Quantitative Analysis Combined with Chromatographic Fingerprint for Comprehensive Evaluation of Xiaoer Chaigui Tuire Granules by HPLC-DAD

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Quantitative analysis of eight major components combined with chromatographic fingerprint based on high performance liquid chromatography coupled with diode array detector (HPLC-DAD) was developed for the quality evaluation of Xiaoer Chaigui Tuire granules (XCTG), a traditional Chinese medicine (TCM) preparation. Each compound was analyzed by comparing its retention time and UV spectrum of each chromatographic peak with the corresponding retention time and UV spectrum of each standard compound. Baseline separation was achieved on an Agilent Zorbax SB-C18 column with gradient elution concerning the fingerprint analysis of XCTG. In this article, a chromatographic fingerprint method based on HPLC-DAD was first established for the quality evaluation of XCTG. Moreover, 12 batches of XCTG were analyzed and eight compounds: puerarin, peoniflorin, daidzin, baicalin, daidzein, cinnamaldehyde, baicalein and wogonin were also simultaneously determined. Three similarity algorithms, cluster analysis and principal component analysis (PCA) were all employed for the quality evaluation of different batches of XCTG.

Introduction

Xiaoer Chaigui Tuire granules (XCTG), a traditional Chinese medicine (TCM) preparation, has the efficacy of promoting sweating to release and reduce interior heat in human body. Consequently, it has a powerful effectiveness in the treatment of many diseases including common cold, acute upper respiratory infection and hand-foot-mouth disease in children. More and more attention has been paid on XCTG since 2005 due to its reliable therapeutic efficacy and low side effects (1). The mean pyretolysis time of XCTG was also shorter than that of antiviral ribonucleoside ribavirin (2). XCTG is a compound preparation of TCMs including Radix Paeoniae Alba, Ramulus Cinnamomi, Herba Spiridela and Periostracum Cicadae. Each of them contains many active compounds that may be relevant to its effectiveness. For example, some components such as puerarin, peoniflorin, daidzin, baicalin, daidzein, cinnamaldehyde, baicalein and wogonin exhibit the actions of pyretolysis, anti-inflammatory and antioxidant (3–6).

Although the chemical identification of the single component of XCTG was reported (7–9), the integrated chemical identification of multi-component was not reported. Additionally, XCTG was produced by different pharmaceutical companies with raw herbs from different areas. Therefore, an effective method, which can analyze as many bioactive constituents in XCTG as possible to ensure stability and efficacy in clinical practice, is necessarily developed for its quality evaluation. In general, the chromatographic fingerprint is widely used to assess the batch-to-batch consistency of chemical constituents and to determine the contents of those bioactive compounds in TCMs (10–12). Among various hyphenated approaches applied to quality evaluation of TCMs over the past decade (13–15), high performance liquid chromatography coupled with diode array detector (HPLC-DAD) was the most widely used approach owing to its convenience, efficiency and popularity (16).

To our best knowledge, there were no research works concerning the fingerprint analysis of XCTG. In this article, a chromatographic fingerprint method based on HPLC-DAD was first established for the quality evaluation of XCTG. Moreover, 12 batches of XCTG were analyzed and eight compounds: puerarin, peoniflorin, daidzin, baicalin, daidzein, cinnamaldehyde, baicalein and wogonin were also simultaneously determined. Three similarity algorithms, cluster analysis and principal component analysis (PCA) were all employed for the quality evaluation of different batches of XCTG.

Experimental

Chemicals and materials

HPLC grade acetonitrile were purchased from Fisher (New Jersey, USA). Deionized water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Other chemicals were commercially available and used as received.

Reference substances including puerarin, peoniflorin, daidzin, baicalin, daidzein, cinnamaldehyde, baicalein and wogonin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The structures of these standards were shown in Figure 1. The purities of all the standards were above 98% by HPLC analysis. Twelve batches of XCTG were produced by two pharmaceutical companies in China (Table 1).

Standard solutions and sample preparation

Stock solutions of eight reference substances were prepared in the concentration ranging from 3.6 to 124.0 µg/mL in methanol and stored at 4°C until use. The mixed standard working solution was obtained by diluting stock solutions to desired concentrations.

Each sample of XCTG was pulverized into fine powder. The powder (ca. 1.0 g) was accurately weighed and extracted ultrasonically with 25 mL of methanol for 30 min. After adding appropriate amount of solvent to make up the lost weight, the extraction was filtrated through 0.45-μm membrane filter into an HPLC sample vial for HPLC analysis.
Results
Optimization of sample preparation and chromatographic condition

To obtain as many peaks as possible in chromatographic fingerprint, extraction time and solvents (ethanol or methanol) were investigated. According to HPLC fingerprints detected, extraction with 25 mL of methanol for 30 min was adopted because the fingerprints had clear characteristics as follows: more peaks with high response, little interference and good peak shape and resolution.

The chromatographic conditions were optimized with respect to the mobile phase systems, the wavelength and the column temperature (25, 30 and 40°C). Methanol or acetonitrile was chosen as the solvent of mobile phase. A small amount of acid was added into the mobile phase to improve the peak shape and restrain the peak tailing. The mobile phases containing different contents (0, 0.1 and 0.2%) of aqueous formic acid or phosphoric acid solutions were compared. It was found that acetonitrile containing with 0.1% phosphoric acid as a solvent had best chromatographic peak shapes and baseline resolution. The detection wavelength of 278 nm was finally selected for HPLC fingerprints because a quite number of chromatographic peaks were able to...
be sufficiently detected. Considering good resolution in the gradient program of mobile phase, the wavelength conversion technique was employed for the quantitative analysis of components in XCTG. The column temperature was maintained at 40°C due to the relatively shorter retention time.

**Identification of chromatographic peaks**

The typical chromatographic fingerprint of XCTG was shown in Figure 2. Peaks 4, 7, 8, 18, 19, 24 and 25 were assigned to puerarin, peoniflorin, daidzin, baicalin, daidzein, baicalein and wogonin by analyzing the corresponding standard compounds. Because cinnamaldehyde presented in *Ramulus Cinnamomi* was not detected in some batches of XCTG under this analytical method, its corresponding chromatographic peak was not involved in the common peaks of fingerprint. Among these chromatographic peaks mentioned above, baicalin (peak 18) was chosen as reference peak. Twenty-five common characteristic peaks in fingerprint were applied to evaluate the similarity of 12 batches of XCTG.

**Method validation of quantitative analysis**

**Linearity, limits of detection and limits of quantification**

Six different concentrations for each analyte were prepared to assess the linearity of this method. The linear calibration curves of eight components were established and calculated in the form of $Y = aX + b$, where $Y$ and $X$ referred to peak area and sample concentration (mg/mL), respectively. The limit of detection (LOD) and limit of quantification (LOQ) were measured on the basis of the signal-to-noise ratio of 3 and 10, respectively. The results of linearity, LOD and LOQ are shown in Table II. Good linear correlation and high sensitivity at the developed chromatographic condition were confirmed by the correlation coefficient, LOD (0.001615–0.4460 μg/mL) and LOQ (0.005382–1.487 μg/mL).

**Precision, repeatability and stability**

The intra- and interday precision was performed on one day and three consecutive days with the same solutions of sample 5 and the relative standard deviation (RSD) ranges of the eight

![Figure 2. Typical HPLC-DAD fingerprint of the extract of XCTG.](image)

**Table II**

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Linear range (mg/mL)</th>
<th>Calibration curve ($n = 6$)</th>
<th>$r^2$ ($n = 6$)</th>
<th>LOD (μg/mL)</th>
<th>LOQ (μg/mL)</th>
<th>Repeatability RSD (%)</th>
<th>Stability RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Puerarin</td>
<td>0.002480–0.4960</td>
<td>$Y = 19,033,424X + 19096$</td>
<td>0.9999</td>
<td>0.03695</td>
<td>0.1232</td>
<td>1.0</td>
<td>0.8</td>
</tr>
<tr>
<td>Peoniflorin</td>
<td>0.005180–0.2072</td>
<td>$Y = 851,937X - 1287$</td>
<td>0.9997</td>
<td>0.4460</td>
<td>1.487</td>
<td>1.8</td>
<td>1.3</td>
</tr>
<tr>
<td>Daidzin</td>
<td>0.0008416–0.1683</td>
<td>$Y = 16,845,196X + 956$</td>
<td>1.0000</td>
<td>0.04151</td>
<td>0.1384</td>
<td>1.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Baicalin</td>
<td>0.001101–0.2201</td>
<td>$Y = 13,853,810X + 17591$</td>
<td>0.9998</td>
<td>0.03691</td>
<td>0.1230</td>
<td>0.7</td>
<td>1.0</td>
</tr>
<tr>
<td>Daidzein</td>
<td>0.0004264–0.08528</td>
<td>$Y = 18,900,715X + 5293$</td>
<td>1.0000</td>
<td>0.08376</td>
<td>0.2792</td>
<td>1.4</td>
<td>1.3</td>
</tr>
<tr>
<td>Cinnamaldehyde</td>
<td>0.0001424–0.02848</td>
<td>$Y = 46,007,154X + 3845$</td>
<td>0.9999</td>
<td>0.03812</td>
<td>0.1271</td>
<td>1.7</td>
<td>1.6</td>
</tr>
<tr>
<td>Baicalein</td>
<td>0.0001597–0.03194</td>
<td>$Y = 10,211,127X - 1458$</td>
<td>1.0000</td>
<td>0.01463</td>
<td>0.04678</td>
<td>1.0</td>
<td>0.9</td>
</tr>
<tr>
<td>Wogonin</td>
<td>0.00007200–0.01440</td>
<td>$Y = 28,354,512X + 399$</td>
<td>1.0000</td>
<td>0.001915</td>
<td>0.005382</td>
<td>1.5</td>
<td>1.1</td>
</tr>
</tbody>
</table>
components were 0.4–1.4% for intraday analysis and 0.5–1.5% for interday analysis (Table III). The repeatability was determined by analyzing six independently prepared solutions of the same sample (sample 5). The RSD values were <2.0% (Table II). Stability was assessed by analyzing the same sample solution at 0, 2, 4, 8, 12 and 24 h, respectively. The results demonstrated that the RSD values of the eight compounds were <1.6%, indicating that the sample solutions were stable within 24 h. The results mentioned above suggested that the developed method had acceptable precision, repeatability and stability.

Recovery

The recovery test was carried out by using the method of standard addition. Three different concentration levels (approximately equivalent to 0.8, 1.0 and 1.2 times of the concentration of the matrix) of the mixed standard solution were added into 0.50 g of sample 5. Then, recoveries were calculated by comparing the determined amount of those standards with the amount originally added. The method was reproducible with recovery ranging from 97.87 to 102.0% and RSD below 2.0% (Table III).

Quantitative determination of eight components in XCTG

The developed method was applied to quantitatively determine eight compounds in 12 batches of XCTG. The contents of eight compounds are shown in Table IV. The four common compounds such as puerarin, peoniflorin, daidzin and baicalin were the high content components in all examined batches of samples. However, the contents of each compound varied in different batches of XCTG, especially in different samples from the different pharmaceutical manufacturers. For example, daidzin was abundant in the batches of samples from G company, but lower in the batches of samples produced from X company. Moreover, the content of daidzin in samples from the former pharmaceutical manufacturer was twice higher than that from the latter pharmaceutical manufacturer, indicating that various processing methods or different raw medicinal materials may be used by different pharmaceutical manufacturers. Cinnamaldehyde, one of the important compounds of *Ramulus Cinnamomi* (18), was hardly detected in batches of samples 8 and 12, while its content was much higher in the batches of samples 3 and 4. The discrepancy in the content of cinnamaldehyde might be due to the variations of cultivation year, harvest time, climate, environment and the extent of processing the *Ramulus Cinnamomi*.

Comparison of different batches of XCTG based on eight component contents

The distribution of eight component contents in different batches was further compared for quality evaluation. To assess the inconsistency between different batches of samples, boxplot was used (Figure 3). As a convenient way to graphically depict the group of data through their quartiles, boxplot, introduced by Tukey, is used as a univariate robust measure for outlier detection (19). The first and third quartiles are used to acquire the robust group of data through their quartiles, boxplot, introduced by Tukey. If outliers of component contents occur in some batches, the consistency of these batches cannot be well controlled. The

### Table III

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Precision Intraday (%)</th>
<th>Precision Interday (%)</th>
<th>Recoveries Initial (μg)</th>
<th>Recoveries Added (μg)</th>
<th>Recoveries Detected (μg)</th>
<th>Recoveries Recovery (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Puerarin</td>
<td>0.6</td>
<td>0.6</td>
<td>1.550</td>
<td>3.566</td>
<td>0.9222</td>
<td>1.2</td>
<td></td>
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<tr>
<td>Peoniflorin</td>
<td>1.3</td>
<td>1.2</td>
<td>0.3749</td>
<td>0.8601</td>
<td>1.0141</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>Daidzin</td>
<td>0.2</td>
<td>0.3</td>
<td>0.2420</td>
<td>0.5689</td>
<td>0.992</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Baicalin</td>
<td>0.7</td>
<td>0.9</td>
<td>0.9254</td>
<td>1.9312</td>
<td>1.022</td>
<td>1.1</td>
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<tr>
<td>Daidzein</td>
<td>0.8</td>
<td>1.0</td>
<td>0.1100</td>
<td>2.1076</td>
<td>1.033</td>
<td>1.3</td>
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<tr>
<td>Cinnamaldehyde</td>
<td>0.7</td>
<td>1.1</td>
<td>0.01025</td>
<td>0.02286</td>
<td>0.970</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>Baicalein</td>
<td>0.6</td>
<td>0.8</td>
<td>0.01796</td>
<td>0.04004</td>
<td>1.04</td>
<td>0.8</td>
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</tr>
<tr>
<td>Wogonin</td>
<td>1.0</td>
<td>0.7</td>
<td>0.01920</td>
<td>0.04034</td>
<td>0.978</td>
<td>1.7</td>
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</tbody>
</table>

### Table IV

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Puerarin (mg/g)</th>
<th>Peoniflorin (mg/g)</th>
<th>Daidzin (mg/g)</th>
<th>Baicalin (mg/g)</th>
<th>Daidzein (mg/g)</th>
<th>Cinnamaldehyde (mg/g)</th>
<th>Baicalein (mg/g)</th>
<th>Wogonin (mg/g)</th>
</tr>
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<tr>
<td>1</td>
<td>5.28</td>
<td>0.7426</td>
<td>0.4263</td>
<td>2.556</td>
<td>0.1402</td>
<td>0.0856</td>
<td>0.1605</td>
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<td>2</td>
<td>5.787</td>
<td>1.338</td>
<td>0.5038</td>
<td>1.377</td>
<td>0.5026</td>
<td>0.05663</td>
<td>0.08069</td>
<td>0.05108</td>
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<tr>
<td>3</td>
<td>4.482</td>
<td>1.134</td>
<td>0.658</td>
<td>1.058</td>
<td>0.2000</td>
<td>0.1038</td>
<td>0.05462</td>
<td>0.03284</td>
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<tr>
<td>4</td>
<td>3.465</td>
<td>0.822</td>
<td>0.4812</td>
<td>0.8299</td>
<td>0.1860</td>
<td>0.2598</td>
<td>0.04950</td>
<td>0.1805</td>
</tr>
<tr>
<td>5</td>
<td>3.984</td>
<td>0.9431</td>
<td>0.6425</td>
<td>1.973</td>
<td>0.1835</td>
<td>0.02483</td>
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<td>0.03772</td>
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<tr>
<td>6</td>
<td>7.981</td>
<td>1.969</td>
<td>1.796</td>
<td>1.590</td>
<td>0.4926</td>
<td>0.00356</td>
<td>0.0315</td>
<td>0.01606</td>
</tr>
<tr>
<td>7</td>
<td>5.277</td>
<td>4.238</td>
<td>1.419</td>
<td>1.616</td>
<td>0.5788</td>
<td>0.008264</td>
<td>0.02926</td>
<td>0.01881</td>
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<tr>
<td>8</td>
<td>7.342</td>
<td>4.058</td>
<td>1.599</td>
<td>1.450</td>
<td>0.3964</td>
<td>-</td>
<td>0.04169</td>
<td>0.01974</td>
</tr>
<tr>
<td>9</td>
<td>8.566</td>
<td>4.053</td>
<td>1.765</td>
<td>1.300</td>
<td>0.4996</td>
<td>0.003255</td>
<td>0.03829</td>
<td>0.02056</td>
</tr>
<tr>
<td>10</td>
<td>7.154</td>
<td>1.527</td>
<td>1.470</td>
<td>1.410</td>
<td>0.3841</td>
<td>0.005227</td>
<td>0.04959</td>
<td>0.02369</td>
</tr>
<tr>
<td>11</td>
<td>7.135</td>
<td>1.403</td>
<td>1.366</td>
<td>1.281</td>
<td>0.4634</td>
<td>0.002415</td>
<td>0.04225</td>
<td>0.02349</td>
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<tr>
<td>12</td>
<td>9.069</td>
<td>3.968</td>
<td>1.665</td>
<td>1.227</td>
<td>0.4718</td>
<td>-</td>
<td>0.03195</td>
<td>0.02514</td>
</tr>
</tbody>
</table>

*Samples 1–5 were produced by X company. Samples 6–12 were from G company.

The data were present as average of triplicates.

Below the limit of detect.
quality of samples from G company was relatively stable in comparison with those from X company. Three samples in the contents of three components such as daidzein, peoniflorin and cinnamaldehyde are thought to be outlier based on boxplot. Two of three samples were from the fourth batch, and one of them was from the first batch. These 'anomalous' samples were all produced by X company, indicating that the consistency of the batches in three component contents could not be well controlled.

**Similarity evaluation of XCTG fingerprints with three algorithms**

HPLC fingerprints of 12 batches of samples after peak alignment are all shown in Figure 4. The reference fingerprint of different batches of samples was generated by the average method provided by similarity analysis software. The similarity values based on three algorithms between these fingerprints and the reference fingerprint are illustrated in Figure 5. It was obvious that samples from G company exhibited the relatively high similarity to the

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Figure 3. Boxplot of eight components in different batches of samples. The cross is a symbol used for outliers and the number indicates the batch number.

Figure 4. HPLC fingerprints of XCTG samples from two manufactures after peak alignment.
reference fingerprint as compared with those from X company. The fluctuation of similarity values between samples from G company was less in comparison with samples from X company, suggesting that sample fingerprints of G company are more consistent and stable. The results shown in Figure 5 indicated that quality consistency of XCTG produced by the two manufactures might be different from each other. For each sample, the similarity values calculated with cosine of angle and correlation coefficient are almost close. For PAR and the other two similarity algorithms, small differences can be observed in samples 5–12, while significant dissimilarities occur in samples 1, 2, 3 and 4. According to results shown in Figure 5, similarity values evaluated by PAR for samples 1, 3 and 4 are <0.900, indicating that the fingerprints of these samples may be significantly different from the reference fingerprint in peaks that have small area.

Hierarchical clustering analysis

In order to illustrate the comparison of the similarity of these fingerprints with the closeness of eight-component contents, hierarchical cluster analysis was performed. Between-groups the average-linkage method was applied due to its superior performance (20). Euclidean distance was used to measure the closeness of eight-component contents between different batches. The dendrograms are shown in Figure 6. Compared with clusters based on cosine of angle and correlation coefficients, the clusters based on PAR and eight-component contents were different. For example, when using cosine of angle to measure the similarity, HPLC fingerprint of sample 4 was similar to that of samples 6–12 because they were grouped in the same class (shown in Figure 6A). However, different clustering results occurred in Figure 6D. Sample 4 was grouped with samples 1, 2, 3 and 5 into one class according to the closeness of eight-component contents. As shown in Figure 6, there were two apparent clusters which corresponding to actual manufactures of samples except the clusters shown in Figures 6A and B. Because small area peaks and large area peaks had the same contribution to final similarity in PAR algorithm, similarity based on PAR may be a better reflection of the discrepancy in samples by different manufacturers in
comparison with cosine of angle or correlation coefficient. As the results shown in Figure 6D, eight components could be used as markers for distinguishing the XCTG samples produced by different companies.

Quality evaluation of XCTG by PCA

Although cluster analysis can give the category information on samples, it is not easy to find which viable/component greatly contributes to classification. PCA is a data reduction approach, which is very useful for observing the distribution of samples in multivariate dimensional space. Before performing PCA, the data of eight-component contents were standardized to put the content of each component on the approximately same scale (21). Therefore, the components of low content assume equal significance to those of high content. The scores plot of PCA-based eight-component contents is shown in Figure 7A.

The principal components PC1 and PC2 could explain over 85% of variance. It was obvious that the samples were classified into two classes corresponding to sample manufacturers. Samples produced by G company were almost gathered in the same region and exhibited a distinct separation from ones made by X company, which were sparsely distributed. The sample distribution suggested that an evident fluctuation occurred in the quality of samples manufactured by X company. Seen from loadings plot of PCA shown in Figure 7B, eight compounds were grouped into three classes. Each compound was roughly the same contribution to separation of samples produced by two manufacturers. The first component of PCA explained the compounds including puerarin, peoniflorin, daidzin, daidzein, cinnamaldehyde, baicalin, baicalein and wogonin. The compounds such as baicalin and baicalein were mainly explained by the second component. According to the loadings plot (Figure 7B) and Table IV, there was an evident difference in the average content of each component between G and X companies.

Discussion

The HPLC-DAD method was developed for simultaneous determination of eight major components and chromatographic fingerprint of XCTG. The method was validated by its linearity, precision, stability, reproducibility and recovery.

By studying all the samples of XCTG, the quality of samples from G company was more stable and consistent than that of samples from X company. The variation in component contents may be due to different geographic environment, hereditary character, various cultivation models, varying processing technologies and other factors and accordingly might lead to potential inconsistency in the efficacy of different products. By comparison of different batches of XCTG based on eight component contents, the consistency of samples in the contents of daidzein, peoniflorin and cinnamaldehyde could not be well controlled. Therefore, the stability of component contents should be strengthened to ensure the quality of XCTG.

For similarities of HPLC fingerprints, PAR results were different from those of cosine of angle and correlation coefficient due to different features of them. Because each similarity algorithm has its own perspective to evaluate the similarity, discrepancies in similarity based on different algorithms are reasonable. Cosine of angle and correlation coefficient are both sensitive to the large peak area. Hence, peaks that have large area greatly contribute to the similarity (22). In other words, the similarity value calculated by the two algorithms (i.e., cosine of angle and correlation coefficient) is decided by a large area peak. However, according to PAR, each peak to be compared has equal contribution to final similarity. Therefore, the contribution of some small area peaks in fingerprints to similarity is exaggerated, which eventually leads to discrepancies in similarity between PAR and the other two algorithms.

Recently, quality evaluation of TCMs has focused on HPLC fingerprints with simultaneous determination of active components. Generally, cosine of angle was usually used for fingerprint similarity. However, similarity between fingerprints of samples by cosine of angle did not necessarily indicate the closeness of component contents of samples (Figures 6A and D). Our results suggested that chromatographic fingerprint analysis and simultaneous determination of multiple components should be both combined for the quality evaluation of XCTG.
Conclusion
An efficient and sensitive HPLC fingerprint method combined with eight-component contents was developed. In addition, similarity analysis with three algorithms, hierarchical cluster analysis and PCA were all applied to evaluate the quality of 12 batches of XCTG. Compared with the current quality standards, HPLC fingerprint combined with quantitative analysis of multiple components may be a rational and informative method for quality assessment of XCTG.

Acknowledgments
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