Comparative analysis of four active compounds of Baikal skullcap and its classical TCM prescriptions according to different clinical curative effects

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ABSTRACT

Objective: A sensitive HPLC-DAD detection method was established for the comparative analysis of the four active compounds (including baicalin, baicalein, wogonoside and wogonin) of Baikal Skullcap and its classical TCM prescriptions according to different clinical curative effects. And analyze the relationship between compatibility of medicines, content and clinical curative effect.

Methods: Water extracts were analyzed by high-performance liquid chromatography-diode array detection (HPLC-DAD), using an Agilent extend-C18 (4.6mm×250mm,5µm) column by using solvent A (0.2% phosphoric acid water) and solvent B (methanol) in the ratio of 52:48 (v/v) at a flow rate of 1 ml/min. The column temperature was maintained at 25°C and the detection wavelength was set at 280 nm.

Results: In quantitative analysis, the four selected markers showed good regression (R^2=0.999) within test ranges. The average recoveries were between 99.75~100.60% and their RSD values were between 0.97%~1.71%. The proportions of the four chemical compounds for different efficacy were 33.51~35.76: 6.78~6.87: 3.45~3.64: 1; 18.6~23.64: 3.63~4.27: 1.36~1.53: 1; 29.94~31.78: 5.14~5.23: 3.28~3.56: 1; 33.99~41.91: 5.83~7.42: 2.83~3.39: 1.

Conclusion: Clinical practice of thousands of years have proved that the curative effect of Traditional Chinese Medicine depends on the compatibility of different kinds of medicine material crude slices. According to this study we found that different compatibility of medicines leads to different proportions of the chemical compounds. We assume that there is a certain correlation between therapeutic effect of TCM and compatibility of medicines.

Key words: Baikal skullcap, Couplet medicine, Clinical curative effect, Compatibility of medicines, Proportions of the chemical compounds

1 Introduction

Traditional Chinese Medicine (TCM) experienced thousands of years, and was proved to have stable and reliable clinical efficacy[1-3]. TCM has the characteristics of reliable curative effect and high safety, which have been recognized by the world medical[4]. The curative effect of TCM is greatly influenced by the compatibility of medicines and processing procedure. Different compatibility of medicines and processing procedure leads to different pharmacodynamic material basis and eventually produces different clinical curative effects[5-8]. So far, the research of chemical composition is the main means to study the pharmacodynamic material basis[9-11]. Therefore, this study mainly through the research of the four active compounds of Baikal Skullcap and its classical TCM prescriptions and comparatively analyses the correlation between the four active compounds and different clinical curative effects including heat clearing, fire purging, stanching, and miscarriage preventing.

Baikal skullcap is a famous Traditional Chinese Medicine in common use, and it was processed goods of the dry root of Scutellaria baicalensis Georg., including Hollow Root of Baikal Skullcap (HRBS) and Solid lateral Root of Baikal Skullcap (SRBS)[12]. The former lived more than 3 years with a hollow root, the latter lived about 1 year with a solid lateral root. Not only their shapes were different, but also they had markedly different clinical curative effect in traditional Chinese medical science. HRBS were good at clearing heat especially lung-heat[13], while SRBS were adept in purging fire[14], stanching and preventing miscarriage[15] [Table 1]. The main reason is that there are significant differences in chemical composition between them. At present, however, they were used in chaos in market due to no apparent discrimination shown in Chinese Pharmacopoeia. There was no obvious limit to the use of these two kinds of medicinal herbs. They are commonly used interchangeably, which has resulted in a great difference in clinical therapeutic effect.

Therefore, it was urgent to determine the active compounds of Baikal skullcap associated with clinical effects and apply Baikal skullcap distinguished. In order to meet the need of research and clinical application, Baikal skullcap was processed into different processed products, such as Charred Baikal Skullcap (CBS) and Stir-baked Baikal Skullcap (SBS), which were also different in shape and clinical effects.

Couplet Medicine was the application pattern of TCM, which indicated that herbs matched into pairs, and produced
Table 1. Compatibility of medicines, ratio and therapeutical effect of Baikal skullcap in different periods.

<table>
<thead>
<tr>
<th>References</th>
<th>Period</th>
<th>Compatibility of medicines</th>
<th>Ratio (W/W)</th>
<th>Clinical curative effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gu Jia Yi Jian</td>
<td>In the Ming dynasty</td>
<td>Baikal skullcap-Bulbus Fritillaria</td>
<td>1:1</td>
<td>Clearing heat</td>
</tr>
<tr>
<td>Wai Tai Mi Yao</td>
<td>In the Tang dynasty</td>
<td>Baikal skullcap-Fructus gardeniae</td>
<td>1:1</td>
<td></td>
</tr>
<tr>
<td>Yi Xue Xin Wu</td>
<td>In the Qing dynasty</td>
<td>Baikal skullcap-Cochinchnine asparagus</td>
<td>1:1</td>
<td></td>
</tr>
<tr>
<td>Ming Yi Zhi Zhang</td>
<td>In the Ming dynasty</td>
<td>Baikal skullcap-Angelica dahurica</td>
<td>2:1</td>
<td></td>
</tr>
<tr>
<td>Shang Han Lun</td>
<td>In the Han dynasty</td>
<td>Baikal skullcap-Herba schizonepetae</td>
<td>1:1</td>
<td></td>
</tr>
<tr>
<td>Shang Han Lun</td>
<td>In the Han dynasty</td>
<td>Baikal skullcap-Coptis chinensis</td>
<td>3:1</td>
<td>Purging fire</td>
</tr>
<tr>
<td>Shang Han Lun</td>
<td>In the Han dynasty</td>
<td>Baikal skullcap-Bupleurum falcatum</td>
<td>1:1</td>
<td></td>
</tr>
<tr>
<td>Shang Han Lun</td>
<td>In the Han dynasty</td>
<td>Baikal skullcap-Pinellia termata</td>
<td>1:1</td>
<td></td>
</tr>
<tr>
<td>Shang Han Lun</td>
<td>In the Han dynasty</td>
<td>Baikal skullcap-White Paony Root</td>
<td>1:1</td>
<td></td>
</tr>
<tr>
<td>Gu Jin Yi Che</td>
<td>In the Qing dynasty</td>
<td>Baikal skullcap-Mangnolia officinalis</td>
<td>1:1</td>
<td></td>
</tr>
<tr>
<td>Jin Kui Yao Yue</td>
<td>In the Han dynasty</td>
<td>Baikal skullcap-rehmnnia root</td>
<td>1:1</td>
<td>Stanching</td>
</tr>
<tr>
<td>Nu Ke Qiye Yao</td>
<td>In the Qing dynasty</td>
<td>Baikal skullcap-Ros Sophorae stir-fried</td>
<td>1:1</td>
<td></td>
</tr>
<tr>
<td>Shang Han Lun</td>
<td>In the Han dynasty</td>
<td>Baikal skullcap-Monkshhood</td>
<td>1:1</td>
<td></td>
</tr>
<tr>
<td>Jing Yue Quan Shu</td>
<td>In the Ming dynasty</td>
<td>Baikal skullcap-white attractyodes rhizome</td>
<td>1:1</td>
<td>Preventing miscarriage</td>
</tr>
<tr>
<td>Pu Ji Fang</td>
<td>In the Ming dynasty</td>
<td>Baikal skullcap-Fructus Aurantii</td>
<td>1:1</td>
<td></td>
</tr>
<tr>
<td>Jing Yue Quan Shu</td>
<td>In the Ming dynasty</td>
<td>Baikal skullcap-Fructus amomni</td>
<td>1:1</td>
<td></td>
</tr>
<tr>
<td>Pu Ji Fang</td>
<td>In the Ming dynasty</td>
<td>Baikal skullcap-Dioscorea Opposita</td>
<td>1:1</td>
<td></td>
</tr>
</tbody>
</table>

2.1 Chemicals and reagents

The reference compounds of Baicalin, Baicalein and Wogonin (purity ≥ 98%) were obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Wogonoside (purity ≥ 98%) was purchased from Victory Biotechnology Co., Ltd. (Sichuan, China). Medicinal materials were purchased from Qixin Medicine Co., Ltd. (Hebei, China). HPLC-grade methanol was purchased from Fisher (Leics, UK). H3PO4 is analytical grade and was obtained from Beijing Chemical Works (Beijing, China) and high purity water was obtained from Wahaha Co., Ltd. (Hangzhou, China).

2.2 Instrumentation and separation conditions

The HPLC system was Agilent 1260 System equipped with a model series, HP quaternary pump, HP Low pressure gradient flow controller valve, 0-100µL HP automatic injector, HP column oven, and an HP DAD detector. Data acquisition was performed on class-VP software. The column was a reversed-phase Agilent extend-C18 (4.6mm×250mm, 5µm). The elution system consisted of solvent A (0.2% phosphoric acid water) and solvent B (methanol) in the ratio of 52:48(v/v) within 60 minutes. The flow rate was set at 1 ml/min, and the injection volume was 10µL and separation was performed isothermally at 25°C. The raw data was detected by an HPLC-DAD (Agilent1260, USA) and the wavelength was set at 280nm.

2.3 Sample preparation

9g of Baikal skullcap was used in all Couplet Medicines. They were immersed in distilled water (1:10, v/v) and were boiled twice in 30min and 20min respectively [Table 2]. The filtrates from each decoction were mixed and concentrate to 1/2 of its original volume, the concentrate solution was thoroughly mixed and then centrifuged again at 6000 rpm for 15 min at 4°C. For HPLC analysis, the solution with the volume of 1 ml was dissolved in methanol at a final volume of 10ml. Before injection, it was filtered through a 0.22 µm filter.

2.4 Preparation of standard solutions

The appropriate amount of Baicalin, Baicalein, Wogonoside and Wogonin were separately weighed and were dissolved in methanol respectively to achieve six standard working solutions with the concentration of 328µg/mL, 80µg/mL, 42µg/mL, 25µg/mL, 16µg/mL and 5µg/mL respectively. All solutions were prepared in dark brown calibrated flasks and stored at 4°C. For calibration standards, the standard solutions were diluted with methanol to make the standard curve cover the range of 0.984–5.904µg for Baicalin, 0.224–1.344µg for Baicalein, 0.432–2.592µg for Wogonoside and 0.052–0.312µg for Wogonin.
2.5 Method validation
The HPLC method was validated in terms of linearity, precision, stability, repeatability and recovery. The validation was performed based on the Relative Peak Areas (RPAs). Linear regression analysis was employed to construct calibration curves. The data was expressed as X±S. And Relative Standard Deviation (RSD) was used to evaluate precision, stability, repeatability and recovery.

2.6 Ethical approval
The conducted research is not related to either human or animals use.

3 Results
3.1 Chromatography
The HPLC chromatogram of test sample and standard solutions were shown in Figure 1. In chromatograms A and B, the four peaks marked with 1-4 are Baicalin, Baicalein, Wogonoside and Wogonin. The retention time is 19.752min, 22.685min, 28.747min and 37.355min respectively.

3.2 Calibration curves
Linearity was evaluated by analyzing four standard working solutions at the same volume of six different concentrations, and then the calibration curves were constructed by plotting the peak areas at the injection quantity of 10µl of each compound. Linear regression equation, correlation coefficient and linear range of four standard substances are given in Table 3.

3.3 Precision, stability, repeatability, and recovery
The precision of this method was validated for intra-day and inter-day precision which performed by six replicate determinations of the standard working solution. Stability was evaluated by analyzing the solutions stored at room temperature (about 25°C) at different time points (0, 4, 8, 12 and 24 h after preparation). The solutions for stability test included mixed solutions of reference standard and the sample solutions. Six replicates were performed for the test. These data confirmed that the four compounds were stable within 24h at 25°C and their RSD values were between 0.43%~1.92%. The repeatability was examined by six replications of a sample. In the recovery test, samples were prepared at one concentration level by spiking known quantities of each of the four standards into the sample, and then extracted and analyzed according to the described procedures. The validation data are shown in Table 4 and Table 5.

3.4 Contents of the four compounds of Baikal Skullcap in different Couplet Medicines
The HPLC data demonstrated that different compatibility of medicines has different contents. The contents of the four compounds of Baikal Skullcap in different Couplet Medicines are shown in Figure 2~Figure 6.

4 Conclusions
Baikal Skullcap is a very famous traditional Chinese Medicine. Medicine material crude slices of Baikal Skullcap and its couplet medicines have been used to treat diseases for thousands of years. This therapeutic effect has also been proved by modern medicine. However, few research has explained the correlation between the therapeutic effect of Baikal Skullcap and its couplet medicines.
In this paper, the four primary clinical effects of various kinds of Baikal Skullcap and its couplet medicines were searched in traditional Chinese medical science based on records in historical materials. They were divided into four groups including clearing heat, purging fire, stanching and preventing miscarriage based on the four primary clinical effects. The research indicated that different compatibility of medicines leads to different proportions of the chemical compounds and different proportions of the chemical compounds eventually leads to different therapeutical effects. The therapeutical effect of TCM is influenced by compatibility of medicines.

According to the analysis of the chemical composition. We can only determine that different treatment effects are related to different chemical compositions. We can’t come to the conclusion that the therapeutic effect is exactly related to a specific chemical composition or the combination of several chemical compositions. Further study on the pharmacology, pharmacokinetics and toxicology of Baikal Skullcap and its couplet medicines is going on and it will be reported in the following manuscripts.

## 5 Discussion

### 5.1 The content of the four active compounds in Baikal Skullcap and its different processed products

The content of chemical compounds of Traditional Chinese Medicine is mainly influenced by its growing years and processing procedure. The baikal skullcap (hollow root of Scutellaria baikalensis Georgi) lived more than 3 years with a hollow root and the Baikal skullcap (solid lateral root of Scutellaria baikalensis Georgi) lived about 1 year with a solid lateral root. They were different in growing years, so they have different chemical compounds. And also they had markedly different clinical effects in traditional Chinese medical science. Compare Baikal skullcap (hollow root of Scutellaria baikalensis Georgi) and Baikal skullcap (solid lateral root of Scutellaria baikalensis Georgi), the former has much Baicalin and Wogonoside but little Baicalein and Wogonin. We also found that the charred baikal skullcap has the most amount of the four active compounds but they can be hardly detected in stir-baked Baikal skullcap.

### Table 3. Linear regression equation, Correlation coefficient and Linear range of four standard substances.

<table>
<thead>
<tr>
<th>Standard substance</th>
<th>Linear regression equation (y=ax+b)</th>
<th>Correlation coefficient (r^2)</th>
<th>Linear range ((\mu g))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baicalin</td>
<td>(y=1063.30x-17.33)</td>
<td>0.9998</td>
<td>0.984-5.904(\mu g)</td>
</tr>
<tr>
<td>Baicalein</td>
<td>(y=599.48x+15.02)</td>
<td>0.9997</td>
<td>0.224-1.344(\mu g)</td>
</tr>
<tr>
<td>Wogonoside</td>
<td>(y=970.36x-379.61)</td>
<td>0.9996</td>
<td>0.432-2.592(\mu g)</td>
</tr>
<tr>
<td>Wogonin</td>
<td>(y=1,254.05x+44.19)</td>
<td>0.9996</td>
<td>0.052-0.312(\mu g)</td>
</tr>
</tbody>
</table>

\(a\) \(y\) and \(x\) stand for the peak area and the injection quantity \((\mu g)\) of each standard substance.

### Table 4. Precision, repeatability and stability of four standard substances.

<table>
<thead>
<tr>
<th>Standard substance</th>
<th>Intra-day precision RSD (%)</th>
<th>Inter-day precision RSD (%)</th>
<th>Repeatability RSD (%)</th>
<th>Stability RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baicalin</td>
<td>0.58%</td>
<td>0.63%</td>
<td>1.43%</td>
<td>1.44%</td>
</tr>
<tr>
<td>Baicalein</td>
<td>1.75%</td>
<td>1.92%</td>
<td>1.56%</td>
<td>1.87%</td>
</tr>
<tr>
<td>Wogonoside</td>
<td>0.43%</td>
<td>0.75%</td>
<td>1.67%</td>
<td>1.27%</td>
</tr>
<tr>
<td>Wogonin</td>
<td>1.69%</td>
<td>1.88%</td>
<td>2.12%</td>
<td>2.31%</td>
</tr>
</tbody>
</table>

Table 5. Recovery of four standard substances.

<table>
<thead>
<tr>
<th>Standard substance</th>
<th>Original amount ((\mu g))</th>
<th>Spiked amount ((\mu g))</th>
<th>Determined amount ((\mu g))</th>
<th>Recoveries(^{(a)}) (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baicalin</td>
<td>317.08</td>
<td>316.75</td>
<td>644.22</td>
<td>100.18%</td>
<td>1.23%</td>
</tr>
<tr>
<td>Baicalein</td>
<td>19.39</td>
<td>19.28</td>
<td>38.56</td>
<td>99.75%</td>
<td>1.71%</td>
</tr>
<tr>
<td>Wogonoside</td>
<td>91.42</td>
<td>91.39</td>
<td>184.26</td>
<td>99.79%</td>
<td>0.97%</td>
</tr>
<tr>
<td>Wogonin</td>
<td>12.17</td>
<td>12.21</td>
<td>24.29</td>
<td>100.60%</td>
<td>1.14%</td>
</tr>
</tbody>
</table>

\(^{(a)}\) Recovery (%) = \(100\times(\text{amount found-original amount})/\text{amount spiked}.\)
The modern clinical use of Baikal skullcap (hollow root of Scutellaria baicalensis Georgi) and Baikal skullcap (solid lateral root of Scutellaria baicalensis Georgi) is very common. Most of the time, however, there was no obvious distinction between the two kinds of Chinese herbal slice, often used interchangeably. Therefore, it is necessary that we need to be awakened by the chemical composition analysis, clinical effects analysis and compatibility analysis to explain the difference between the two mentioned earlier.

Figure 2. The content of Baicalin, Baicalein, Wogonoside and Wogonin in single medicine of Scutellariae radix (mg/g) (n=3).

Figure 3. The content of Baicalin, Baicalein, Wogonoside and Wogonin in Heat-clearing couplet medicines (mg/g) (n=3).
Figure 4. The content of Baicalin, Baicalein, Wogonoside and Wogonin in Fire-purging couplet medicines (mg/g) (n=3).

Figure 5. The content of Baicalin, Baicalein, Wogonoside and Wogonin in stanching couplet medicines (mg/g) (n=3).
5.2 The content of the four active compounds in "Couplet Medicine" according to different clinical effects

The content of chemical compounds of traditional Chinese Medicinal Prescription is mainly influenced by the dosage and compatibility of medicines. The dosage of herbs remains constant while the compatibility of herb medicines is changed, and different compatibility of herb medicines can lead to different content of compounds. In this research, the average content of Baicalin, Baicalein, Wogonoside and Wogonin in heat-clearing couplet medicines, fire-purging couplet medicines, stanching couplet medicines and miscarriage-preventing couplet medicines were (70.95 ±7.25mg/g, 4.87±0.53mg/g, 17.27±2.89mg/g, 1.84±0.37mg/g), (65.27±3.06 mg/g, 3.48±1.56mg/g, 16.89±0.42mg/g, 1.6±0.36mg/g), (31.62±4.59mg/g, 1.18±1.14 mg/g, 7.17±2.20mg/g, 0.82±0.59mg/g) and (69.92±6.28mg/g, 6.06±0.58mg/g, 18.14±1.42 mg/g, 2.34±0.21mg/g), data shown in Figure 7.

The average ratio of Baicalin, Baicalein, Wogonoside and Wogonin were (38.54: 2.65: 9.38: 1.00), (40.79: 2.17: 10.55: 1.00), (38.80: 1.45: 8.80: 1.00) and (29.90: 2.59: 7.76: 1.00) correspondingly.

5.3 The analysis of the four active compounds in Heat-clearing group

Comparing the four active compounds of HRBS and SRBS in Heat-clearing group, the contents of Baicalin and Wogonoside in HRBS were more than those in SRBS, but the contents of Baicalein and Wogonin were opposite, which were corresponded with the antipyretic activity of Baicalin in modern pharmacological study. Furthermore, the correspondence between “HRBS was good at clearing away heat” in traditional Chinese medical science theory and the content and ratio of the four active compounds could also be clearly indicated. Moreover, comparing with the single herb, the contents of Baicalin and Wogonoside in the two groups of HRBS and SRBS were increased, while the contents of Baicalein and Wogonin were decreased when they were paired with other herb in clearing away heat of “Couplet Medicine” (Tab.4).

The essence of “attenuation and synergism” in traditional Chinese medical science theory was suggested. The variation of the content of active compounds was also corresponded with the traditional Chinese medical science theory, especially the clinical efficacy.

5.4 The analysis of the four active compounds in Fire-purging group

Comparing with the single herb, the changes of the contents of the four active compounds were found in both groups of HRBS and SRBS after they were paired with other herb in reducing fire of “Couplet Medicine”. Generally, the content of the four active compounds in SRBS was decreased in the certain proportion in a slight variation range after paired. However, no obvious regularity was found in the control group of HRBS. It also validated the fact that HRBS and SRBS were applied separately according to their different clinical effects. The correspondence of clinical therapeutic effects and active compounds, as well as the accuracy of the theory basis of Chinese medicine theory were deeply indicated.

5.5 The analysis of the four active compounds in Stanching group

The research showed that the content of the four active compounds of SRBS group were (60.32±1.62mg/g, 3.26±0.33mg/g,
14.53±1.61mg/g, 2.16±0.33mg/g, 1.42±0.21mg/g, 12.57±1.73mg/g, 1.39±0.38mg/g, 65.04±0.56mg/g, 1.42±0.21mg/g, 12.57±1.73mg/g, 1.39±0.38mg/g, 64.35±2.32 mg/g, 2.42±0.37 mg/g, 15.93±1.68mg/g, 1.34±0.46 mg/g). Interestingly, they can not be found in the “Couplet Medicine” of CBS group. Compared with the single herb of 9g of Baikal skullcap (charred) (Table 5), the content of the four active compounds were all lower, except wogonin in group of 9g of Baikal skullcap (solid lateral root of Scutellaria baicalensis Georgi) and 9g of rehmannia (raw) group. Compared with the single herb of 9g of Baikal skullcap (stir-baked) (Table 5), only a small amount of Baicalin and Baicalein were found, and Wogonoside and Wogonin could not be detected. The most probable cause is that, on one hand, it was difficult to detect low content of chemical compounds with relatively low sensitivity of HPLC, most of which was destroyed by high temperature. On the other hand, dissolution was decreased by compatibility.

5.6 The analysis of the four active compounds in Miscarriage-preventing group
According to traditional Chinese medicine, SRBS was good at miscarriage prevention while Baikal Skullcap (stir-baked) was a good medicine for the tocolysis. Therefore, SRBS and SBS were chosen in this group of miscarriage-preventing. The content of the four active compounds in these groups were analyzed to clarify correlation between compatibility and therapeutic effect. The content of the four active compounds of SRBS for the tocolysis were [(46.81±1.63)mg/g, 4.78±1.07)mg/g, (10.92±1.07) mg/g, (1.64±0.41)mg/g, [(60.69±2.03)mg/g, (7.13±0.73)mg/g, (16.15±1.86)mg/g, (2.34±0.23)mg/g], [(59.16±2.01)mg/g, (6.45±0.31)mg/g, (13.54±1.03)mg/g, (2.18±0.65)mg/g], [(61.66±2.25) mg/g, (7.46±0.53)mg/g, (16.49±1.46)mg/g, (2.29 ±0.79) mg/g].

Due to the lack of literatures about comparison of the two common medicinal slices, we could not hastily judge which one was better for the tocolysis. Further study on comparison of the two slices should be carried on, especially pharmacology and pharmacokinetics.

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7 Conflict of interest
The authors declare that there are no conflicts of interest.

Reference


