Relationship between HPLC fingerprints and in vivo pharmacological effects of a traditional Chinese medicine: Radix Angelicae Dahuricae

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We report the fingerprint development of a traditional Chinese medicine Radix Angelicae Dahuricae root and the correlation of the fingerprint peaks with its in vivo pharmacological effects. The high-performance liquid chromatography (HPLC) methods with the computer aided similarity evaluation were validated and used in serial pharmacological studies in mice. The major constituents of R. Angelicae Dahuricae were successfully separated by the HPLC methods, and the effects of sedation and analgesia were mainly related to the chromatographic peaks of group II. The anti-inflammatory, anti-heat stroke and anti-endotoxemic effects were mainly related to the peaks in group III. These results indicated a correlation between the HPLC fingerprints in groups and the pharmacological effects of R. Angelicae Dahuricae. This simple and accurate method can be used for the identification of the active components of R. Angelicae Dahuricae and for the quality control of its pharmaceutical preparations.

Keywords: Radix Angelicae Dahuricae; fingerprint chromatograms; HPLC; fingerprint-effect correlation

1. Introduction

There is an increasing need for better quality control of traditional Chinese medicine (TCM) in order to ensure its efficacy and safety. The dried root of Angelica dahurica (Fisch. ex Hoffm.) Benth. et Hook. f. var. formosana (Boiss.) Shan et Yuan (R. Angelicae Dahuricae), or the Baizhi herb, has been widely used as an antipyretic and analgesic medicine for colds, headaches and toothaches (Pharmacopoeia Commission of the People’s Republic of China, 2005). The active constituents in R. Angelicae Dahuricae include coumarin components, such as imperatorin and isoimperatorin (Liu, Li, & Sun, 2004; Mammen, Kleiner, DiGiovanni, Sutter, &

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Strickland, 2005). This herb is grown and used all over China, but the levels of each component vary significantly due to differences in geographic origin, climate, environment and other factors.

For efficacy evaluation and quality control, it is not adequate to identify only one or a few components (Shao et al., 2003; Yu, Wu, & Cheng, 2002). The fingerprint analysis has been introduced and accepted by the World Health Organisation (WHO) (World Health Organization, 1991). Compared with thin layer chromatography, X-ray and capillary electrophoresis, high-performance liquid chromatography (HPLC) is often the first choice (Jiang, Tao, & Shao, 2007; Li et al., 2007; Zhang, Yang, & Deng, 2009). However, the HPLC technique also has its limitations, such as the lack of correlation between chemical composition and pharmacodynamic action. In this study, we developed a HPLC method to investigate the fingerprint-effect correlation for coumarin-containing *R. Angelicae Dahuricae*. Our results not only provide a practical method for the identification of the active components, which will be useful for future investigation, and the quality control of this TCM, but also contribute to the development of optimal methods for quality control for TCMs in general.

2. Results and discussion

2.1. Method validation

For precision testing, the RSDs of relative retention time and relative peak area were found to not exceed 1% and 3%, respectively. The repeatability testing showed the RSDs of the relative retention time and relative peak area were also below 1% and 3%, respectively. The sample stability test was determined with one sample over 24 h and the RSDs of the relative retention time and relative peak area were less than 1% and 3%, respectively.

2.2. Standardisation of fingerprints

In this fingerprint analysis, chromatographic peaks with the same relative retention time were selected as the common peak fractions in different samples. There were 13 common peaks in 10 analysed batches of *R. Angelicae Dahuricae* (Figure 1). The areas of all 13 peaks accounted for more than 81% of the total peak area. As shown in Figure 1, peak 11 is the standard compound imperatorin; peak 13 is the standard compound isoimperatorin. The fingerprint can be separated into three parts: group I (peaks 1, 2), II (peaks 3–10) and III (peak 11–13). The correlation coefficients of the similarity of the 13 batches were above 0.81. Figure 2 illustrates representative HPLC fingerprinting of various extracts.

2.3. Results of pharmacological experiments

In the hot-plate tests, the cyclohexane extract increased the pain threshold of the mice ($p < 0.05$) 15 min after drug treatment. Two hours after treatment, the cyclohexane extract had a significant analgesic effect compared to the Tween-80
The effects of the acetoacetate and supercritical CO₂ fluid extracts were weaker than that of the cyclohexane extract.

In the experiment of acetic acid-induced abdominal constrictions, the cyclohexane extract had a significant anti-inflammatory effect compared with the Tween-80 group ($p < 0.01$). The effects of the acetoacetate extract and the acetone extract were weaker than that of the cyclohexane extract ($p < 0.05$).

In the sedation experiment, the cyclohexane extract had the highest effect ($p < 0.01$) in comparison with the other extracts, while the effects of the supercritical CO₂ fluid and acetoacetate extracts were weaker compared to the Tween-80 group.

In the anti-inflammatory test, the acetoacetate extract had the highest anti-inflammatory effect ($p < 0.01$) in comparison with the other extracts.
In the heat-stroke testing, the acetoacetate and acetone extracts had the greatest effects on limiting the rate of increase in rectal temperatures and lengthening the lifetime of mice in comparison with the other extracts or controls ($p < 0.01$ versus the model control group).

In the endotoxemia testing, the acetoacetate extract had the greatest effect on lengthening the lifetime of mice in comparison with the other extracts ($p < 0.01$ versus the Tween-80 control group).

2.4. The relationship between characteristic fingerprints and pharmacological effects of the extracts

Our comparative studies of pharmacological actions were used to identify the active components in the extracts and establish the HPLC fingerprint–pharmacological effect relationship. The active components for anti-inflammation and anti-endotoxemia were mainly found in the acetoacetate extract. Comparisons of the chromatographs from various extracts indicated that the effects of sedation and analgesia were mainly attributable to peaks 11–13 (group II) of the ethanol extracts, which contained isoimperatorin and imperatorin, with retention times ranging from 30 to 40 min. The results also suggested that the effects of anti-inflammation, anti-heat stroke and anti-endotoxemia were attributable to substances that had retention times ranging from 20 to 30 min (group III).

3. Experimental

3.1. Materials and reference compounds

The dried root of *R. Angelicae Dahuricae* was obtained from 13 different herbal markets in seven provinces of China, including Sichuan (available with/without sulphur), Jiangsu, Yunnan, Zhejiang, Anhui, Henan and Hebei provinces. The species are all *Angelica dahurica* (Fisch. ex Hoffm.) Benth. et Hook. f.var. *formosana* (Boiss.) Shan et Yuan. All herbal samples were authenticated by Professor Ma Yuying in the Department of Pharmacognosy, Chengdu University of TCM, according to the *Chinese Pharmacopoeia* (Pharmacopoeia Commission of the People’s Republic of China, 2005). The voucher specimen (no. 05030202) was deposited at the Herbarium Center of Jinan University College of Pharmacy, Guangzhou, China. The samples were cut into smaller pieces, ground into powder, passed through a 20-mesh (0.9 mm) sieve and stored at $-4^\circ$C until use. Imperatorin (no. 11826-200410) and isoimperatorin (no. 1827-200407) were provided by the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China. The ethanol used for sample extraction was of analytical reagent grade, and the methanol was of chromatographic grade. Deionised water was obtained using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

3.2. Sample preparation

Sample powder (20 g) was added into a 250 mL volumetric flask, to which 160 mL of 95% alcohol was added. The extracts were evaporated until dry after a 2 h
recirculation in a heated water bath, and then redissolved in methanol. The suspended particles were removed by filtration through a 0.45 \( \mu \text{m} \) membrane filter. A volume of 20 \( \mu \text{L} \) of the filtered sample solution was then injected onto an HPLC column and separated under the chromatographic conditions described below.

The dried roots of *R. Angelicae Dahuricae* (1 kg) were treated with 2 L of each of the following solvents in sequence: cyclohexane, ethyl acetate, acetone, methanol, 95% ethanol and water under reflux for 3 h. The extracts in each solvent were filtered and dried at 50\(^\circ\)C. The yields of prepared extract (w/w) were 1.14\%, 0.71\%, 1.73\%, 3.94\%, 2.11\% and 1.80\%, respectively. The extract from *R. Angelicae Dahuricae* using the supercritical CO\(_2\) fluid extraction method was provided by the Guangzhou Institute of Pharmaceutical Industry, Guangzhou, China.

### 3.3. Instrumentation

An Agilent/HP 1100 Series high-performance liquid chromatography–diode array detectors (HPLC–DAD) system with a DAD (Agilent, Palo Alto, CA, USA) was used for all experiments. The columns were Lichrospher-C\(_{18}\) columns (250 mm x 4.6 mm, 5 \( \mu \)m; Jiangsu Hanbang Science & Technology Co., Ltd) and Zorbax 300SB C\(_{18}\) columns (250 mm x 4.6 mm, 5 \( \mu \)m; Agilent). The mobile phase was composed of a mixture of water and methanol using a gradient program: 95–80:5–20 v/v for 0–5 min, 80:20 v/v for 5–15 min, 80–30:20–70 v/v for 15–20 min, 30:70 v/v for 20–30 min and 30–10:70–90 v/v for 30–40 min. The flow rate was 1.0 mL min\(^{-1}\), and the column temperature was maintained at 30\(^\circ\)C. The DAD detector was set at 254 nm for monitoring elutes. The presence of imperatorin and isoimperatorin in each extract were confirmed by comparing their retention times and ultraviolet (UV) spectra with those of reference standards. The quantification of these components was calculated using the corresponding standard curves based on the peak areas of the samples. The Computer-Aided Similarity Evaluation System used in this study was a chemo metrics-based computing program developed recently by the National Institute for the Control of Pharmaceutical and Biological Products of Beijing, China.

### 3.4. Method validation and sample analysis

Samples from the same batch of preparation were analysed six times to determine precision and the relative standard deviation (RSDs) of the relative retention times and the relative peak areas of the 13 characteristic peaks. Samples from the same batch were separated into eight aliquots by the quarter separation method. Six of these aliquots were analysed for reproducibility; one aliquot was analysed for stability at 0, 2, 4, 8, 12 and 24 h after the preparation. Three batches of *R. Angelicae Dahuricae* samples from Sichuan province were analysed in this experiment.

### 3.5. Animals

Male and female Kunming mice (superplastically formed (SPF) class, Certificate No. SCXK2004-0011) were obtained from the Central Animal Facility at Sun Yat-Sen University, Guangzhou, China. They were housed in single-sex cages under a 12 h
light: 12 h dark cycle in a temperature-controlled room (22 ± 2°C). They had free access to food and water. The animals were randomly divided into either test or control groups. All experiments were carried out in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann, 1983).

3.6. Pharmacological experiments

3.6.1. Hot-plate testing in mice

The method previously described by Turner (Xu, 2002) was employed using a hot-plate apparatus (YLS-6A, Shandong, China), and the testing temperature was maintained at 55 ± 0.5°C. Mice, 64 in number, were randomly divided into six groups (10 per group). The experimental reaction time of a mouse to the thermal stimulus was measured as the interval between the time the animal reached the hot plate and the time it licked its hind paw. The pain threshold was measured prior to extract administration or drug treatment. The extracts (20 mg kg⁻¹) were administered by oral gavage. Only the cyclohexane, acetoacetate, acetone and supercritical CO₂ fluid extracts were administered. The methanol extracts and water extracts had no effects in the preliminary testing and therefore were not included in this study. The negative control group was administered by using distilled Tween-80 at the same volumes as the test groups, while the positive control group was treated with pethidine at 50 mg kg⁻¹ intraperitoneal (i.p.). The reaction times were again measured at 15, 30, 60, 90, 120 and 150 min after the treatment. All experiments were carried out in a blinded fashion. To avoid possible tissue damage to the mouse paws, the cut-off time for response to the thermal stimulus was set at 60 s. The percentage increase in reaction times was calculated for each extract, drug-treated and control group.

3.6.2. Acetic acid-induced abdominal constrictions in mice

The method of Xu (2002) was employed, with modifications to the timing of observations. Sixty mice were randomly divided into six groups (five males and five females in each group) and treated with extracts from various preparations: cyclohexane, acetoacetate, acetone and supercritical CO₂ fluid (20 g kg⁻¹). The positive group received pethidine (150 mg kg⁻¹, i.p.) and the control group received Tween-80 per oral (p.o.). At 30 min after the treatment, each animal was administered 0.6% of an aqueous solution of acetic acid (10 mL kg⁻¹) and then placed in transparent observation boxes for observation. Beginning 5 min after the administration of acetic acid, the number of abdominal constrictions was counted for 15 min. The experiments were performed in a blinded fashion. The percentages of inhibitions of constrictions compared with the control group were calculated for the extract-treated and pethidine-treated groups.

3.6.3. Sedation experiment

Mice were randomly divided into five groups (five males and five females per group) and treated by gavage with extracts with cyclohexane, acetoacetate, acetone and supercritical CO₂ fluid at 20 g kg⁻¹, or Tween-80 for the control group, in
appropriate volumes. At 45 min after the treatment, each mouse was put into an activity box. After a 15 min accommodation period, the frequency of the autonomic activities of each mouse in its activity box was recorded.

3.6.4. **Anti-inflammatory test**

Anti-inflammatory activity was evaluated by the vascular permeability increase induced by 0.6% acetic acid in mice. Mice were randomly divided into six groups (five males and five females in each group), and subjected to the treatment of extracts as above. The positive control group received aspirin (0.2 g kg$^{-1}$), and the negative control group received Tween-80. At 1 h after the treatment, 0.6% acetic acid and Evans blue dye were injected through the vena caudalis, and 20 min later, the mice were killed and the abdominal cavity was subjected to three rounds of clysis using 2 mL of distilled water each time. The optical density (OD) value of the collected elutes (6 mL) was determined at 590 nm with distilled water as the reference.

3.6.5. **Heat-stroke testing**

Mice were randomly divided into six groups (four males and four females in each group). The extract-treated groups were treated as above. The positive control group received Chuanhunin (0.05 g kg$^{-1}$), and the heat-stroke model control group received Tween-80. The mouse model of heat-stroke was established by placing the mice into a warm temperature chamber (36 ± 0.5°C) with 60 ± 5% relative humidity. The aforementioned treatments were administered to the mice 30 min before they were put into the chambers. The rectal temperatures of the mice were measured every 30 min until death. The duration in which the mice survived, the highest rectal temperature for each mouse, and the time at which each mouse reached the highest rectal temperature were also recorded.

3.6.6. **Endotoxemia testing**

Mice were randomly divided into six groups (eight per group, equal number of female and male) and treated with extracts as above. The positive control group received Chuanhunin (0.05 g kg$^{-1}$), and the negative control group received Tween-80 in appropriate volumes, all administered by gavage. The endotoxemia model of 40 mice was duplicated with lipopolysaccaride (20 g kg$^{-1}$) and D-galactosamine (800 mg kg$^{-1}$) abdominal injection, respectively. The survival time of endotoxemic mice 24 h after injection was then observed.

3.7. **Data analysis**

The correlation coefficients of the entire chromatographic patterns across samples were analysed using the Computer-Aided Similarity Evaluation System. The relative retention time and relative peak area of each characteristic peak were also calculated with respect to the references (Li & Wang, 2005; Xie, 2005). Data from pharmacological experiments were expressed as the mean ± SD and analysed by using the SPSS 10.0 software. The student’s $t$ test and one-way analysis of
variance were used as appropriate. Differences were considered significant when $p < 0.05$.

4. Conclusions
A total of 13 authentic samples of *R. Angelicae Dahuricae* were collected and used to create a comprehensive fingerprint, representing the entire chemical profile of the herb. The fingerprints of the genuine herb showed 13 ‘common peaks’ that are likely to represent the herb’s major constituents. The fingerprint-effect correlation was clearly established according to HPLC fingerprint analysis and pharmacological testing. The effects of sedation and analgesia are mainly attributable to the chromatographic peaks of group II of the ethanol extract, and the anti-inflammatory, anti-heat stroke and anti-endotoxemic effects are mainly related to group III of the ethanol extract. These results indicate that this is a simple, reproducible and accurate method for the identification of the active sections and quality control of pharmaceutical preparations of *R. Angelicae Dahuricae*.

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References


