Simultaneous Determination of Chlorogenic Acid, Caffeic Acid, Alantolactone and Isoalantolactone in *Inula helenium* by HPLC

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A rapid and sensitive high-performance liquid chromatographic (HPLC) method was developed for the simultaneous separation and determination of chlorogenic acid, caffeic acid, alantolactone and isoalantolactone in *Inula helenium*. The HPLC separation was performed on an Elite Hypersil C18 column (200 × 4.6 mm i.d., 5 µm particle size) with a gradient elution of solvent A (acetonitrile) and solvent B (0.1% phosphoric acid in water) at a flow rate of 1.0 mL/min. Detection was monitored at 225 nm. The recovery of chlorogenic acid ranged from 95.6 to 107.7%, the recovery of caffeic acid ranged from 95.4 to 104.2%, the recovery of alantolactone ranged from 95.8 to 100.8% and the recovery of isoalantolactone ranged from 96.5 to 102.3%. The retention times for chlorogenic acid, caffeic acid, alantolactone and isoalantolactone were 5.2, 7.1, 25.6 and 26.6 min with the limits of detection of 0.069, 0.021, 0.039 and 0.051 µg/mL, respectively. Relative standard deviation for the intra-day and inter-day was ≤2.5%. The validated method is reliable for the routine control of these four compounds in *I. helenium*.

Introduction

*Inula helenium* L. (Compositae) is a perennial herb frequently found in Europe, Asia, North America and North Mongolia (1). The roots of *I. helenium* L. have been widely used as a diuretic, diaphoretic, expectorant and anthelmintic remedy (2). Moreover, it can also be used for the treatment of various ailments including asthma, abdominal distention, vomiting, diarrhea and lung disorders (3, 4). In addition, modern pharmacological studies have shown that the extract of *I. helenium* possessed antibacterial, antitumor, antiproliferative, anti-inflammatory and antistressor activities (5–8).

Up to now, the focus is primarily on the alantolactone and isoalantolactone (9, 10). However, to our surprise, a high content of chlorogenic acid and caffeic acid was also found in *I. helenium* (11). Research studies have shown that chlorogenic acid and caffeic acid demonstrated a wide range of pharmacological activities including antioxidant (12, 13), antimicrobial activity (14), anti-obesity (15), anti-inflammatory, antiinociceptive and antipyretic activities (16). The molecular structures of chlorogenic acid, caffeic acid, alantolactone and isoalantolactone are displayed in Figure 1.

The process of the separation and identification of alantolactone and isoalantolactone was relatively complicated. Numerous analytical methods such as high-performance capillary electrophoresis (17) and gas chromatography (18) were used to separate and identify two isomers. These methods were accurate and the result was credible, but expensive or time consuming. An improved high-performance liquid chromatographic (HPLC) method was used for the separation and determination of alantolactone and isoalantolactone (10, 19). In contrast, the separation and determination of chlorogenic acid and caffeic acid are simpler, and many analytical methods are reported in the literature (20–22). However, to the best of our knowledge, there are no reports on the simultaneous separation and determination of these four studied components. The main purpose of this study is to develop a simple and convenient HPLC method for the determination of chlorogenic acid, caffeic acid, alantolactone and isoalantolactone in *I. helenium*.

Experimental

Materials and chemicals

The roots of *I. helenium* were purchased from Hebei Province, Henan Province, Sichuan Province and Gansu Province, China. The specimen (no. 2011–10) was deposited in the Department of Pharmacy, Hebei North University.

Alantolactone (purity 98%, w/w) and isoalantolactone (purity 98%, w/w) were purchased from Nanjing ZeLang Medical Technology Co., Ltd. (Nanjing, China). Caffeic acid and chlorogenic acid were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC grade acetonitrile was kindly supplied by the Adams (Shanghai, China). All other reagents used in this study were of analytical grade.

Apparatus

Quantitative HPLC was performed on an Agilent HP 1100 series HPLC system (Agilent Technologies, USA) consisting of a quaternary pump solvent delivery system, an auto degasser, a variable-wavelength UV detector and a PC for data processing. Ultrasound-assisted extraction (UAE) was performed using an ultrasonic cleaning bath (KQ-250V; Kun-Shan Ultrasonic Instruments Co., Ltd, Kun-Shan, China).

Preparation of stock solution

The stock solution was prepared by dissolving 2.32 mg of chlorogenic acid, 3.36 mg of caffeic acid, 2.10 mg of alantolactone and 2.02 mg of isoalantolactone in 10 mL of methanol, respectively. All the stock solutions were stored at 4°C.

Extraction solvents, ultrasonic time and solid–liquid ratio

Air-dried roots of *I. helenium* were ground to a powder and passed through a 60-mesh sieve. The powder (5.0 g) was
mixed with solvent in a 300-mL conical flask. Three different solvents, water, ethanol and methanol, were used to extract chlorogenic acid, caffeic acid, alantolactone and isoalantolactone. Ultrasound equipment operated at a frequency of 40 kHz, power of 100 W and temperature of 25 ± 8°C. After extraction, the mixture was centrifuged for 15 min at 3,000 rpm (4–15 high speed centrifuge; Sigma-Aldrich, Germany) for deposit suspension particle. The supernatant solution was filtered through a 0.22-μm membrane and utilized for further analysis. To optimize the extraction efficiency, different solid–liquid ratios (1:10, 1:20, 1:30 and 1:40) and ultrasonic times (30, 40, 50 and 60 min) were compared.

Calibration curve, limit of detection and precision
The calibration curves for chlorogenic acid, caffeic acid, alantolactone and isoalantolactone were constructed by dilution of a mixed standard solution to different concentrations with methanol. Standard solution for chlorogenic acid (4.35, 8.70, 17.40, 34.80 and 69.60 μg/mL), caffeic acid (2.10, 4.20, 8.40, 16.80 and 33.60 μg/mL), alantolactone (3.938, 7.875, 15.75, 31.50 and 63.00 μg/mL) and isoalantolactone (3.788, 7.575, 15.15, 30.30 and 60.60 μg/mL) were prepared. The calibration curves were constructed by plotting the mean peak areas versus concentration. Limits of detection (LODs) were the lowest analyte concentration at which the signal-to-noise ratio (S/N) is equal to 3:1. The precision was determined by the intra-day and inter-day analysis of standards.

Results
Optimization of chromatographic conditions
Selection of chromatography column
In this section, several chromatography columns including Merck Purospher Star C18, Agilent Eclipse XDB-C18, Elite Hypersil C18 and Dikma Diamonsil C18 columns were tested to separate the four compounds. The results showed that the separation performance was better in the Elite Hypersil C18 column. Further studies indicated that a better resolution and a shorter retention time were achieved at the column length of 200 mm.

Selection of detection wavelength
To achieve maximum sensitivity, the UV spectrum of these four compounds was scanned. It can be seen in Figure 2 that all the compounds have a greater absorbance at 225 nm. Therefore, the detection wavelength was set at 225 nm for the simultaneous determination of these compounds.

Chromatographic conditions
For the quantification of chlorogenic acid, caffeic acid, alantolactone and isoalantolactone, RP-HPLC was conducted using an Elite Hypersil C18 column (200 × 4.6 mm i.d., 5 μm particle size); the mobile phase consisted of acetonitrile (solution A) and 0.1% H3PO4 (solution B). The flow rate and column temperature were set to 1 mL/min and 30°C, respectively. The gradient elution technique was employed because of the different polarities of four compounds, and the gradient elution procedure is shown in Table I. The HPLC chromatogram is shown in Figure 3.

Optimization of extraction procedure
Effect of extraction methods
A study and a comparison of Soxhlet extraction, heat reflux extraction and UAE for four compounds (chlorogenic acid, caffeic acid, alantolactone and isoalantolactone) were carried out in order to establish an effective extraction method. The experimental conditions and the yield of four compounds are shown in Table II. UAE for 0.5 h got a higher extraction yield than Soxhlet extraction for 4 h and heat reflux extraction for 1 h, respectively (Table II). Compared with the reflux extraction and Soxhlet extraction, higher extraction efficiency and reduced extraction time were obtained by using UAE.

Effect of extraction solvent
Methanol, water and ethanol were used to evaluate the efficiency of the extraction solvent. The yield of chlorogenic acid, caffeic
acid, alantolactone and isoalantolactone, as shown in Figure 4A, was reached a maximum when the solution was methanol. The main reason may be attributable to the four compounds that have greater solubility in methanol.

Effect of ultrasonic time
In order to obtain the maximum yield of four compounds from the root of *I. helenium*, different extraction times (30, 40, 50 and 60 min) were experimented. As shown in Figure 4B, the higher yield was obtained with an extraction time of 40 min.

Effect of solid–liquid ratio
To determine the effect of the 'solid–liquid' ratio on the extraction yield, experiments were carried out at the ratio ranging between 1:10 and 1:40 (g/mL). As shown in Figure 4C, the extraction yield was initially increased when the ratio increased from 1:10 to 1:20 and then remained fairly constant. Similar observations have also been made in other UAE experiments (11, 23). Therefore, the final extraction conditions were as follows: extraction method, UAE; extraction solvent, methanol; extraction time, 40 min and solid–liquid ratio, 1:20 (g/mL).

Method validation

Linearity and range
A mixed standard solution was prepared from individual stock solutions and the final concentration is 69.60 µg/mL of chlorogenic acid, 33.60 µg/mL of caffeic acid, 63.00 µg/mL of alantolactone and 60.60 µg/mL of isoalantolactone. Then, this standard solution was diluted at five different concentrations and analyzed in triplicate per level. Table III shows the linear calibration regression equations and correlation coefficients for chlorogenic acid, caffeic acid, alantolactone and isoalantolactone.

Sensitivity
The LOD and limit of quantitation (LOQ) of each compound using the above-mentioned chromatographic conditions are also shown in Table III. The S/N is a measurement that compares the level of background noise. It is defined as the ratio of signal power to the noise power. The lowest analyte concentration is the content detected when the S/N is equal to 3:1.

Precision
The repeatability test was conducted by repeatedly measuring a standard solution. The intra-day precision was determined by performing six aliquots at each sample level during a single day, and the inter-day precision was determined by performing six aliquots at each sample level in different days. The relative standard deviation (RSD), also termed coefficient of variation (CV), was used as a statistic parameter. The intra- and inter-day precision RSD were < 2.47%, indicating that the proposed method is highly precise (Table IV).

Recovery
To verify the accuracy of the method, different amounts of known standards were added to the sample solution. The samples were prepared in triplicate at each level and the accuracy was expressed as recovery percentage. Good percentage recoveries were obtained and the results are shown in Table V.

Discussion

Optimization of chromatographic conditions
Chromatographic conditions determine the retention time and resolution of each analyte. Under the above-mentioned conditions, the four studied compounds were separated from the baseline. The chromatograms of four components in the standard solution (A) and UAE (B) are shown in Figure 3. The retention time of chlorogenic acid, caffeic acid, alantolactone and isoalantolactone was about 5.2, 7.1, 25.6 and 26.6 min, respectively. In all extraction samples, no component was observed interfering with the quantification of four components.

Method validation
Method validation was carried out in accordance with the ICH guidelines (24). The method was evaluated in terms of linearity, range, and precision. The results showed that the method was sensitive, precise, and accurate.
specificity, accuracy, precision, LODs and quantitation. The method validation for the quantification of four components showed good linearity and sensitivity ($R^2 > 0.999$).

To determine the LOD and LOQ, a sample solution was injected into the HPLC instrument. The LOD is the lowest analyte concentration at which the S/N is equal to 3:1, while the LOQ is defined as the S/N of 10:1. Precision was expressed as RSD.

### Table III
Linear Regression Data and Validation for Four Compounds ($n = 3$)

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Regression equation</th>
<th>$R^2$</th>
<th>Linear range (cL)</th>
<th>LOQ ($\mu$g/mL)</th>
<th>LOD ($\mu$g/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorogenic acid</td>
<td>$Y = 23.317X - 9.817$</td>
<td>0.9996</td>
<td>4.35 – 69.60</td>
<td>0.218</td>
<td>0.069</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>$Y = 84.128X - 8.888$</td>
<td>0.9995</td>
<td>2.10 – 33.60</td>
<td>0.084</td>
<td>0.021</td>
</tr>
<tr>
<td>Alantolactone</td>
<td>$Y = 38.349X + 4.886$</td>
<td>0.9999</td>
<td>3.938 – 63.60</td>
<td>0.158</td>
<td>0.039</td>
</tr>
<tr>
<td>Isolantolactone</td>
<td>$Y = 32.537X - 0.4826$</td>
<td>0.9988</td>
<td>3.788 – 60.60</td>
<td>0.189</td>
<td>0.051</td>
</tr>
</tbody>
</table>

*Y, peak area; X, concentration ($\mu$g/mL).*

### Table IV
Intra- and Inter-Day Precision for the Four Compounds ($n = 6$)

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration ($\mu$g/mL)</th>
<th>Intra-day precision (RSD, %)</th>
<th>Inter-day precision (RSD, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorogenic acid</td>
<td>8.70</td>
<td>2.13</td>
<td>1.22</td>
</tr>
<tr>
<td></td>
<td>34.80</td>
<td>1.83</td>
<td>1.47</td>
</tr>
<tr>
<td></td>
<td>69.60</td>
<td>1.25</td>
<td>1.36</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>4.20</td>
<td>2.47</td>
<td>1.75</td>
</tr>
<tr>
<td></td>
<td>16.80</td>
<td>1.77</td>
<td>1.44</td>
</tr>
<tr>
<td></td>
<td>33.60</td>
<td>0.96</td>
<td>1.03</td>
</tr>
<tr>
<td>Alantolactone</td>
<td>3.94</td>
<td>1.63</td>
<td>1.26</td>
</tr>
<tr>
<td></td>
<td>15.75</td>
<td>0.88</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>31.50</td>
<td>0.71</td>
<td>0.49</td>
</tr>
<tr>
<td>Isolantolactone</td>
<td>3.79</td>
<td>1.28</td>
<td>1.35</td>
</tr>
<tr>
<td></td>
<td>15.15</td>
<td>0.69</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td>30.30</td>
<td>0.94</td>
<td>0.68</td>
</tr>
</tbody>
</table>

### Table V
Recoveries for the Four Compounds ($n = 6$)

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Original level ($\mu$g/mL)</th>
<th>Added ($\mu$g/mL)</th>
<th>Measured ($\mu$g/mL)</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorogenic acid</td>
<td>10.72</td>
<td>5.22</td>
<td>5.62</td>
<td>107.7</td>
<td>1.84</td>
</tr>
<tr>
<td></td>
<td>10.44</td>
<td>10.27</td>
<td>98.37</td>
<td>1.12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20.88</td>
<td>19.96</td>
<td>95.59</td>
<td>1.76</td>
<td></td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>3.88</td>
<td>4.37</td>
<td>4.23</td>
<td>96.79</td>
<td>1.47</td>
</tr>
<tr>
<td></td>
<td>8.74</td>
<td>9.11</td>
<td>104.2</td>
<td>1.62</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17.48</td>
<td>16.68</td>
<td>95.42</td>
<td>1.35</td>
<td></td>
</tr>
<tr>
<td>Alantolactone</td>
<td>25.94</td>
<td>12.18</td>
<td>11.67</td>
<td>95.81</td>
<td>1.26</td>
</tr>
<tr>
<td></td>
<td>24.36</td>
<td>24.01</td>
<td>98.56</td>
<td>1.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48.72</td>
<td>49.14</td>
<td>100.9</td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td>Isolantolactone</td>
<td>14.37</td>
<td>11.23</td>
<td>10.84</td>
<td>96.53</td>
<td>1.37</td>
</tr>
<tr>
<td></td>
<td>44.92</td>
<td>45.35</td>
<td>100.9</td>
<td>0.77</td>
<td></td>
</tr>
</tbody>
</table>

### Table VI
The Contents of Four Components from Different Sources (mg/g) ($n = 6$)

<table>
<thead>
<tr>
<th>Province</th>
<th>Chlorogenic acid</th>
<th>Caffeic acid</th>
<th>Alantolactone</th>
<th>Isolantolactone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hebei Province</td>
<td>1.04 ± 0.11</td>
<td>0.39 ± 0.05</td>
<td>25.87 ± 1.29</td>
<td>14.22 ± 0.76</td>
</tr>
<tr>
<td>Henan Province</td>
<td>0.87 ± 0.09</td>
<td>0.34 ± 0.05</td>
<td>22.22 ± 1.31</td>
<td>13.16 ± 0.68</td>
</tr>
<tr>
<td>Sichuan Province</td>
<td>0.95 ± 0.13</td>
<td>0.26 ± 0.04</td>
<td>21.63 ± 1.17</td>
<td>13.77 ± 0.72</td>
</tr>
<tr>
<td>Gansu Province</td>
<td>0.97 ± 0.11</td>
<td>0.29 ± 0.06</td>
<td>22.86 ± 1.14</td>
<td>12.63 ± 0.66</td>
</tr>
</tbody>
</table>
while accuracy was expressed as the percent recovery and RSD. As shown in Tables IV and V, these four compounds have good precision and accuracy, with satisfactory recoveries and small relative errors.

Sample analysis
After method validation, the improved HPLC method was successfully used for the simultaneous determination of chlorogenic acid, caffeic acid, alantolactone and isoalantolactone in *I. belenium*. As shown in Table VI, there is a large difference among the contents of four components from different sources. The higher amounts of four components were observed that the herbs were collected in Hebei Province, China.

Conclusion
A simple, fast and accurate HPLC method was established for the separation and quantitation of four active ingredients in *I. belenium*. The chromatographic conditions were optimized to achieve satisfactory resolution, accuracy and linearity. The results showed that the proposed method could be used for the quality control of *I. belenium*.

Acknowledgments
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