Screening of Bioactive Ingredients in Ligusticum Chuanxiong Hort for Protection against Myocardial Ischemia

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Ligusticum Chuanxiong Hort • Myocardial ischemia, spectrum-effect relationship

Abstract
Background/Aims: To study the spectrum-effect relationship and effective components of Ligusticum Chuanxiong Hort. (LCH) on the protection of canine myocardial ischemia. Methods: Fingerprint spectrum of LCH extracts was developed using high performance liquid chromatography (HPLC), and a canine model of acute myocardial ischemia was established by ligating the coronary artery. Bivariate correlation analysis and multivariate regression analysis were used to correlate the pharmacodynamics of LCH extract and its common peaks in HPLC. Results: The bioactive components of LCH were ligustrazine, ferulic acid, cnidilide and ligustilide. Ligustrazine and ferulic acid could significantly reduce serum lactic acid in canine model of acute myocardial ischemia, while ligustilide could significantly reduce the elevation of serum free fatty acid. Conclusions: The spectrum-effect relationship study shows that the effective components of LCH are ligustrazine, ferulic acid, cnidilide and ligustilide, which have protective effect on myocardial ischemia.

Introduction
Chuanxiong Rhizoma (CR) is the dried rhizome of Ligusticum chuanxiong Hort (LCH). Umbelliferae is a major cardiovascular protective herb that has been widely used in traditional Chinese medicine for a long time [1-3]. It is frequently prescribed to treat angina pectoris, cardiac arrhythmias, hypertension, stroke, as well as myocardial ischemia [4-7]. It is believed to help in blood circulation and reverses blood stasis [8, 9]. CR also exhibits neuroprotective, anti-fibrotic, anti-nociceptive, anti-inflammatory and antineoplastic activities [10-14]. The essential biological active components of LCH are volatile oils, organic acids, alkaloids,
phthalides and other ingredients, which make it difficult to identify individual components, especially the determination of biologically active ingredients [15-19]. Meanwhile, fingerprint analysis based on chromatography has been widely used for authentication and quality control of herbal drugs [20-22]. LCH extracts have been widely studied in animal models, both in vivo and in vitro to determine their cardiovascular function [23, 24]. It has been found that, some traditional Chinese medicines such as Panax Quinquefolium Saponin can reverse myocardial ischemia [25]. Lu et al. have shown that the effect of Suxiao Jiuxin Pill (SX), a traditional Chinese medicine including LCH extract, on reversing acute myocardial ischemia caused by coronary occlusion in anesthetized dogs, are significant [26]. In addition, Tetramethylpyrazine is an extract of the traditional Chinese medicine Chuanxiong, which can exert protective effects against myocardial ischemia-reperfusion injury (MIRI) in multiple ways [27]. However, LCH extract used in in vitro animal models needs to be explored further. Interestingly, the HPLC fingerprint analysis and drug active substances model can provide the complexity and integrity to study traditional Chinese medicine, objectively and this might become a powerful tool for the rapid identification of the constituents in herbs. The spectrum-effect relationship is a rational approach to screening effective compounds, reflecting the quality of Chinese herbal medicine. Therefore, this study aims to investigate the bioactive ingredients of CR by HPLC coupled with a canine model of acute myocardial ischemia and attempts to reveal the relationship between the active ingredients of Ligusticum Chuanxiong Hort. and cardioprotection.

Materials and Methods

This study was approved by the ethics committee of No.401 Hospital of PLA, Qingdao, China.

Materials, Reagents and chemicals

High performance liquid chromatograph was purchased from Agilent technology (Quaternary pumps, 1100 chromatography workstation). Chuanxiong Rhizoma was identified by crude drug laboratory in the College of Pharmacy at Shandong University (batch number: 14051702), which was performed according to “First edition of Chinese Pharmacopoeia 2010”. Chuanxiong Rhizoma was comparable to the standard and the survey report is shown as an attachment. Ligustrazine (batch number: 0803-0803, purity: 99%), ferulic acid (batch number: 905-201215, purity: 99%), cnidilide (batch number: 1006-201201, purity: 99%), ligustilide (batch number: 0708-201210, purity: 99%) and the reference substances above were purchased from the pharmaceutical and biological products office of China and acetonitrile was supplied by Merck KGaA (chromatography pure, 64271 Darmstadt, Germany). SC - M5 anesthesia ventilator was obtained from Shanghai Medical Instrument Factory, 752 type grating spectrophotometer was supplied by Analysis Instrument Factory of Shanghai and LD5-2 type centrifuge was purchased from Beijing Medical Centrifuge Factory. Creatine kinase (CK) kit (batch number: 20111010) and lactate dehydrogenase (LDH) kit (batch number: 20111230) were provided by Nanjing Institute of Biological Engineering Production. Fluorescein-luciferase buffer was obtained from Shanghai vegetative institute of the Chinese academy of sciences, RANDOX company kit (CAT NO.: ENR:U90625, FA115; ENR:T77192, LC2389; ADM:J64951, CK112) and compound Danshen dripping pill (positive control, was purchased from Tianjin Tasly Pharmaceutical co., LTD, batch number 120820).

Animals: 30 Healthy mongrel canines, weight: 10 ~ 15 kg, both male and female, were purchased from the Laboratory Animal Center in Qingdao Medicine Inspecting Institute, (experimental animal certificate number: 2012 A030).

Methods

HPLC fingerprint. (1) Chromatographic condition and the system suitability. BDS (200 mm × 4.6 mm, 5 microns) C18 column was used for the analysis, with A: methanol - B: 1% ice acetic acid aqueous solution as mobile phase, gradient elution [10:90 (10min)→20:80 (20min)→30:70 (30min)→30:70 (60min)]/ν/ν and the flow rate was 1 ml/min. The column temperature was set to room temperature, and the UV detection wavelength was 322 nm. Under these conditions, Ligustrazine, ferulic acid, cnidilide A and ligustilidein
Chuanxiong Rhizoma achieved baseline separation and separation of chromatographic peak of adjacent degree >1.5. Trailing factor was between 0.95-1.1, theoretical plate number calculated by ligustrazine, ferulic acid, not less than 4500 and the peak symmetry factor as to meet the requirements of Chinese pharmacopoeia.

(2) Sample preparation of Chinese herbs. Precise weight for Chuanxiong Rhizoma (40 mesh) was about 100 mg, taken in a 10 ml volumetric flask, after adding 0.1 ml glacial acetic acid and 90% methanol diluted to scale. According to the preliminary experimental screening condition, the ultrasound time was 18 min and the microporous membrane filter was 0.45 µm. These samples would be used for HPLC analysis after leaching the liquid.

(3) Fingerprint determination. 20 µl of the test sample solution was injected into the HPLC instrument, and chromatogram was recorded for 60 min. Since Ligustrazine was found in abundance in the sample and its chromatogram showed a stable peak, it was chosen as the reference peak. The integral value of the retention time and the area of the chromatographic peak was 1 and the ratio of other peaks of retention time and peak area integral value was calculated using the benchmark peak retention time and its peak area integral values.

(4) Sampling precision test. Test of the sample solution was repeated 5 times within 2 h and the HPLC of each common peak relative standard deviation (RSD) to relative peak area was less than 1.5%. The RSD of relative retention time was less than 0.75%, which satisfied the quantitative analysis of the samples.

(5) Reproducibility of the test. All experiments were repeated 5 times and the determination of control HPLC chromatograms was within one day. The results of each common peak RSD to relative peak area were less than 1.5% and the RSD of relative retention time was less than 0.95%, with good reproducibility.

(6) Stability test. Test sample solutions from Chuanxiong Rhizoma were measured at 0, 2, 4, 6, 8 and 10 h to determine the HPLC fingerprint. Calculations were performed to ensure that the peak area of the RSD was less than 1.95% and the RSD of relative retention time was less than 0.98%, showing that the test sample maintained solution stability up to 10 h.

The effect of Ligusticum chuanxiong Hort extract on Canine ischemia model

Preparation. Extracts from Ligusticum chuanxiong were prepared in saline with incremental concentrations of 7.5, 15, 30 mg/ml with a 2 ml/kg lavage [28-30]. Animals were given intravenous anesthesia with 3% pentobarbital sodium (30 mg/kg). Endotracheal intubation was performed using a SC-M5 anesthesia apparatus and the thoracic cavity was opened after mechanical ventilation (respiratory frequency: 16~18 times/min, tidal volume: 350-550 ml). Thoracotomy was performed along the left sternal border between the 4th and 5th ribs to expose the heart, the pericardium was cut and a pericardial hammock was made. Between the second and the third branch of the left anterior descending coronary artery, two silk threads were introduced, which acted as the standby in two step ligation. A single dose of lidocaine 5 mg/kg was administered through the femoral artery before ligation, using Harris ligation method, to prevent arrhythmia [31]. For the initial ligation a steel wire of 1 mm diameter was inserted to make a loose knot around the coronary artery and then the steel wire was pulled out. The second junction ligation was completed after 30 minutes. Following ligation, a reference electrocardiogram was recorded for 10 min and lavage was performed at a volume of 2 ml/kg. The negative control group received same volume of saline.

3 h after ligation of the heart, the whole heart was weighed, following which the atrium right and left ventricles were weighed. Then coronary artery ligation was performed parallel to the coronary groove and the left ventricle was cut into 5 pieces, washed with physiological saline and stained using 0.05% nitro blue tetrazolium chloride monohydrate (N-BT) at 37°C for 30 minutes on a shaker. Excess dye was flushed immediately after dying. The infarction area was stained dark blue, after washing, the weight of the heart was recorded and the ratio of left ventricle weight to the total heart was calculated.

3 ml of coronary sinus venous blood and femoral arterial blood were collected before and after ligation to measure biochemical index. 200 µl of blood was directly injected into the precooling buffer containing 0.4 ml Tris-HCl, the total volume was 3 ml, immediately shaken in boiling water for 5 min, then centrifuged 5 min at 3000 RPM and placed on an ice bath. 20 ml was taken in a small glass tube and placed in a FGF - 91 light spectrophotometer and 0.4 ml fluorescein - luciferase buffer was added. The luminous intensity was measured immediately and the experiment was repeated 3 times. The standard curve was prepared with reference to relevant specifications.
**Dose Settings.** The canine dose was calculated according to the formula human dose * 104/100 * 1/3 (70/11.5). Positive control was given isosorbide dinitrate tablet at a dose of 20 mg/day with 70 kg body weight (0.29 mg/kg) calculation. Compound Danshen dripping pill was given at a dose of 750 mg/day (10.7 mg/kg). Canine dose conversion, according to body surface area, was equivalent to 0.54 mg/kg and positive control received a drug dose equivalent to that of the reagent (2 mg/kg). Compound Danshen dripping pill was given at a dose of 750 mg/day (10.7 mg/kg). Canine dose conversion, according to body surface area, was equivalent to 20.3 mg/kg and the positive control received a drug dose equivalent to the reagent dosage of 60 mg/kg. Vehicle control at baseline (no coronary artery ligation) was defined as control group. Vehicle group after coronary artery ligation was defined as model group. Drug delivery was by lavage and delivery was limited to a single dose.

**Observation indices and observation time.** Canine epicardial electrocardiogram, quantitative histology as well as quantitative analysis of ATP, FFA, serum CK and blood lactic acid were performed. Observation time was 0h (before drug administration) and 3h after drug administration.

**Screening of spectrum-effect relationship**
Spectrum correlation effect was determined by using bivariate correlation analysis to establish the mathematical statistical model. Using multiple regression analysis, correlation analysis, grey correlation mathematical methods, such as for Chuanxiong Rhizoma fingerprint and pharmacodynamic investigation was made on the correlation between them, the internal relation of fingerprint and efficacy study [32-34]. Regression analysis is the study of one or more independent variables and a dependent variable to determine whether there is a linear or nonlinear relationship between the statistical methods [35]. Bivariate correlation analysis is the study of variables between the drug HPLC fingerprint peak area of each peak in the treatment of myocardial ischemia with regression analysis and correlation analysis, using the statistical software SPSS17.0. The composition of Ligustrazine, ferulic acid, cnidilide and ligustilide by HPLC are independent variables in this analysis.

**Statistical analysis**
The data are presented as mean±SD. Differences between the groups before and after drug administration at each time point was performed and compared to the saline group at the same time point. Statistical differences between the groups were analyzed by one-way analysis of variance (ANOVA) using SPSS18.0 statistical software. A value of p < 0.05 was considered significant. p < 0.01 and p < 0.001 were considered highly significant.

**Results**

**Screening HPLC fingerprint**
According to the method described, we measured the HPLC fingerprints of 10 batches of drugs for peaks appearing at the same retention time. Retention time of cnidilide A was 8.45, lactone retention time was 15.01, the retention time of ligustrazine was 19.56 and that of ferulic acid was 25.79. Be precise according to the reference substance each 10 mg. 90% methanol diluted to scale was added to a 100 ml volumetric flask and shaken well, under the condition of the chromatographic detection and the reference substance graph is shown in Fig. 1. In order to establish quality control of Ligusticum chuanxiong evaluation model conveniently and quickly, the fingerprint of 7 peaks, including the above four chromatographic peaks were selected as examining index. The relative retention time of the RSD was 0.6% to 0.95% and the relative peak area integral value ratio of 1.16% to 1.16% (Fig. 2).

**Effect of Extract of CR on the canine model of acute myocardial ischemia**
15 and 30 mg/kg dose of extracts from Ligusticum chuanxiong could reduce myocardial ischemic injury in a dose dependent manner. N-ST value (changed value of ST segments on electrocardiogram) was also lower and the sustainable time was 120 min. N-ST at each time point of the low dose group (15 mg/kg) was reduced and there were significant differences when compared with the saline group (control) at 60, 90 and 120 min time points (p < 0.01 or p < 0.05). N-ST value in the 30 mg/kg dose group was significantly lower compared to
the saline control at 60, 90 and 180 min time points (p < 0.01 or p < 0.05). N-ST value in the 60 mg/kg dose group was significantly lower compared to the saline control at 60, 90, 120 and 180 min time points (p < 0.01). Positive control group which was given isosorbide dinitrate showed a significant (p < 0.01) decrease at 15 min, following administration, when compared with the saline group, however there was no significant difference at 30 min. It might be due to the fact that isosorbide dinitrate worked effectively only for a short duration.
Danshen dripping pill group showed significant difference following administration, at 15, 30, 60, 90 and 120 min, in reducing myocardial ischemia injury compared to the saline group (p < 0.01 or p < 0.05). The experimental results are shown in Table 1.

Quantitative histological detection showed that Ligusticum chuanxiong extract’s effect on myocardial infarction was consistent with the results of epicardial electrocardiogram. Extracts from Ligusticum chuanxiong 15~60 mg/kg dose lavage can reduce myocardial infarction. The high dose group demonstrated a significant reduction in the infarction area ratio of the whole heart as well as the left ventricular infarction ratio compared to the saline group (p < 0.01). Isosorbide dinitrate, positive control group also demonstrated a significant reduction in the myocardial infarction area (p < 0.01). The experimental results are shown in Table 2.

Serum CK, FFA and lactic acid levels were elevated following coronary artery ligation. However, they were decreased in the Ligusticum chuanxiong extract (15 ~ 60 mg/kg)
Interestingly, the effect of middle and high dose group of Ligusticum chuanxiong extract (30 and 60 mg/kg) was highly significant. The experimental results are shown in Tables 3 and 4.

### Screening of spectrum-effect relationship and effective components

The results of bivariate correlation analysis on relative peak areas of common peak and pharmacological data are shown in Table 5. In peaks 1, 2, 3, 5 and 7, the relative peak...
area was positively correlated with CK and FFA concentrations. In peaks 1, 3, 5 and 7, the relative peak area was correlated with lactic acid concentration. The fingerprint peaks from stepwise regression analysis were as follows: for CK, the correlated peak numbers were 1 and 2; for FFA, the correlated peak numbers were 2 and 5; for lactic acid, the correlated peak numbers were 1, 3 and 5. The correlation coefficients between relative peak area and effective fingerprint peaks of Ligusticum Chuanxiong Hort are shown in Table 6. In peaks 1, 2, 3, 4 and 5, the relative peak area was positively correlated with effect of fingerprint peaks of Ligusticum Chuanxiong Hort.

Discussion

Optimization of Chromatographic conditions

Methanol-water and acetonitrile-water act as the mobile phase and were separated. We found that methanol-water showed less dosage of organic phase in the column than the acetonitrile-water system. Chuanxiong Rhizoma contains plenty of medicinal chemical components and has a wide range of polarities. However, the different proportions of methanol on the separation effect of different gradients were screened. According to the selection, considering the conditions of separation comprehensively, the methanol-water gradient elution procedure was selected to be appropriate and suitable glacial acetic acid was added to adjust pH value, which effectively improved the chromatographic peak tailing phenomenon.

Detection wavelength of 200-400 nm was compared via diode array detector. We found that when the detection wavelength was less than 280 nm, the baseline drifted significantly. Interestingly, the chromatographic peak number decreased when the detection wavelength was greater than 325 nm. Each chromatographic peak at 322 nm showed strong ultraviolet absorption, rich chromatographic information and good degree of separation. Hence, that wavelength was selected to be the detection wavelength.

Pharmacological mechanism

Coronary artery ligation can lead to cardiac cells ischemia, hypoxia, and aerobic oxidation process. The cell membrane stability is reduced, resulting in leakage of enzymes, creatine phosphokinase (CK) and lactate dehydrogenase (LDH) that are released into the blood and cause necrosis of myocardial cells and its surrounding tissues [36, 37]. Experimental results showed that CR could significantly lower serum CK activity in canines with acute myocardial infarction. Prompt administration of Chuanxiong Rhizoma extract can prevent the overflow of enzymes from the cytoplasm, reduce the degree of damage to a certain extent and improve myocardial metabolism. Aerobic oxidation process of acute myocardial infarction leads to fatty acid oxidation and reduction and significantly increased serum FFA, which causes further myocardial damage by increasing myocardial ischemia. The experimental results demonstrate that the extracts from rhizoma ligustici wallichii can significantly inhibit myocardial ischemia and FFA metabolic disorder thereby having a protective effect on myocardial cells.

Analysis of spectrum-effect

The chemical compositions of traditional Chinese medicines are complex and research and characterization of its active ingredients is difficult. This study used bivariate correlation analysis as well as regression analysis and the test results were found to be identical. Peak of cnidilide, ligustilide, ligustrazine and ferulic acid were associated with significant pharmacological activities and the compounds could significantly inhibit myocardial ischemia caused by coronary artery ligation. The pharmacological effects of this kind of the extract are consistent with previous studies [38-41]. Ligustrazine can significantly reduce serum lactic acid and ferulic acid, the rise of chaff this can significantly reduce serum FFA lactone is the main active ingredient of the traditional Chinese medicine Chuanxiong
Rhizoma in treatment of myocardial ischemia. The effect of Chuanxiong Rhizoma extracts in the treatment of myocardial ischemia is the result of multiple components working together. Experiments show that the bivariate analysis and multivariate regression analysis are effective methods for studying multivariate correlation, through chromatographic peak, which can aid in identifying the complex and active components of traditional Chinese medicine [42].

The active ingredients in natural compounds are the essence of traditional Chinese medicine (TCM). Using modern technology and advanced analysis to establish TCM material database laid the foundation for this experiment [43, 44].

Conclusion

Ligustrazine, ferulic acid, cnidilide and ligustilide are the main bioactive ingredients of Ligusticum Chuanxiong Hort., according to the analysis, and they show positive correlation with protection against myocardial ischemia. This study used HPLC fingerprint technology to identify the complex composition of Ligusticum Chuanxiong Hort. extract. This was achieved by the analysis of correlation of HPLC fingerprint information and its medicinal activity. Choosing a representative composition as base material, not only can overall characterization of the ingredients be performed, one could also study individual, pharmacologically active, ingredients such as ligustrazine, ferulic acid, cnidilide and ligustilide. The study of the individual compound could lead to the development of novel therapies for other pathologies.

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Disclosure Statement

The authors declare that they have no conflict of interest.

References


