Development and validation of HPLC method for Z-ligustilide in *Angelica sinensis* (Danggui) root capsules

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**Keywords:** Danggui roots, *Angelica sinensis*, HPLC, Z-ligustilide

**Introduction**

“Danggui root capsules”, developed from Danggui root extract, is a herbal nutraceuticals for menopausal symptom relieving. This product has passed efficacy and safety evaluation in animal testing. Danggui root (*Angelica sinensis*), a herb of the Apiaceae family, has been used in Chinese medicine to treat menstrual disorders. Over 70 compounds have been identified from Danggui, including essential oils such as ligustilide, butylphthalide and senkyunolide A, phthalide dimers, organic acids and their esters such as ferulic acid, coniferyl ferulate, polyacetylenes, vitamins and amino acids. Z-ligustilide (water insoluble and heat stable) is thought to be the most biologically active components in *Angelica sinensis* and is often used in quality control and pharmacokinetic studies of Danggui. The objective of this research is to develop and validate the analytical method of Z-ligustilide for quality control of this product. The chemical structures of Z-ligustilide are shown in Figure 1.

![Chemical Structures of Z-ligustilide](image)

**Methods**

**A. Reagents and samples**

Z-ligustilide was purchased from Phytolab, Germany. Acetonitrile and formic acid were HPLC grade from Lab-Scan, Thailand. All the water used in this study was Ultrapure, obtained from a Milli-Q RO system (Milipore Corporation, France). The Danggui root capsules were developed in our research from Danggui root extract.

**B. Preparation of sample solution**

The granules 865 mg was weighted and extracted with 30 ml methanol by sonicator for 10 minutes (3 times). The solution was filtered through a Whatman No.1. The filtrate was evaporated to less than 50 ml, then transferred to 50 ml volumetric flasks and the volume of each was adjusted to 50 ml with methanol. After filtering through a 0.2 µm syringe filter, the final sample was injected directly.

**C. Preparation of standard solution**
Z-ligustilide (10 mg) was dissolved with methanol (10 ml), to get stock solution containing 1000 µg/ml.

The stock solutions were diluted to create the five-point standard curves of Z-ligustilide using concentration at 30-90 µg/ml.

D. Instrumentation and chromatographic conditions

The analytical method of Z-ligustilide was performed on a Waters Alliance e2695 LC system connected with a Waters model 2996 photodiode-array detector. Data collection and processing were carried out using an Empower workstation. The optimum HPLC system was comprised of a C18 reverse phase column (Luna C18, 250x4.6 mm i.d., 5 µm particle size). The gradient was eluted with acetonitrile and 0.5% formic acid at a flow rate of 1.0 ml/min and PDA detection at 321.0 nm. The mobile phase consisted acetonitrile and 0.5% formic acid and all solutions were degassed and filtered through a 0.20 µm pore size filter (Millipore, USA).²

E. Method validation

The analytical method was validated on specificity, precision, accuracy, linearity, range, and limits of detection and quantification.

F. Statistical calculations

Standard regression curve analysis was performed by using Micro-soft Office Excel 2007 software (Microsoft, USA), without forcing through zero. Means and standard deviations were calculated by using SPSS software version 9.5 (SPSS, Cary, NC, USA).

Results and Discussions

A. Specificity of the developed method

The specificity of this method was determined by analysis of the blank, placebo, and sample solution chromatograms (Figures 2-5). Good separation between Z-ligustilide and matrix was achieved, with the retention times, 33.196 min by comparing chromatograms of blank, placebo, standard and sample, there was no interference observed from the peaks of the blank and placebo. It showed that the method is high specificity.

B. Linearity and range of the developed method

For linearity studied, five solutions in the ranges of 30-90 µg/ml for Z-ligustilide were analyzed. Each concentration was made and analyzed in triplicate. The peak areas obtained from each concentration of the analytes were used to build a linear regression equation as well as determined the value of correlation coefficient (Table 1). Good linearity was observed over the above mentioned range with linear regression equation Y = 514444x - 34477 (x is concentration of analytes in µg/ml and Y is peak area). The values of correlation coefficient were 0.9973.

C. Accuracy of the developed method

This study was performed by adding known amounts of Z-ligustilide to the placebo samples. A level of solutions were made and having concentrations at 60 µg/ml. The recovery range for Z-ligustilide was 93.06 % (limit 80 to 110% ) (3, 4)

D. Precision of the developed method

Repeatability was studied by calculating the relative standard deviation (RSD) from six determinations of the 100% concentration of sample. The studied was performed on the same day and under same experimental conditions. The concentrations of Z-ligustilide determinations in the sample solution with the relative standard deviation were calculated (Table 3). The RSD value obtained for Z-ligustilide was 2.75. (limit less than 3.7%). (3, 4) The result showed that the developed method was precise.

E. Sensitivity of the developed method

LOD were calculated by using the following equations. LOD = 3.3 x SD/S and LOQ = 10 x SD/S, where SD = the standard deviation of the response, S = Slope of the calibration curve. The LOD values were 4.28 µg/ml and the LOQ values were 14.28 µg/ml of Z-ligustilide, respectively. Method validation following ICH guidelines indicated that the developed method had high sensitivity.
Figure 2 HPLC Chromatogram of blank solution

Figure 3 HPLC Chromatogram of Placebo Solution

Figure 4 HPLC Chromatogram of Z-ligustilide (standard solution)

Figure 5 HPLC Chromatogram of sample solution

Table 1  Linearity and range for Z-ligustilide by HPLC

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Z-ligustilide</th>
<th>Concentration (µg/mL)</th>
<th>Peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>30</td>
<td>1,500,674</td>
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<tr>
<td>2</td>
<td></td>
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<td></td>
<td>60</td>
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<tr>
<td>4</td>
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<td>3,719,844</td>
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<tr>
<td>5</td>
<td></td>
<td>90</td>
<td>4,741,735</td>
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</table>
Figure 6 Calibration Curve of Z-ligustilide by HPLC

Table 2  Accuracy data of Z-ligustilide by HPLC

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Amount (µg/mL)</th>
<th>% Recovery</th>
<th>% RSD</th>
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<tbody>
<tr>
<td>Z-ligustilide</td>
<td>60</td>
<td>93.06</td>
<td>1.77</td>
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</table>

Table 3  Precision studies of Z-ligustilide by HPLC

<table>
<thead>
<tr>
<th>N</th>
<th>% W/W</th>
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<td>5</td>
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<tr>
<td>% RSD</td>
<td>2.75</td>
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</table>

Conclusion

Using this method, Z-ligustilide could be determined and the validity of the method was also verified. The proposed analytical method for estimation of Z-ligustilide in the Danggui root capsules is accurate, precise, linear, robust, reproducible and within the range.

Acknowledgements

The authors would like to acknowledge financial support from Thailand Institute of Scientific and Technological Research.

References