Quantitative analysis of ligustilide in roots and stems of
Angelica sinensis (Oliv.) Diels.

Pattra Ahmadi Pirshahid*, Thongchai Hemthanon*, Yaowaluk Khamphan*,
Phatsuda Chueboonmee*, Chuleratana Banchonglikitkul*

*Pharmaceutical and Natural Products Department, Thailand Institute of
Scientific and Technological Research, 35 Moo 3, Klong 5, Klong Luang, Pathum Thani, Thailand 12120

ABSTRACT

Medicinal herb, Angelica sinensis (Oliv.) Diels, belongs to Umbelliferae
family, is indigenous plant in the eastern country such as China, Korea,
and Japan. Northern part of Thailand, people always use A. sinensis as
for cooking. In Asia, the plants have been cultivated for medicinal
purpose and was recorded for treating a wide variety of female
disorders. Currently, the popular use of A. sinensis is the treatment of
menopausal symptoms, hot flashes, promote a healthy menstrual
cycle and painful during menstruation. Ligustilide is the most abundant
active component in A. sinensis and was reported that the therapeutic
pharmacological activity is most often attributed to the Ligustilide
content. In this paper, the quantitative analysis of Ligustilide in the
ethanol extract of separated whole root (RE) and aerial stems
including leaves (SE) are investigated. The result of Ligustilide analysis
will be used as a marker compounds and could be served as a
fingerprint for quality control of A. sinensis. The content of
Ligustilide can lead us to utilize the A. sinensis extract as a
dietary supplement products for hormone replacement from both
whole root and aerial stems including leaves. This analysis had
performed by High Performance Liquid Chromatography, method
validation; specificity, linearity, range, Limit of Detection (LOD),
Limit of Quantitation (LOQ), precision as well as accuracy (%recovery)
are reported.

Keywords: Angelica sinensis (Oliv.) Diels, Ligustilide, Menopause


1. INTRODUCTION

The deficiency of estrogen hormone is one of the main causes of women menopause such as sweating, mood swing, hot flashes [1] and contributes to adverse health problems such as osteoporosis or the loss of bone density [2]. Hormone replacement therapy (HRT) has been shown as an effective method in relieving menopause symptoms [3]. However, HRT is generally not recommended due to the risk of breast cancer [4, 5]. Medicinal plants are a natural alternative to conventional HRT to treat menopause [6]. In traditional Chinese medicine, the hot water extract of the herb mixture of *Radix Astragali* and *Radix Angelica sinensis* (Oliv.) Diels. has been used to treat blood circulation and immune system [7]. Health benefits of the herbal extract are attributed to active compounds found in *A. sinensis*, mainly ligustilide that is largely responsible for the pharmacological activity [8]. Ligustilide is a bioactive marker for the authentication and standardization of *A. sinensis*. The objective of the present study was to develop a reversed-phase high performance liquid chromatography (RP-HPLC) method for quantitative determination of Ligustilide from the ethanol extracts of separated whole roots (RE) and aerial stems including leaves (SE) of *A. sinensis*. Validation of the analytical method was performed in terms of specificity, linearity, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ).

![Figure 1. Structure of ligustilide ((3Z)-3-butylidene-4,5-dihydro-2-benzofuran-1-one)](image)

2. MATERIALS AND METHOD

**Materials**

About six month old *Angelica sinensis* (Oliv.) Diels. plants were obtained in June 2013 from The Agricultural, Forest, Environmental Demonstration and Transfer Station Project at Baan Lee Sor, Paek Sam, Chiang Mai province. Commercial grade ethanol 95% was provided by The Liquor Distillery Organization. HPLC grade methanol and phosphoric acid were obtained from RCI Labscan Ltd. Ligustilide reference standard was purchased from Standlord Chemicals, Co., Ltd.

**Preparation of plant material**

The whole roots and aerial stems including leaves were separately rinsed with water, sliced into small pieces, and then oven-dried at 45°C for 15-24 h. The dried roots and stems were ground and stored in a plastic bag at room temperature for subsequent extraction.

**Preparation of extracts**

1 gm of the dried ground sample of each part (roots and stems) of *A. sinensis* was extracted with 95% ethanol under stirring at ambient temperature for 20 min. The solid was separated from the liquor by filtration, followed by re-extraction for 4 times. The filtered solution was combined together, and then evaporated under vacuum at 40°C to yield the crude extracts of roots (RE) and stems (SE) of *A. sinensis*.

**HPLC analysis**

Chromatographic analysis was performed with a Waters 600 controller equipped with a Waters 486 tunable absorbance detector. Data were analyzed using Clarity chromatography software (Data Apex, Czech Republic). Chromatographic analysis was performed with a Waters Xterra reversed-phase C18 column (5 µm, 3.9 x 150 mm) with a mobile phase gradient consisting of acetonitrile and 0.05% phosphoric acid (pH 5.0) at a flow rate of 1 ml/min and a detection wavelength at 288 nm.

**Preparation of standard solution**

An accurate weight of 5 mg of ligustilide reference standard was transferred into a 25 ml volumetric flask, sonicated for 15 min and adjusted to the final volume of 50 ml with methanol. The stock solution was then diluted to concentrations of 10, 20, 30, 40, 50 and 60 µg/ml. These serial different dilutions were filtered through 0.2 µm cellulose membrane before use.
Preparation of sample solutions

An accurate weight of 0.1 g each of RE and SE of *A. sinensis* was dissolved with 20 ml methanol in a 50 ml volumetric flask, and sonicated for 15 min. The mixture was then adjusted to the final volume of 50 ml with methanol. The solution was filtered through a 0.45 µm cellulose membrane prior injects to HPLC.

Validation of HPLC method

The validation of HPLC method was examined in terms of specificity, linearity, precision, accuracy, limit of detection (LOD), and limit of quantification (LOQ).

Specificity

The standard Ligustilide solution and sample solutions of RE and SE were prepared in acetronitrile. A volume of 10 µl was injected into the HPLC column individually.

Linearity

The linearity was validated by preparing the standard ligustilide solutions at least 5 concentrations. A volume of 10 µl of each concentration was injected to the HPLC column. Triplicate determinations were performed. The standard curve was analyzed using the linear least-squares regression equation derived from the peak area.

Precision and sample preparation

The precision of the analytical method was validated by determining the content of ligustilide in RE and SE of *A. sinensis*. Concentrations of the standard ligustilide were calculated with the linear equation of standard curve. Triplicate determinations were performed. Coefficient of variation (CV) was calculated as a standard deviation (SD) to the mean value from the results obtained and not more than 2%.

Accurately weighted 0.1 g of RE in a volume flask 50 ml for 6 samples sonicated for 15 min then adjusted to final volume with methanol and filter through cellulose membrane 0.2 µm prior to HPLC.

Accuracy and sample preparation

The standard ligustilide with the determined amount was spiked to the RE sample solution. Triplicate injections for each concentration were performed. Recovery (%) of the standard ligustilide was calculated.

Accurately weighted 0.1 g of RE for 9 samples sonicate for 15 min, added standard ligustilide at concentration 20, 40 and 60 ppm to each 3 sample of RE respectively, then adjust to final volume of 50 ml by methanol, filter through 0.2 µm cellulose membrane prior to HPLC injection for triplicate.

LOD and LOQ

For the limit of detection (LOD) and limit of quantification (LOQ), serial dilutions of ligustilide were prepared and analyzed using the HPLC method. LOD and LOQ were obtained as the ratio of signal to noise equal to 3 and 10, respectively.

Figure 2. *Angelica sinensis* (Oliv.) Diels.
3. RESULTS

Specificity validation
HPLC chromatograms for specificity validation are shown in Figure 3. Ligustilide was identified as the main compound in the ethanol extracts of the whole roots (RE) and aerial stems including leaves (SE) of *A. sinensis*, with retention time of 32.2 min.

Figure 3. HPLC chromatograms for specificity validation: (1) ligustilide reference standard; (2) RE and (3) SE.

Linearity and Range
The standard curve of ligustilide was examined from the results of Table 1. The curve is linear at the concentrations range of 10-60 µg/ml. The linear equation of $Y = 28.34X + 21.04$ and correlation coefficient ($R^2$) of 0.999 were obtained as shown in Figure 4.

Table 1. Peak areas of standard ligustilide at the concentrations range of 10 to 60 µg/ml for the linearity

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Peak area 1</th>
<th>Peak area 2</th>
<th>Peak area 3</th>
<th>Mean peak area</th>
<th>SD</th>
<th>% CV (%RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>299.84</td>
<td>299.702</td>
<td>295.545</td>
<td>298.362</td>
<td>2.441</td>
<td>0.818</td>
</tr>
<tr>
<td>20</td>
<td>588.83</td>
<td>589.408</td>
<td>585.109</td>
<td>587.782</td>
<td>2.333</td>
<td>0.397</td>
</tr>
<tr>
<td>30</td>
<td>880.792</td>
<td>879.079</td>
<td>878.300</td>
<td>879.390</td>
<td>1.275</td>
<td>0.145</td>
</tr>
<tr>
<td>40</td>
<td>1159.515</td>
<td>1157.976</td>
<td>1159.993</td>
<td>1159.161</td>
<td>1.054</td>
<td>0.091</td>
</tr>
<tr>
<td>50</td>
<td>1437.161</td>
<td>1434.986</td>
<td>1436.18</td>
<td>1436.109</td>
<td>1.089</td>
<td>0.076</td>
</tr>
<tr>
<td>60</td>
<td>1711.782</td>
<td>1717.532</td>
<td>1722.824</td>
<td>1717.379</td>
<td>5.523</td>
<td>0.322</td>
</tr>
</tbody>
</table>

Coefficient of deviation (CV) = SD/mean x100%

Figure 4. The standard linear curve of ligustilide: $Y = 28.34X + 21.04$, $R^2 = 0.999$, $Y$ is peak area, $X$ is the concentration of the analyzed sample.
Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ for ligustilide are 0.63 and 2.12 µg/ml, respectively. The LOD and LOQ values are low, indicating the good sensitivity of the HPLC method.

Precision

Comparing the measured concentration with the standard concentration, the coefficient variations for ligustilide in RE and SE of *A. sinensis* were 0.257. All the coefficient variations were less than 2%, indicating that the HPLC method for quantitative determination of ligustilide from the ethanol extracts of *A. sinensis* has good precision.

Table 2. Precision validation of the HPLC method for determination of ligustilide from RE of *A. sinensis* (Ang khang area)

<table>
<thead>
<tr>
<th>Samples</th>
<th>Standard concentration (µg/ml)</th>
<th>Measured concentration (µg/ml)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RE</td>
<td>46-53</td>
<td>2.269 ± 0.006</td>
<td>0.257</td>
</tr>
</tbody>
</table>

Values are mean ± SEM as obtained by triplicate determinations, Coefficient of deviation (CV) = SD/mean x100%

Accuracy

As presented in Table 3, the percentage recovery of ligustilide ranged from 99.52 to 101.17%, with 0.54 to 1.40% of coefficient variations. The results demonstrate that the HPLC method has good accuracy.

Table 3. Accuracy validation of the HPLC method for ligustilide

<table>
<thead>
<tr>
<th>Spiked level (µg/ml)</th>
<th>Nominal (%)</th>
<th>Recovery (%) (n = 3)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>50</td>
<td>101.17</td>
<td>0.54</td>
</tr>
<tr>
<td>40</td>
<td>100</td>
<td>99.52</td>
<td>1.34</td>
</tr>
<tr>
<td>60</td>
<td>150</td>
<td>99.94</td>
<td>1.40</td>
</tr>
</tbody>
</table>

Coefficient of deviation (CV) = SD/mean x100%

Determination of ligustilide contents in ethanol extracts of *A. sinensis*

The amounts of ligustilide found in ethanol extracts of the whole roots (RE) and aerial stems including leaves (SE) of *A. sinensis* were 0.562 and 0.887 mg/g as shown in Table 4.

Table 4. Ligustilide contents (mg/g) in ethanol extracts of *A. sinensis*

<table>
<thead>
<tr>
<th></th>
<th>RE</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.562 ± 0.001</td>
<td>0.887 ± 0.001</td>
</tr>
</tbody>
</table>

Values are mean ± SEM as obtained by triplicate determinations

4. CONCLUSION

A reversed-phase high performance liquid chromatography (HPLC) method was used for analysis of Ligustilide from the ethanol extracts of separated whole roots (RE) and aerial stems including leaves (SE) of *A. sinensis*. The analytical method was validated and showed good linearity, precision, accuracy and low LOD and LOQ. This method is suitable for quality control of ligustilide from the ethanol extracts of *A. sinensis*, which will be further developed to dietary supplement product.
ACKNOWLEDGEMENTS

The author would like to thank Mr. Kanchit Laochai, Royal administration officer, The Agricultural, Forest, Environmental Demonstration and Transfer Station Project at Baan Lee Sor Paek Sam, Chiang Mai province, whom support the raw material of *Angelica sinensis* (Oliv.) Diels. throughout this project. This study was supported by Thailand Institute of Scientific and Technological Research (TISTR), Ministry of Science and Technology on research funds.

REFERENCES