Simultaneous determination of 10 components in traditional Chinese medicine Dachaihu Granule by reversed-phase-high-performance liquid chromatographic-diode array detector

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ABSTRACT

Background: Dachaihu Granule, commonly used for treating cholecystitis, is derived from a famous traditional Chinese formula named Dachaihu Decoction. No analytical method has been reported for simultaneous determination of 10 bioactive compounds for quality control in Dachaihu Granule so far. Objective: To develop a high-performance liquid chromatographic (HPLC) method with diode array detector (DAD) for simultaneous determination of 10 bioactive compounds (paeoniflorin, aloe-emodin, rhein, emodin, chrysophanol, physcion, naringin, hesperidin, neohesperidin, and baicalin) in traditional Chinese medicine Dachaihu Granule. Materials and Methods: The samples were separated on a Kromasil C18 (250 × 4.6 mm,i.d. with 5.0 µm particle size) column with multi-wavelength detection method by a gradient elution using acetonitrile (A) and 0.2% acetic acid (B) as the mobile phase. The column temperature was maintained at 30°C and the detection wavelength was set at 230 nm for paeoniflorin, 254 nm for aloe-emodin, rhein, emodin, chrysophanol, and physcion, 280 nm for naringin, hesperidin, neohesperidin, and baicalin. Results: The developed method provided satisfactory precision and the accuracy of this method was in the range from 94.0% to 103.1%, all of the 10 compounds showed good linearity (r > 0.999) in a detected concentration range. Conclusion: The validated method was successfully applied to the simultaneously of these active components in Dachaihu Granule from different production batches. Key words: Dachaihu granule, high-performance liquid chromatographic-diode array detector, multi-components, multi-wavelength, quality control

INTRODUCTION

Dachaihu Granule (DCHG) is derived from a well-known traditional Chinese formula named “Dachaihu decoction”, which was founded by Zhongjing Zhang, a famous medical scientist during the Eastern Han Dynasty of China. The formula is a combination of eight medicinal materials, including Baptisia Radix, Scutellariae Radix, Rhei Radix et Rhizome, Aurantii Fructus Immaturus, Paeoniae Radix Alba, Pinelliae Rhizoma Praeparatum Cum Zingibere et Alumine, Zingiberis Rhizome Recens, and Jujubae Fructus. Recent study showed that DCHG has a significant effect on curing acute or chronic cholecystitis in clinical practice.[1-2] Pharmacological studies and clinical practice also have demonstrated that DCHG has many biological functions, such as antibacterial, antiulcer, preventing atherosclerosis, and protecting liver and gallbladder.[3-7] Moreover, it is also an effective adjunctive therapy for the cure of pediatric high fever, gastritis, and pancreatitis.[8-10] However, it is not yet clear what the bioactive constituents and mechanisms of DCHG are, although it is known that DCHG has numerous and diverse compounds, including flavone, saponin, terpene, coumarin, anthraquinones, etc.

As we know, paeoniflorin (PA) has a well known effect on treating inflammation.[11] Aloe-emodin (AE), rhein (RH), emodin (EM), chrysophanol (CH), and physcion (PH), which were extracted from Rhei Radix et Rhizome, were proved to be effective components of antiinflammatory, protecting liver and gallbladder.[12-13] Flavone compounds including naringin (NA), hesperidin (HE), neohesperidin (NE), and baicalin (BA)[14-17] also have bioactivity, and...
their chemical structures are shown in Figure 1. Generally, NA, HE, and BA were believed to be the main active constituents and were chosen as marker compounds for the quality evaluation and standardization of DCHG. However, due to multiple compounds that might be associated with the therapeutic functions, a single or a few marker compounds could not be responsible for the overall pharmacological activities of DCHG. Therefore, it is urgently needed to establish a comprehensive quality evaluation method based on analysis of the whole bioactive compounds in order to accurately control the quality of this herbal drug.

Previously, BA from DCHG was analyzed and three marker constituents (NA, HE, and BA) from Dachaihudocetion were simultaneously analyzed with iso-gradient method for the quality control of the medicine.\(^{18-19}\) Although HPLC methods have been applied to determine some of the constituents in crude drugs and Chinese patented medicine,\(^{20-22}\) no analytical method has been reported for simultaneous determination of 10 major constituents in DCHG. Hence, it is very important to establish a method for quality control of these bioactive compounds, which could help to evaluate the quality of the herbal formula. In this study, the method of HPLC-DAD has been developed for the simultaneously qualitative and quantitative analysis of 10 bioactive compounds: PA, AE, RH, EM, CH, PH, NA, HE, NE, and BA.

**MATERIALS AND METHODS**

**Chemical and reagents**

Sample DCHG was supplied by Nantong Essence Pharmaceutical Co., Ltd. (Jiangsu, China). Standard substances including PA, AE, RH, EM, CH, PH, NA, and BA were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Standard substances including NA and NE were purchased from the Nanjing ZeLang Medicine Photochemistry Technology Co., Ltd. (Jiangsu, China), the purity of these reference compounds were determined to be more than 98% by HPLC analysis. The HPLC grade methanol and acetonitrile were purchased from Tedia (Fairfield, USA). Acetic acid (analytical reagents) was purchased from LingFeng Chemical Reagent Company (Shanghai, China). Other reagents were all of analytical grade. Ultra pure water was obtained in a Milli-Q system (Milford, MA, USA).

**Instrument and chromatographic conditions**

The HPLC system 1200 series (Agilent Technologies, USA) equipped with the Chem Station software (Agilent Technologies) and comprised of a double solvent delivery pump, an online vacuum degasser, an auto sampler, a thermostatic compartment and a diode array detector, were used for the chromatographic analysis. All separations were carried out on a Kromasil C\(_{18}\) column (250 × 4.6 mm i.d. with 5.0 \(\mu\)m particle size) from Hanbang Science and Technology (Jiangsu, China). Mobile phase A was 0.2% (v/v) acetic acid aqueous solution and phase B was acetonitrile. The elution was performed using a linear gradient of 5–19% B at 0–13 min, 19–26% B at 13–28 min, 26% B at 28–36 min, 26–40% B at 36–50 min, 40–85% B at 50–69 min, 85% B at 69–75 min. The flow rate was 1.0 mL/min and column temperature was maintained at 30°C, detect wavelength was set at 230 nm for PA, 254 nm for AE, RH, EM, CH, and PH, and 280 nm for NA, HE, NE, and BA. The injection volume was 10 \(\mu\)L. The peak identification was based on the retention time and the DAD spectrum against the standard presented in the chromatogram.

**Standard solution preparation**

The standard stock solutions of PA 0.1254, AE 0.0092, RH 0.0212, EM 0.0097, CH 0.0191, PH 0.0101, NA 0.1938, HE 0.1096, NE 0.1287, and BA 0.4525 mg/mL were prepared in methanol and stored away from light at 4°C. Working solutions of the low concentration were prepared by appropriate dilution of the stock solution.
Sample solution preparation
The powder of DCHG (about 1.0 g) was extracted with 25.00 mL methanol for 30 min in an ultrasonic bath for one time. Adding up the loss of weight by methanol after cooling, then, the solution was filtered through a syringe filter (0.45 µm) before being injected into the HPLC system for analysis.

RESULTS AND DISCUSSION

Chromatographic separation
In order to optimize the extraction conditions for achievement of quantitative extraction, variables involved in the procedure such as solvent, extraction method and extraction time were optimized. Pure and aqueous methanol or ethanol solutions also were tried as the extraction solvent, the best solvent was found to be pure methanol which gave rise to optimum extraction of all the 10 components. Compared with refluxing extraction, the ultrasonic treatment procedure was found to be the better extraction method for all the 10 components. In order to investigate extraction time, powdered DCHG samples were extracted with 25 mL pure methanol 20, 30, 45, and 60 min, respectively. The results suggested that all the 10 components were almost completely extracted by pure methanol at one time for 30 min.

Due to the complex composition of the sample solution, different mobile phases (methanol–water, methanol–water–acetic acid, acetonitrile–water, and acetonitrile–water–acetic acid) were attempted to elute the investigated 10 components. In order to enhance resolution and eliminate tailing of the peaks of the target compounds, formic acid and acetic acid were added in the mobile phase. Considering the total resolution of the chromatographic separation, the running time and solvent consumption, the mobile phase acetonitrile–water–acetic acid was chosen for the separation. The typical chromatographic profiles of the blank, standard solution, and the real sample solution were shown in Figure 2. On the basis of the absorption maxima of the 10 compounds in UV spectra acquired by use of the diode array detector, the monitoring wavelength was set at 230 nm for PA, 254 nm for AE, RH, EM, CH, and PH, and 280 nm for NA, HE, NE, and BA, respectively. The detections at three wavelengths were carried out to improve the sensitivity and selectivity for the quantitative analysis.

Linearity, range, and limits of detection
Integrated chromatographic peak areas (Y) were plotted against the corresponding concentrations (X µg/mL) of the 10 constituents in the standard solutions to obtain calibration curves based on linear regression analysis. The regression curves were obtained from six concentration levels and all of them had good linearity (r >0.999) in the investigated ranges [Table 1]. The working solutions of the analysis were further diluted with methanol to yield a series of appropriate concentrations. The limits of detection (LOD) and the limits of quantization (LOQ) for each investigated compounds can be seen in Table 1.

Precision, repeatability, and stability
The injection of continuous six times using the same sample was employed for the injection precision, and the injection of six different samples, which were obtained through the same sample preparation procedure, was used for the analysis repeatability. Furthermore, the test of injection precision was determined by the standard solutions, and the test of analysis repeatability was investigated by the real sample solution. The instrument precision was examined by the performance of the intra-day and inter-day assays by six injections of the mixture standard solutions used above. The intra-assay precision was performed with the interval of 2 h in 1 day, and the inter-assay precision was performed over 3 days. The precision result of the solution is presented in Table 2, and it was shown that the RSD(relative standard deviation) values of retention time were lower than 0.25%, while the RSD values of peak area were lower than 2.5% both for the intra-assay and inter-assay precision. Results of
injection repeatability of the solution are shown in Table 3, and the RSD values were lower than 3%. The same real sample was analyzed within 24 h at 30°C for the stability test, and the solution was found to be stable while RSD values of the retention time and peak area were both lower than 2.0%.

**Accuracy**

Neither a standard method for the determination of these active components nor a standard reference had been established because of the complexity of Chinese medicines. So accuracy of the standard from samples is generally used to evaluate the accuracy of the newly developed analytical method. Three different quantities (low, medium, and high) of the authentic standards were added into the known real sample. The mixtures were extracted as sample preparation described above, and was analyzed using the developed HPLC method mentioned above. Then the quantity of each component was subsequently achieved from the corresponding calibration curves. The recovery of the investigated components ranged from 94.0% to 103.1%, and their RSD values were all less than 3.0% [Table 4]. It was known from the recovery tests that this developed method manifested the reliability and accuracy for the measurement of these components.

**Sample analysis**

For the simultaneous determination of 10 major components in Chinese medicine DCHG from different production batches, the developed HPLC method was used by comparing the retention time with those of standards. The amounts of the 10 compounds in the samples were then calculated. The results shown in Table 5 indicate that the content of anthraquinones was lower than other components between batches. However, the content of some components such as NA, HE, NE, and BA were higher than other compounds. Therefore, the simultaneous determination of all the components contained in DCHG is necessary to improve the quality control of this drug.

**CONCLUSION**

RP-HPLC with DAD was found to be effective for the simultaneous detection and determination of 10 major

| Component | Regression equation | $r^2$ | Liner range (µg/ml) | LOD (µg/ml) | LOQ (µg/ml) |
|-----------|---------------------|-------|---------------------|-------------|-------------|
| PA        | Y = 7.0667 X + 17.367 | 0.9990 | 25.1–250.9         | 0.04        | 0.12        |
| AE        | Y = 18.617 X + 2.8467 | 0.9990 | 1.83–18.3          | 0.01        | 0.03        |
| RH        | Y = 14.698 X - 0.9508 | 0.9999 | 4.23–42.3          | 0.23        | 0.61        |
| EM        | Y = 15.885 X - 0.0522 | 0.9999 | 1.93–19.3          | 0.12        | 0.37        |
| CH        | Y = 19.943 X + 2.8487 | 0.9999 | 3.83–38.3          | 0.24        | 0.65        |
| PH        | Y = 12.398 X + 1.7793 | 0.9999 | 2.02–20.2          | 0.14        | 0.41        |
| NA        | Y = 8.9243 X - 1.4227 | 0.9990 | 38.7–387.6         | 0.02        | 0.07        |
| HE        | Y = 7.1781 X + 7.7818 | 0.9999 | 21.9–219.2         | 0.04        | 0.13        |
| NE        | Y = 9.4416 X + 11.054 | 0.9999 | 25.7–257.4         | 0.03        | 0.10        |
| BA        | Y = 12.324 X + 50.139 | 0.9999 | 90.5–905.0         | 0.01        | 0.03        |

Note: All the abbreviations as seen in Figure 1.

| Components | RSD of retention time (%) | RSD of peak area (%) |
|------------|---------------------------|----------------------|
| PA         | 0.14                      | 0.23                 |
| AE         | 0.09                      | 0.22                 |
| RH         | 0.03                      | 0.17                 |
| EM         | 0.04                      | 0.23                 |
| CH         | 0.05                      | 0.14                 |
| PH         | 0.04                      | 0.04                 |
| NA         | 0.03                      | 0.06                 |
| HE         | 0.07                      | 0.12                 |
| NE         | 0.05                      | 0.14                 |
| BA         | 0.08                      | 0.11                 |

Note: All the abbreviations as seen in Figure 1.
bioactive constituents (PA, AE, RH, EM, CH, PH, NA, HE, NE, and BA) in Chinese traditional medicine DCHG. This is also the first report of an accurate and reliable analytical method for the simultaneous determination. High linearity, repeatability, precision, accuracy and reliability were presented in the method validation procedure. The proposed method is promising to improve the quality control of DCHG.

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