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A Fast HPLC Quantitative Determination of Ligustrazine in Rhizome of *Ligusticum chuanxiong*

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A simple and quick method is described for the determination of ligustrazine in rhizomes of *Ligusticum chuanxiong*. The ligustrazine in the sample was extracted using methanol and analyzed by reversed-phase high performance liquid chromatography. Chromatography separation was performed using Agilent 1100 series HPLC system with a waters symmetry C_{18} column and isocratic elution with a mixture of three solvents: acetonitrile, methanol and 1 % aqueous acetic acid (20: 40: 40, v/v/v). The effluent was monitored using a VWD detector set at 294 nm. The average recoveries were 84.93 - 88.65 % (n = 3). The limits of detection and quantification were 0.044 and 0.133 μg mL^{-1}, respectively. The method has been successfully applied to the analysis of *L. chuanxiong* samples. For 37 samples, the ligustrazine content varied from 0.22 to 1.06 mg g^{-1}.

Keywords: Ligustrazine, HPLC, *Ligusticum chuanxiong*, Quantitative determination, Method validation.

INTRODUCTION

Ligusticum chuanxiong Hort. is one of the vegetable and traditional Chinese herbal medicines (TCM), which has been widely used as food and medicine in China for many centuries. It is mainly used to do cold dishes, stewed chicken and duck. The essential biological active ingredients of *L. chuanxiong* are ligustrazine, ferulic acid and volatile oil[1-5]. Ligustraline is mainly used for the treatment of lung injury, pancreatitis, endothelial dysfunction, amnesia, antioxidant, apoptosis, nephric, hepatic, cardiovascular and cerebrovascular diseases[6-18].

Ligustrazine is one of the most important ingredients in *L. chuanxiong* and necessary index of products quality control. Quality control is important in guaranteeing the safety, efficacy and stability of a product[5,19].

High performance liquid chromatography is currently the most commonly used separation technique and quantitative determination in combination with detection by ultraviolet and diode array[20-26]. So far, to our best knowledge, few detailed HPLC method is reported for the quantitative determination of ligustrazine in rhizomes[3,4], but this method has a number of shortcomings, such as lowered determination efficiency, great consumption of solvents, long determination time. Therefore developing of a simple and quick HPLC method for the determination of ligustrazine in rhizomes is necessary for further studies.

The purpose of this study is to develop a simple and quick method for the quantitative analyses of ligustrazine in rhizomes of *L. chuanxiong*. Ligustrazine was extracted with methanol and analyzed by HPLC. The validated method has been successfully utilized for determining ligustrazine content and investigating the range of ligustrazine content in rhizome of *L. chuanxiong* samples.

EXPERIMENTAL

Seven commercial samples were purchased from markets, with the number of YP01-YP07. Thirty other samples, YP08-YP37, were obtained from cultivated fields of major *L. chuanxiong* producing zone (Dujiangyan County, Chengdu City, Sichuan Province of China, 31 ° 48' N, 99 ° 10' W) during harvest time. All the samples were sun-dried and grounded into powder before analysis and then were shifted through a 60 mesh sieve and kept in an air-tight container until being used.

Ligustrazine (standard sample) was obtained from National Institutes for Food and Drug Control, Beijing, China. Acetonitrile (HPLC grade) and methanol (HPLC grade) were purchased from Fisher Scientific, Inc. Acetic acid (AC grade) was purchased from Chengdu Kelong Chemical Factory (Chengdu, China). De-ionised water was prepared using Millipore Milli-Q Plus system (Millipore, Bedford, MA, USA).

High performance liquid chromatography (HPLC): HPLC analysis were carried out using Agilent 1100 LC system

(Agilent, USA), which included a quaternary pump, an ALS auto injector, column oven and a VWD detector, connected to LC Chem. Station. A symmetry C_{18} column (250 mm × 4.6 mm, 5 µm) from waters (USA) was used. The column temperature was maintained at 35 °C. The standards and samples were separated using isocratic mobile phase consisting of 1 % aqueous acetic acid, methanol and acetonitrile (40: 40: 20, v/v/v). The flow rate was set at 1 mL min^{-1} and the injection volume was 10 µL. The detection wavelength was set at 294 nm. Identification of ligustrazine was based on retention time when co-injected with standards.

Sample extraction procedure: The extraction of ligustrazine was performed by adding 0.5 g powdered material into 25 mL methanol in a 50 mL tube. The tubes were then partially immersed into the ultrasonic bath. The working frequency was 40 kHz, the bath power rating was 200 W, the temperature was 40 °C and the extraction time was 60 min at the beginning. After extraction, this solution was filtered through 0.45 µm membrane filter and was collected into 1.5 mL vial before HPLC analysis.

Preparation of the standard solution: Quantification was based on the external standard method. A stock solution of ligustrazine standard (0.15 mg mL^{-1}) was prepared by dissolving in methanol. The working standard solutions for linear calibration were prepared by diluting the stock solution to produce a concentration sequence of 0, 15, 22.5, 30, 75, 150, 225 and 300 µg mL^{-1}.

Validation procedure: Validation of the analytical method was done according to the International Conference on Harmonization guideline[27]. The method was validated for linearity, precision, accuracy, limit of detection (LOD) and limit of quantitation (LOQ).

Linearity: Linearity of the method was studied by injecting eight known concentrations of the standard in the range of 0-300 µg mL^{-1} in triplicate. The calibration curves were obtained by plotting the peak area *versus* the amount of the standards.

Precision: The measurement of intra-day and inter-day precision was done by analyzing five samples extracted solution to determine the precision of the method. The intra-day precision (repeatability) was examined by analyzing three times within 1 day, while the inter-day precision (reproducibility) was examined for 3 consecutive days by the proposed method. The precision was expressed as percent relative standard deviation (% RSD).

Accuracy: Recovery study of ligustrazine was performed using the method of standard addition for measuring accuracy of method. Known amount of ligustrazine was added to five raw materials extraction solution and carried out the extraction procedure, the concentration of standard reached 3.82 µg mL^{-1}. Spiked samples were prepared in triplicate. The recovery was calculated as follows:

$$\text{Recovery (\%)} = (\text{amount found-original amount})/\text{amount spiked} \times 100.$$

Limit of detection (LOD) and limit of quantitation (LOQ): Determination of signal-to-noise ratio was calculated under the proposed chromatographic condition. LOD was considered as 3:1 and LOQ as 10:1.

RESULTS AND DISCUSSION

The new HPLC method was established for the quantitative analysis ligustrazine in rhizomes of *L. chuanxiong* extracts. The method is newly studied and it is a simple and quick method for the determination of ligustrazine in rhizomes, which has achieved the optimal separation with the peaked earlier and reduced runtime. The peak time is 5 min, compared with the results of Ru Yan and Su Yan-Li, saves almost 7 and 9 min[3,4], respectively. Wen weiyu was reported for determination of ligustrazine method, but this method determined samples are human plasma and not suitable for determination of raw *L. chuanxiong* materials[28]. In this study, from various mobile phase trials, their effects on the enhancement of separation selectivity have been determined, with increasing efficiency and elimination of peak tailing of the ligustrazine. As a result, a mobile phase containing aqueous acetic acid and methanol were selected together with acetonitrile giving a stable baseline, symmetric peak and the most efficient separation rate and speed. Finally, a mobile phase consisting of acetonitrile, methanol and 1% aqueous acetic acid (10: 40: 40, v/v/v) was chosen for the determination of ligustrazine in rhizomes of *L. chuanxiong*. The ultraviolet spectrum of the ligustrazine reference showed maximum absorbance at 294 nm. Thus, in this study, 294 nm was selected as the detection wavelength. Typical HPLC chromatograms of ligustrazine standard and actual samples are presented in Fig. 1.

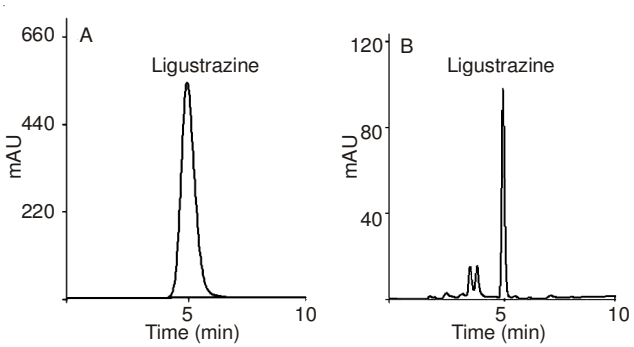

Fig. 1. A and B are typical HPLC chromatograms of ligustrazine standard and actual samples, respectively

Method validation: The developed method was validated for its linearity, precision, accuracy, LOD and LOQ. Calibration plots for ligustrazine content the linear relationship Y = 3630.1X + 7.3414, where Y and X are the peak area (mAU) and concentration of the standard solution (µg mL^{-1}), respectively. Linear regression showed good linearity in the range of 0.15-300 µg mL^{-1} with a correlation co-efficient of 0.9999. This allows the determination of ligustrazine over a wide range of concentrations. Precision of the method was determined using the 5 samples solutions. The results showed acceptable precision of the method with percent relative standard deviation (% RSD) values lower than1.2 % (Table-1). The recovery of ligustrazine, representing accuracy of the method, ranged between 84.93 and 88.65 (Table-2). The LOD and LOQ for ligustrazine were found to be 0.044 and 0.1332 µg mL^{-1}, indicating high sensitivity of the method. These results show that the method is accurate and precise as evidenced by the high recovery and low % RSD.

TABLE-1
INTRA-DAY AND INTER-DAY PRECISION OF LIGUSTRAZINE; RESULTS ARE SHOWN AS RSD %

Samples	Inter-day			Intra-day
	Day 1	Day 2	Day 3	
1	0.01	0.05	0.32	0.09
2	0.40	0.54	0.08	0.30
3	0.08	0.83	0.38	0.44
4	0.38	0.82	0.67	0.46
5	0.67	1.14	1.14	0.01

TABLE-2
RECOVERY OF LIGUSTRAZINE (n = 3)

Samples	Theoretical (mg)	Found (mg)	Recovery (%)
1	0.0159	0.0137 ± 0.0000	84.9266 ± 0.3550
2	0.0160	0.0143 ± 0.0001	88.6498 ± 0.3549
3	0.0159	0.0139 ± 0.0001	86.4473 ± 0.3549
4	0.0160	0.0139 ± 0.0000	86.7024 ± 0.3550
5	0.0159	0.0139 ± 0.0001	86.5666 ± 0.3549

Application of the method: The proposed HPLC method was used for the quantification of the contents of the ligustrazine in thirty seven samples. The ligustrazine content ranged from 0.22 to 1.06 mg g^{-1}. The details are summarized in Table-3. According to the results, some *L. chuanxiong* samples were rich in ligustrazine, but there was a large variation amongst the samples.

TABLE-3
CONTENT OF LIGUSTRAZINE (mg g^{-1}) IN ACTUAL RHIZOMES OF *Ligusticum chuanxiong* SAMPLES (n = 3) AND EXPRESSED AS MEAN ± SD

Sample No.	Content	Sample No.	Content
YP01	0.49 ± 0.05	YP20	0.60 ± 0.01
YP02	0.44 ± 0.06	YP21	0.56 ± 0.01
YP03	0.47 ± 0.05	YP22	0.60 ± 0.01
YP04	0.22 ± 0.07	YP23	0.60 ± 0.01
YP05	1.06 ± 0.21	YP24	0.58 ± 0.02
YP06	0.98 ± 0.02	YP25	0.41 ± 0.02
YP07	0.96 ± 0.07	YP26	0.46 ± 0.03
YP08	0.57 ± 0.01	YP27	0.42 ± 0.01
YP09	0.59 ± 0.01	YP28	0.43 ± 0.06
YP10	0.60 ± 0.01	YP29	0.44 ± 0.03
YP11	0.57 ± 0.02	YP30	0.48 ± 0.06
YP12	0.60 ± 0.01	YP31	0.59 ± 0.02
YP13	0.61 ± 0.01	YP32	0.55 ± 0.02
YP14	0.56 ± 0.02	YP33	0.60 ± 0.01
YP15	0.47 ± 0.01	YP34	0.45 ± 0.06
YP16	0.40 ± 0.01	YP35	0.51 ± 0.05
YP17	0.43 ± 0.01	YP36	0.39 ± 0.03
YP18	0.55 ± 0.01	YP37	0.36 ± 0.03
YP19	0.51 ± 0.05	–	–

Conclusion

In this study, a new HPLC method has been established for the quantitative analysis of ligustrazine in rhizomes of *L. chuanxiong*. The proposed method is simple and quick with high precision, sensitivity, accuracy and reliability and is appropriate for the detection work. Thirty seven *L. chuanxiong* samples were investigated for their content of ligustrazine. By comparing the results, we found a substantial variation among different samples. It will be interesting to further investigate the factors that lead to the variation in the ligustrazine content of different *L. chuanxiong* samples in the future.

This HPLC method could be used for quality control and standardization of *L. chuanxiong* materials as well as its final products, improving the quality in the mass production industry.

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