of the four main compounds of HLJDT.

Repeatability – Analysis repeatability

Five samples of HLJDT were injected, each twice, for testing the analysis repeatability of the described RP-HPLC method focusing on geniposide, berberine, baicalin, and crocin as main active ingredients. The statistically calculated RSD values were ≤ 5% (Table 2). Regarding these results an adequate analysis repeat-ability was obtained.

Post-preparative stability of the decoction

The decoction of HLJDT was investigated following a

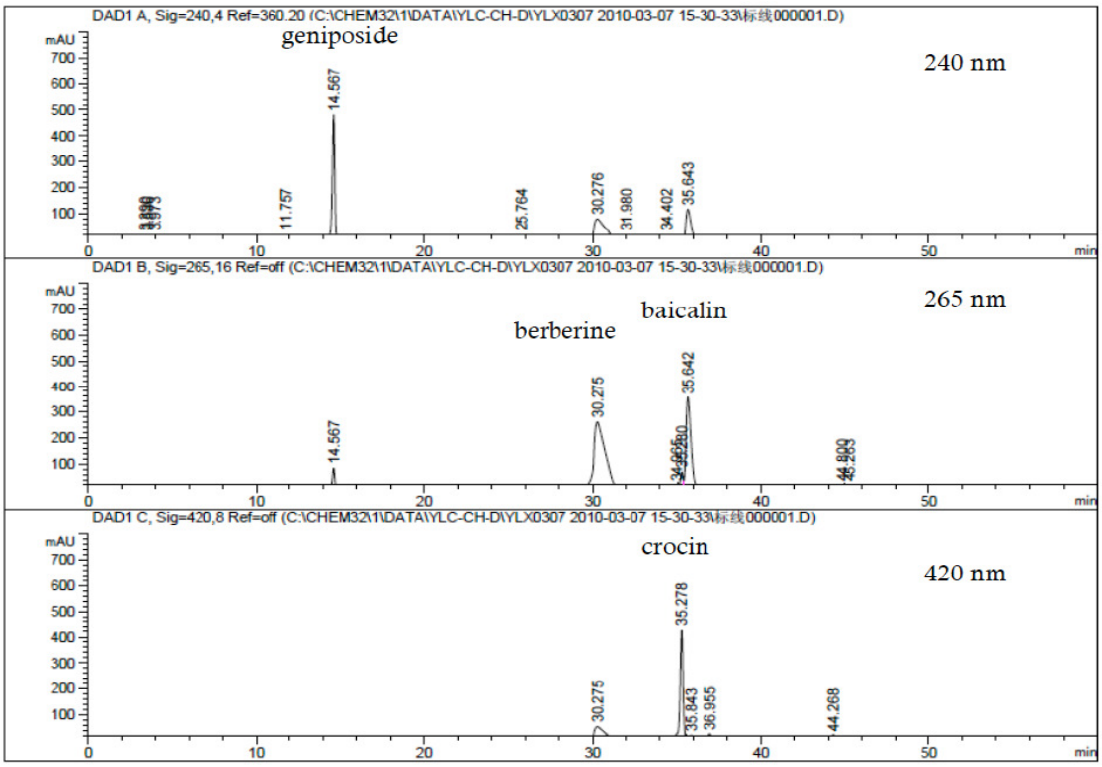

Figure 1. HPLC chromatograms of the pure compounds at different wavelengths GEN = geniposide; BER = berberine; BAI = baicalin; CRO = crocin.

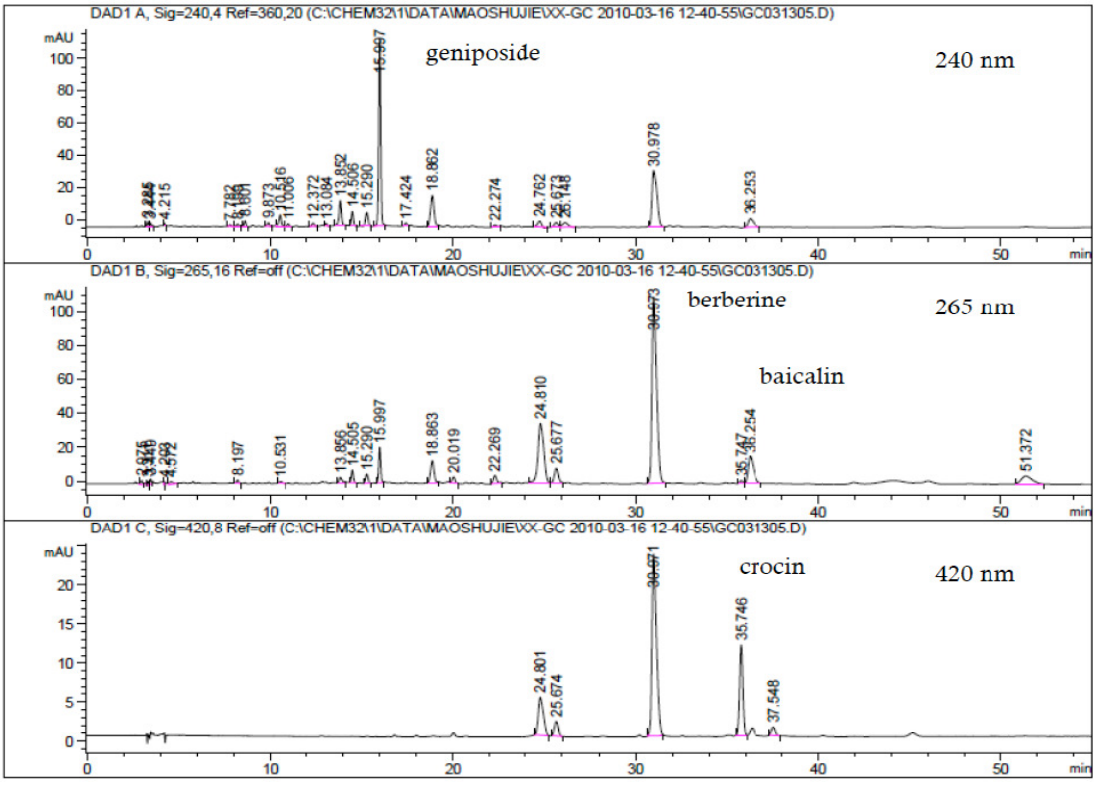

Figure 2. HPLC chromatograms of the decoction HLJDT at different wavelengths. GEN = geniposide; BER = berberine; BAI = baicalin; CRO = crocin.

Table 1. Intra-day precision of the HPLC method (n = 5).

Sample (extract)	Peak areas geniposide	Peak areas berberine	Peak areas baicalin	Peak areas crocin
1	921	1792	369	158
2	922	1808	370	158
3	921	1772	371	159
4	919	1768	371	159
5	909	1748	367	157
Average	918.4	1777.6	369.6	158.2
RSD %	0.584	1.299	0.453	0.529

Table 2. Inter-day repeatability of the HPLC method (n = 5).

Sample (extract)	Average peak areas geniposide	Average peak areas berberine	Average peak areas baicalin	Average peak areas crocin
1	1464	1859	292	199
2	1594	2031	285.5	213
3	1582	1836.5	289.5	197
4	1549.5	1947	295.5	203
5	1594	1796	307	206.5
Average	1556.7	1893.9	293.9	203.7
RSD %	3.528	4.989	2.784	3.122

Table 3. Post-preparative stability test of the decoction (n = 6).

Time (hours)	Average peak areas geniposide	Average peak areas berberine	Average peak areas baicalin	Average peak areas crocin
0	921.5	1800	369.5	158
2	920	1770	371	159
4	910	1749	365.5	155.5
8	918	1751.5	368.5	158
16	919	1753.5	364.5	159
24	919.5	1747.5	365.5	159
average	918	1761.2	367.4	158.1
RSD %	0.445	1.155	0.712	0.858

special study design. The first sample was measured immediately after the preparation of the decoction, followed by five fixed dates within 24 h (after 2, 4, 8, 16, and 24 h). The sample was analyzed twice each time. The calculated RSD values were ≤ 5% (Table 3). So the decoction was considered to be stable for at least 24 h.

Conclusion

The optimized HPLC method provides a simple, accurate and reproducible quantitive analysis for the simultaneous determination of geniposide, berberine, baicalin, and crocin in HLJDT. The application of the described HPLC system demonstrated clearly that the proposed method was reasonable and reliable and it was user-friendly. With its high separation degree it might chemically represent characteristic components of HJLDT. Therefore this RP-HPLC analysis could be utilized as a quality control method for the TCM formulation HLJDT.

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