Short communication

Combinative method using HPLC fingerprint and quantitative analyses for quality consistency evaluation of an herbal medicinal preparation produced by different manufacturers

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1. Introduction

Yiqing is a medicinal preparation derived from the well-known traditional Chinese medicine (TCM) formula named San Huang Xie Xin (SHXX) decoction, and is commonly used in clinical practice for the treatment of pharyngitis, amygdaatitis, constipation, hot flush, stomach disorders, and irritability [1–3]. Yiqing is composed of three Chinese medicinal herbs including Rhizoma Coptidis (Coptis chinensis), Radix et Rhizoma Rhei (Rheum officinale) and Radix Scutellariae (Scutellaria baicalensis). The composition of these herbs can vary depending on geographical source, cultivation conditions, harvest time, storage, and pretreatment. This variation makes it difficult to maintain quality consistency for herbal preparations such as Yiqing both from batch to batch and from manufacturer-to-manufacturer [4].

Unlike the synthetic drugs, medicinal herbs and their preparations exert the curative effects based on the synergic effects of their multi-components and multi-targets. Often, a few chemical markers or bioactive constituents are selected to assess the quality of the complex preparations. This strategy has proved to be insufficient for the quality control of herbs and their preparations because it does not evaluate all chemical components present in the chromatographic profile. Chromatographic fingerprinting has been used over the last decade for the authentication and quality control of herbs [5,6]. Chromatographic fingerprinting techniques can be used to characterize both the marker compounds and the unknown components in a complex system, a strategy recommended to assess the quality and consistency of botanical products by US Food and Drug Administration (FDA), the European Medicines Evaluation Agency (EMEA), and State Food and Drug Administration of China (SFDA) [7–9]. This strategy, however, is not sufficient to control the overall quality of botanical products because it does not quantitate active components [10,11]. Recently, some combinative methods using HPLC fingerprinting and quantitative determination have been developed and validated for quality control of herbal preparations [11,12]. To the best of our knowledge, few reports have been published on quality consistency evaluation of herbal preparations from different manufacturers using such combinative methods [12].

There are over 40 manufacturers that produce Yiqing preparations in either granule or capsule across China. Several HPLC methods so far have been developed for quantitative analysis of...
one or more chemical markers in *Yiqing* capsules [13–16] or SHXX decoction [17]. To date, little is known about the quality consistency of this medicine from different manufacturers. In the present study, a combinative method using HPLC fingerprint and quantitative determination was developed to analyze nine bioactive compounds from *Yiqing* preparations and to assess the quality consistency among 12 manufacturers.

## 2. Experimental

### 2.1. Materials and chemicals

Fifteen samples of *Yiqing* preparations from twelve manufacturers were collected and summarized in Table 1. Chemical standards of berberine, aloe-emodin, rhein, emodin, chrysophanol, baicalin, baicalein and wogonin were purchased from the Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), and wogonoside was obtained from Shanghai Shunbo Bioengineering Co., Ltd (Shanghai, China). HPLC grade methanol and phosphoric acid were purchased from Tedia (Fairfield, OH, USA). Reverse osmosis water was generated from a Millipore ultrapure water system (18 M, simplicity 185, Millipore, France). All other chemicals and reagents were of analytical reagent grade and used without further purification.

### 2.2. Apparatus and chromatographic conditions

An Agilent 1100 series LC system consisting of a G1312A binary pump, a G1322A degasser, a G1313A autosampler, a G1316A thermostatted column compartment, and a G1315A DAD detector (Agilent, Palo Alto, USA) was used to achieve HPLC fingerprints and quantitative chromatograms. The chromatographic separation was carried out on a Calesil C18 column (250 mm × 4.6 mm, 5 μm; Promptar Co., Ltd., China) maintained at 30 °C. The mobile phase was methanol (A) and 0.2% phosphoric acid aqueous solution (B) with a gradient program as follows: 0–5 min, linear gradient 55–60% B; 5–42 min, linear gradient 60–10% B; and 42–60 min, isocratic 10% B at a flow rate of 0.8 mL/min. The UV absorbance was monitored at 254 nm using DAD. All injection volumes of sample and standard solutions were 10 μL.

### 2.3. Sample and standard solution preparation

The sample extracts were prepared by the method of weight relief, where the weight loss of solvent during the extraction procedure is compensated for. In brief, 0.5 g powder of *Yiqing* capsule (or 0.75 g powder of Yiqing granule) was extracted by ultrasonication for 30 min with 25 mL methanol. Additional solvent was then added to bring the weight to the pre-extraction weight, and the extracts were filtered with a 0.45 μm membrane filter prior to HPLC analysis.

Each standard was accurately weighed and then dissolved in absolute methanol to produce stock standard solutions. A mixed working standard solution was then prepared in methanol from the individual standard stock solutions. All the solutions were stored in a refrigerator at 4 °C and brought to room temperature before use. The sample extraction and standard solutions were filtered through 0.45 μm filters prior to HPLC analysis.

### 2.4. Similarity calculation of the HPLC fingerprints

All determinations were carried out in triplicate, and the data were analyzed by the Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine software (Version 2004A) which was developed by Chinese Pharmacopoeia Committee. This software evaluates similarity based on correlation coefficient calculations for fingerprint chromatograms [5,18].

## 3. Results and discussion

### 3.1. Optimization of extraction conditions

Sample pretreatment conditions were first optimized by investigating the effect of extraction solvents and methods on the extraction efficiencies for different classes of chemical markers used for HPLC fingerprinting and quantification. For example, rhein, aloe-emodin, emodin and chrysophanol are anthraquinones; baicalein and wogonin are flavones; wogonoside and baicalin are flavone glycosides; and berberine is an alkaloid (Fig. 1). In the preliminary studies for the selection of extraction solvents (20%, 70%, 100% methanol, and 0.1% HCl in methanol; 50%, 100% ethanol, 0.1% HCl in 50% aqueous ethanol, and 0.1% HCl in ethanol), methanol was found to be the most effective solvent for extracting all compounds based on the HPLC results (data not shown). Extraction methods including ultrasonication and heat-reflux were then investigated for extraction efficiencies for marker compounds using methanol as the extraction solvent. The HPLC peak areas of the marker compounds obtained from these two techniques were comparable, and ultrasonication was chosen because of its technical simplicity and rapid performance. Extraction time under ultrasonication was also investigated, and the results showed that all the marker compounds were extracted within 30 min, and that longer period of ultrasonication did not increase the contents significantly. The optimal
3.2. Optimization of HPLC chromatographic conditions

HPLC conditions including mobile phase and detection wavelength were investigated for optimization of chromatographic separations for marker compounds. The effect of mobile phase composition on chromatographic separation was first investigated. No differences were observed between methanol–water and acetonitrile–water. Considering the higher toxicity and price of acetonitrile, the binary mixture of methanol–water was chosen, and 0.2% (v/v) phosphoric acid was added to improve the peak shape.

The wavelength for the detection of the target compounds in the preparation was selected by a DAD full wavelength scan (200–400 nm) and data reported in the literature [14]. Three wavelengths, 254 nm (for anthraquinones), 278 nm (for flavones) and 345 nm (for alkaloids) were selected for comparison of peak numbers and resolution. Most chemical constituents in Yiqing had the best responses at 254 nm, especially for 30–60 min in which six marker compounds showed greatest absorbance. Therefore, 254 nm was chosen to be the best wavelength for the detection of all nine investigated compounds. Optimal HPLC condition used in this study are shown in Section 2.2.

3.3. HPLC fingerprints of Yiqing preparations

Using the optimized HPLC method, separation of the nine chemical markers and a typical HPLC chromatogram are shown in Fig. 2. The major peaks showed satisfactory resolution except peaks 16 and 17. Chromatographic fingerprint were generated for 15 Yiqing samples from 12 manufacturers, and about 40 peaks were found in each individual sample (Fig. 3A). A simulative median fingerprint, also known as a mean chromatographic fingerprint, was generated by the professional software by analyzing all the 15 samples (Fig. 3B). The simulative median chromatogram of Yiqing had 22 well-resolved “characteristic peaks”. Peak 6 (baicalin) is an important active component of Yiqing with a consistently high content and was chosen to calculate the relative retention time (RRT) and relation peak area (RPA). RRT and RPA of the 22 characteristic peaks in 15 samples are shown in Table 2.

Chromatographic profiles were generally consistent although the absorption intensity of some peaks and the number of peaks were slightly different for some samples. The similarity of each chromatograph against simulative median chromatogram was cal-
Fig. 2. The typical HPLC chromatographic profile of nine standards. The peaks marked with 3, 6, 10, 15, 16, 17, 19, 21 and 22 are berberine, baicalin, wogonoside, baicalein, wogonin, aloe-emodin, rhein, emodin and chrysophanol, respectively. The separation condition was described in Section 2.2.

culated (Table 1). Most of the similarity values in Table 1 were in the range of 0.992–0.997 for samples S1–S9, S12, S13, and S15, indicating that similar chemical components were present in these samples regardless of manufacturer. In contrast to these samples, three samples (S10, S11, and S14) demonstrated low similarity values of 0.871, 0.884 and 0.780, suggesting that these three products were different from those with a high similarity value.

In addition to the inter-manufacturer similarity comparison for Yiqing samples, intra-manufacturer similarity comparisons were also performed by the software. Four batches of Yiqing capsules (S1–S4) obtained from Chengdu Kanghong Pharmaceutical Co., Ltd were analyzed, and the similarity values of S1–S4 were all above 0.90 (Table 1), indicating that the quality of Yiqing capsules produced by this manufacturer was relatively consistent. No obvious difference was observed between the Yiqing capsule and granule preparations. Overall, the data from the chromatographic fingerprints indicated that the principal components of Yiqing preparations were relatively stable.

3.4. Method validation

Calibration curves were generated by plotting peak area (Y) versus the concentrations (X, mg/mL) of the mixed standard solutions. Results, expressed as the values of the correlation coef-

Fig. 3. HPLC chromatographic fingerprints of 15 Yiqing samples (A) and simulative median chromatogram obtained by Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine software (B). The chromatograms marked with S1–S15, and R represents 15 Yiqing samples and the simulative median chromatogram, respectively. The peaks marked with 1–22 in the simulative median chromatogram represent 22 characteristic peaks. The separation condition was described in Section 2.2.
The intra-day and inter-day precision of the method were assessed by six replicate injections of a mixed standard solution in one day and twice a day over three consecutive days, respectively. The RSD values of retention time and peak area of the nine markers were found in the range of 0.08–0.65% and 0.27–1.51%, respectively, in the intra-day precision test. Similar results were obtained in inter-day precision assay. The method repeatability was determined by analyzing six independently prepared solutions of sample S1. The RSD values of retention time and peak area were less than 0.02% and 5.76%, respectively. Recovery was determined by adding an accurately known amount of the corresponding marker compounds at three concentration levels (0.5, 1.0 and 1.2 times of the concentration of the matrix), to a sample of Yiqing that had previously been analyzed, and re-analyzing the sample. Recovery was between 93.1% and 108.5% with RSD values of less than 3.79% for all the nine marker compounds. These validation results indicated that the conditions used in the quantitative determination were acceptable.

3.5. Quantitative determination of the nine marker compounds in Yiqing samples

To determine which herb each marker compound originated from, a comparative study was conducted by using the individual extracts of three herbs. The results of this study and literatures showed that flavonoids including baicalin, wogonoside, baicalein, and wogonin were from S. baicalensis [19], anthraquinone derivatives including rhein, aloe-emodin, emodin, and chrysophanol were from R. officinale [20], and the predominant alkoloid, berberine was from C. chinensis [21]. These components are typically usually used as the markers for quality control of these herbs or their herbal preparations.

In this study, the proposed HPLC-DAD method was successfully applied to the simultaneous determination of the nine markers in Yiqing samples. The identity of the marker compound peaks in the chromatogram was confirmed by their retention times and their UV profiles. The contents of the nine marker compounds in 15 samples from 12 manufacturers were summarized in Table 3. Significant variances among the contents of these markers were observed within one sample. For example, the highest content (61.13 mg/g) was of baicalin and the lowest (0.38 mg/g) was of aloe-emodin in sample S1.
The concentrations of the nine markers were in the order of baicalin > wogonoside > berberine > baicalein > wogonin > rhein > emodin > chrysophanol > aloe-emodin determined in sample S1, which was similar to those previously reported [14]. The same trend was also obtained in samples S2, S3 and S4. In addition, we found that the contents of the nine compounds were consistent in four batches of Yiqing capsules (S1–S4) produced by the same manufacturer. These data suggested that the batch-to-batch consistency of Yiqing preparations was good under the same manufacturing conditions, consistent with results from the chromatographic fingerprints. However, the contents of each individual marker determined between manufacturers, were significantly different with RSD values beyond 100% except for baicalein (36.53%), indicating the large variations in their quality. A possible explanation of the result is that the herbal materials and manufacturing processes applied in manufacturers were quite different. It is noteworthy that quality consistency may not only be monitored by chromatographic fingerprints. For example, relatively higher similarity values (0.995, 0.997, 0.985, and 0.987) were obtained for samples S5, S6, S7, S8, respectively, indicating the good quality consistency of Yiqing produced by four different manufacturers. However, in the quantitative study, the contents of five major compounds such as berberine, baicalein, baicalin, wogonin and wogonoside were significantly different among these four Yiqing samples. The RSD values for contents of the five markers fell in the range of 18.83–50.70%, which indicated that quality consistency of Yiqing was different from factory to factory. Thus, more attention should be paid to quality consistency of Yiqing to ensure its clinical efficacy and safety. Results from this study agree with a previous study that found chromatographic fingerprinting combined with quantitative techniques for determining marker compounds a better tool for quality consistency evaluation of herbal preparations compared to chromatographic fingerprinting alone [22].

4. Conclusions

The proposed HPLC fingerprint method combined with quantitative analyse, is an efficient and comprehensive tool for quality consistency evaluations of Yiqing preparations. HPLC fingerprint of the chromatographic profiles could serve as the first tool for quality consistency of Yiqing by similarity comparison. Furthermore, simultaneous quantification of nine marker compounds from each individual herbas present in Yiqing can be carried out in a single HPLC chromatogram, and used as a supplemental tool for quality consistency evaluation. This is the first report for the manufacturer-to-manufacturer consistency assessment of Yiqing preparations. Overall, this study sets a good example for quality consistency evaluation by using a combination of HPLC fingerprint and quantitative analyses.

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