Quality Evaluation of Semen Oroxyli Based on the Determination of Multiple Components with a Single Reference Standard

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The determination of multiple components is considered to be one of the key methods to control the quality of traditional Chinese medicine (TCM), because the clinical efficacy of TCM is derived from the synergistic action of multi-components. When simultaneously determining multi-components in TCM with multiple reference standards (MRS), the limited availability and high expense of various reference standards is a major obstacle. Currently, a practical method determining multi-components with a single reference standard (SRS) is needed to solve the problem, in which the contents of those components without reference standards can be calculated using relative conversion factor. In the present paper, both MRS and SRS methods were established for the simultaneous quantitative determination of seven bioactive flavonoids in Muhudie (MHD), the seed of Oroxylum indicum L., a traditional Chinese herb that has been used for centuries. Forty samples were assayed and data obtained from the two methods were compared, and no statistically significant difference was observed by a T-test (P > 0.05), thus, the SRS method can be applied for quality analysis of MHD. At the same time, a certain correlation was discovered between the contents of the bioactive components and the morphological character of MHD.

Introduction

Oroxylum indicum L. belongs to the Bignoniaceae family (1). It grows naturally in south and southeast Asia (2). The root, root bark and stem bark of the plant have been used as traditional medicines for centuries in India, Thailand, Bangladesh and other Asian countries. Semen Oroxyli, the seed of O. indicum, which is named Muhudie (MHD) in Chinese, is a traditional Chinese medicine (TCM) and is famous for its beautiful butterfly shape. MHD has widely been used for the symptomatic treatment of coughs (3), chronic pharyngitis, acute bronchitis, upper respiratory tract infections (4) and other respiratory disorders. Studies have shown that in respiratory diseases such as asthma and chronic obstructive pulmonary disease (COPD), oxidative stress plays a key role in deleterious and inflammatory responses (5, 6). Polyphenols, such as flavonoids possessing antioxidant and anti-inflammatory abilities by directly scavenging free radicals and/or modulating important cellular signaling pathways, are beneficial to lung inflammations and related diseases (7, 8). In this way, the mechanism of using MHD to treat respiratory diseases may be attributed to the antioxidant flavonoids it contains.

Chemical investigations on MHD have demonstrated that flavonoid glycosides and aglycones are the primary components (9–15). Baicalin is usually chosen as a marker compound for the quality control of MHD (16–18); baicalein, chrysin (19) or chrysin-7-O-glucuronide (CGL) have also been used sometimes (20) as marker compounds. Recently, a high-performance liquid chromatography–ultraviolet detection (HPLC–UV) method was developed for simultaneously quantifying 13 flavonoids in MHD (21). The determination of multiple components is considered to be one of the key methods to control the quality of TCM, because the clinical efficacy of TCM is derived from the synergistic action of multi-components. When simultaneously determining multi-components in TCM with multiple reference standards (MRS), the limited availability and high expense of various reference standards is a major obstacle. Currently, a practical method for determining multi-components with single reference standard (SRS) is needed to solve the problem, in which the contents of those components without reference standards can be calculated using the relative conversion factor (RCF). The SRS method has been adopted in the United States and European Pharmacopoeia (22, 23), and has been successfully used to analyze the chemical composition of TCM containing coumarins (24), anthraquinones (25), steroidal saponins (26) and phenantherenequinones (27).

In the authors’ previous work, 22 flavonoids were isolated from MHD, and the antioxidant activities of all isolated compounds were evaluated using a 1,1-diphenyl-2-picrylhydrazyl (DPPH) and an oxygen radical absorbance capacity (ORAC) assay. Among all of the compounds, baicalein-7-O-gentiobioside (BGE), chrysin-7-O-gentiobioside (CGE), baicalein-7-O-glucoside (BGL), baicalein (BAI), chrysin-7-O-glucuronide (CGL), baicalein (BGE) and chrysin (CHR) (Figure 1) exhibited potent antioxidant activities, and HPLC analysis of the herb showed that these seven compounds were also the primary ingredients of MHD (28). Therefore, in the present paper, an HPLC method was established to determine the contents of the seven bioactive compounds, and data obtained from the SRS method was compared to that from the MRS method. At the same time, the relationship between the contents of the active components in MHD and its morphological character were studied; according to the result, a preliminary estimation of the quality of MHD by its morphological character was possible.
Experiments were performed on a Waters Alliance system, equipped with a binary pump, an autosampler, a column oven and a Waters 2996 DAD. The mobile phase consisted of water–acetonitrile 40:60 at 1.0 mL/min. An Agilent Zorbax Extend-C18 column (5 μm, 4.6 mm × 250 mm, i.d.) was used and column temperature was controlled at 30°C. The wavelength of DAD ranged from 200 to 400 nm.

Colorimetric analysis of samples was conducted by a Hitachi 3010 Chroma instrument. Briefly, three kinds of parameters were assayed for each sample: L, A and B. L stands for the sample’s brightness, the higher the brighter. A represents the color difference between red and green; the higher the value, the closer to red it approximates. B represents color difference between yellow and blue; the higher the value, the closer to yellow it approximates.

Preparation of standards and sample solutions
The standard solution was prepared by dissolving each standard in methanol at the concentrations of 61.00 μg/mL (BGE), 28.80 μg/mL (CGE), 218.00 μg/mL (BGL), 276.00 μg/mL (BAI), 170.00 μg/mL (CGL), 20.70 μg/mL (BAE) and 7.50 μg/mL (CHR).

An accurately weighed powder (40 mesh, 500 mg) was extracted with 25 mL methanol in an ultrasonic bath (40 kHZ, 100 W) for 30 min. After cooling down, the extracted solution was adjusted to the original weight, and then filtered through a 0.22 μm filter before injection. Each extract was injected in duplicate.

Validation of Method
A method validation of quantitative analysis was performed on parameters such as linearity, precision and recovery. Seven different amounts of the standard solution were used to draw up a calibration curve. The limit of detection (LOD) and limit of quantification (LOQ) for each marker compound were determined at a signal-to-noise ratios (S/N) of 3 and 10, respectively. The relative standard deviation (RSD) values were calculated for integration area and considered to be the measure of precision. To assess the intra-day precision, both the sample solution and the standard solution containing the seven analytes were injected five times within a day. The inter-day precision test was assessed by testing over three consecutive days (S2, S4). The accuracy of quantitation in terms of recovery was assessed (S5). A standard solution with the seven analytes was spiked into a sample solution containing half the mass of the plant material (250 mg) with known amounts of the seven analytes, and the sample was extracted according to the procedure of the sample preparation. The amount of each analyte in the standard solution was almost equal to that in the sample solution.

Calculation of RCF and its ruggedness and robustness tests
BAI, the easily available compound, was set as the reference standard. The ratio of the slope of calibration curve between BAI and the other compound was calculated as the RCF. The ruggedness test, concerning the effects of environmental factors, such as analysts (S6), instruments (S7) and column sources (S8), and the robustness test, focusing on the effects of the operational factors such as pH of mobile phase (S10) and precipitation.
column temperature (59), were performed. Three analysts, three HPLC instruments (Waters Alliance, Agilent 1200 and Shimadzu LC-20A) and three columns, including an Agilent Zorbax Extend-C18 (4.6 mm × 250 mm, 5 µm), a Kromasil-C18 (4.6 mm × 200 mm, 5 µm) and a Phenomenex-C18 (4.6 mm × 250 mm, 5 µm) were involved in the ruggedness test. In the robustness test, three pH values (2.0, 2.2 and 2.5) and column temperatures (25, 30 and 35°C) were investigated.

Figure 2. HPLC chromatogram of the methanol extract of MHD, obtained under the optimized sample preparation procedure at 270 nm.

Figure 3. HPLC chromatograms of different pHs of mobile phase A: pH 3.0 (A); pH 4.0 (B); pH 5.0 (C); pH 6.0 (D). Peaks: CGE (1); BAI (2); CGL (3). Detection at 270 nm.
Results and Discussion

Optimization of the chromatographic conditions
During the optimization process, several mobile phases (hybrid systems of organic phase and acid water) were studied, and the best separation result was obtained when using a CH$_3$CN–CH$_3$OH–phosphoric solution (Figure 2). Moreover, the effect of pH in the mobile phase on the retention time of the seven components was studied. BAI and CGL were implicated heavily by pH: their retention time shortened with an increase of pH, whereas few effects were observed on the other five compounds (Figure 3).

Because of their maximum UV absorbance, 270 nm was chosen for the simultaneous detection of CGE, CGL and CHR, and 280 nm was chosen for the other four compounds.

Optimization of the sample preparation
To obtain a high extraction efficiency of the seven components from the sample, several extraction solvents were investigated,
including methanol, 70% ethanol, a mixture of acidic water and methanol (MAWM), acidic water and water. It was found that the contents of BAI, CGL, BAE and CHR varied in different extraction solutions. The peaks of BAI, CGL, BAE and CHR could not be detected when using water or acidic water as the extraction solvent; small peaks of BAI and CGL and big peaks of BAE and CHR could be detected when using 70% ethanol or MAWM as the extraction solvent. When using methanol as the extraction solvent, big peaks of BAI and CGL and small peaks of BAE and CHR were detected (Figure 4). This implied that BAI and CGL, both with a glucuronide moiety, transformed to

Table I
Calibration Curves, LOD, LOQ and RCFs of the Investigated Compounds

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Test range (µg)</th>
<th>Calibration equation*</th>
<th>$r^1$</th>
<th>LOD (ng)</th>
<th>LOQ (ng)</th>
<th>RCF</th>
</tr>
</thead>
<tbody>
<tr>
<td>BGE</td>
<td>0.082–2.050</td>
<td>$y = 2091411x - 44734$</td>
<td>0.9999</td>
<td>1.26</td>
<td>2.52</td>
<td>1.369</td>
</tr>
<tr>
<td>CGE</td>
<td>0.027–0.675</td>
<td>$y = 3135570x - 11872$</td>
<td>1.0000</td>
<td>1.84</td>
<td>9.20</td>
<td>0.949</td>
</tr>
<tr>
<td>BGL</td>
<td>0.261–6.525</td>
<td>$y = 3150827x - 11627$</td>
<td>1.0000</td>
<td>0.92</td>
<td>4.60</td>
<td>0.943</td>
</tr>
<tr>
<td>BAI</td>
<td>0.244–6.100</td>
<td>$y = 2974338x - 108383$</td>
<td>0.9999</td>
<td>0.92</td>
<td>4.60</td>
<td>1.000</td>
</tr>
<tr>
<td>CGL</td>
<td>0.105–2.625</td>
<td>$y = 3191374x - 54834$</td>
<td>1.0000</td>
<td>0.70</td>
<td>1.40</td>
<td>0.553</td>
</tr>
<tr>
<td>BAE</td>
<td>0.023–0.570</td>
<td>$y = 5377748x - 18291$</td>
<td>1.0000</td>
<td>0.70</td>
<td>1.40</td>
<td>0.553</td>
</tr>
<tr>
<td>CHR</td>
<td>0.008–0.189</td>
<td>$y = 6678160x - 5097$</td>
<td>0.9999</td>
<td>0.61</td>
<td>3.15</td>
<td>0.445</td>
</tr>
</tbody>
</table>

*$y$ = peak area, $x$ = amount of standards in µg.

$^1r$ = correlation coefficient for seven data points ($n = 3$) in the calibration curves (linear model).

Table II
Precision Data and Recovery for the Developed HPLC Method

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Intra-day variability</th>
<th>Inter-day variability</th>
<th>Recovery (%)</th>
<th>RSD* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Standard solution</td>
<td>Sample</td>
<td>Standard solution</td>
<td>Sample</td>
</tr>
<tr>
<td>BGE</td>
<td>1.25</td>
<td>0.88</td>
<td>1.58</td>
<td>0.47</td>
</tr>
<tr>
<td>CGE</td>
<td>2.60</td>
<td>0.85</td>
<td>1.05</td>
<td>0.74</td>
</tr>
<tr>
<td>BGL</td>
<td>1.85</td>
<td>0.72</td>
<td>0.78</td>
<td>0.43</td>
</tr>
<tr>
<td>BAI</td>
<td>1.75</td>
<td>0.70</td>
<td>0.72</td>
<td>0.66</td>
</tr>
<tr>
<td>CGL</td>
<td>1.87</td>
<td>0.50</td>
<td>0.75</td>
<td>0.57</td>
</tr>
<tr>
<td>BAE</td>
<td>1.65</td>
<td>1.92</td>
<td>1.77</td>
<td>1.67</td>
</tr>
<tr>
<td>CHR</td>
<td>2.42</td>
<td>2.11</td>
<td>0.58</td>
<td>1.56</td>
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</tbody>
</table>

*RSD values for $n = 6$.

Table III
Ruggedness and Robustness Tests of RCF ($n = 3$)

<table>
<thead>
<tr>
<th>Examined factors</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAI/BGE</td>
<td>2.1</td>
</tr>
<tr>
<td>BAI/CGE</td>
<td>2.0</td>
</tr>
<tr>
<td>BAI/BGL</td>
<td>0.3</td>
</tr>
<tr>
<td>BAI/CGL</td>
<td>3.8</td>
</tr>
<tr>
<td>BAI/BAE</td>
<td>1.9</td>
</tr>
<tr>
<td>BAI/CHR</td>
<td>2.9</td>
</tr>
</tbody>
</table>

*Three operators in the investigation were from the authors’ lab.

**Instruments for investigation were Waters Alliance, Agilent 1200 and Shimadzu LC-20A.

†Columns for investigation were Agilent Zorbax Extend-C18 (4.6 × 250 mm, 5 µm), Kromasil-C18 (4.6 × 200 mm, 5 µm) and Phenomenex-C18 (4.6 × 250 mm, 5 µm).

§Column temperatures for investigation were 25, 30 and 35°C.

**Investigated pHs were 2.0, 2.2 and 2.5.

including methanol, 70% ethanol, a mixture of acidic water and methanol (MAWM), acidic water and water. It was found that the contents of BAI, CGL, BAE and CHR varied in different extraction solutions. The peaks of BAI, CGL, BAE and CHR could not be detected when using water or acidic water as the extraction solvent; small peaks of BAI and CGL and big peaks of BAE and CHR could be detected when using 70% ethanol or MAWM as the extraction solvent. When using methanol as the extraction solvent, big peaks of BAI and CGL and small peaks of BAE and CHR were detected (Figure 4). This implied that BAI and CGL, both with a glucuronide moiety, transformed to
their aglycones BAE and CHR, respectively, when water existed in the extraction solvent. This could be verified by monitoring the peak area change between the pair of BAI and BAE, CGL and CHR in 70% ethanol, MAWM and methanol extraction solutions. Because the water solubility of BAE and CHR is not good, no peaks of these two compounds can be observed in water or acidic water solutions, although BAE and CHR do exist in these two solutions. If the water solution is dried and its residue is dissolved with methanol, then the peaks of these two compounds can be detected (Figure 5). In other words, the seven marker compounds appeared in their original states when using methanol as the extraction solvent.

During optimization, different extraction methods (soaking, ultrasonic and reflux extraction) and time (15, 30, 60 and 90 min) were investigated to determine a convenient and efficient extraction technique.

**Method validation**

Calibration curves were constructed from peak areas versus compound amounts. The seven calibration curves exhibited...
excellent linear regressions of $r > 0.9999$ and the calibration data are shown in Table I. The RSDs of the intra-day and inter-day precision for standard and sample solutions (S2, S4) were all less than 3.00% (Table II). The average recovery rate of the seven analytes (S5) ranged from 97.89 to 101.53%, with RSD values varying between 0.78 and 4.11% (Table II). The RCF of each compound is listed in Table I. It was found that the RCFs of flavonoid glycosides BGE, CGE, BGL and CGL were close to 1.0, while the RCFs of flavonoid aglycones BAE and CHR were approximately 0.5. The results of the ruggedness and robustness tests (S6–S10) are shown in Table III, from which the HPLC instruments was observed to be an important factor that influenced the RCF.

**Sample analysis**

Forty samples of MHD were analyzed using both MRS and SRS methods under the optimized conditions mentioned previously, and data are listed in Tables IV and V. No remarkable differences were observed between data obtained from the two methods using a $T$-test ($P > 0.05$).

Forty samples were divided into two groups by their morphological characters. The surface of the sample in the first group was smooth, and shrank in the second group. Colorimetric analysis showed an obvious difference between the two groups: value $A$ in the samples of the first group was less than 2.00 and more than 4.30 in the second group (Figure 6), which meant that the sample color in the first group is yellowish green and it is reddish brown in the second group. As for quantitative analysis, it was found that for all samples, the concentration of flavonoid glycosides was much higher than that of flavonoid aglycones. Furthermore, the concentrations of CGE, BAE and CHR were lower than 0.6% in all tested samples, while the concentrations of BGE, BGL, BAI and BGL were all higher than 1.0% in the samples belonging to the first group and lower than 1.0% in the second group of samples. Principle component analysis (PCA) was applied on the chemical data and the PC1–PC2 score plot, shown in Figure 7, was obtained. Two patterns were clearly categorized: the samples in the second group are situated together and located separately from the samples in the first group along PC1. This result suggested that a certain correlation exists between the contents of the seven marker components and the morphological character of MHD, and the yellowish green sample with the smooth surface was inferred to have a high content of flavonoids.

**Conclusions**

A simple, accurate and stable HPLC–DAD method was established for the simultaneous quantitative determination of seven bioactive flavonoids in MHD. To the authors’ knowledge, it is the first report for the quantitative analysis of MHD applying not only MRS, but also the SRS method. The validation of the developed method verified its reliability and stability. A comparison shows no obvious distinction between the assay results of the two methods, thus SRS method can be recommended for sample analysis, because apart from its reliable results, it can save reference substances. Additionally, a certain correlation was discovered between the content of the bioactive components and the morphological character of MHD.

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