Development and Validation of an UPLC–DAD–MS Method for the Determination of Leonurine in Chinese Motherwort (*Leonurus japonicus*)

Zhe Chen[1,*], Jian Bing Wu[2], Xiao Juan Liao[1], Wei Yang[1], and **Kang Song[1,*]**

[1]National Clinical Research Base of Traditional Chinese Medicine, Zhejiang Hospital of Traditional Chinese Medicine, Zhejiang Chinese Medical University, Hangzhou 310006, China and [2]Institute of Biotechnology, Zhejiang University, Hangzhou 310029, China

Abstract

In the present study, an ultra-performance liquid chromatography method with diode array detection (UPLC–DAD) was developed for the analysis and determination of leonurine from motherwort (Leonurus japonicus), a traditional Chinese medicinal plant. This method was validated in terms of specificity, linearity (R^2 = 0.9995, linear range: 0.005 ~ 0.5 mg/mL), precision (< 5.0% RSD), and recovery (103.2%). The extracted amount of leonurine is 0.15 mg/g. Moreover, the target analyte was identified or tentatively characterized using UPLC coupled with electrospary tandem mass spectrometry (UPLC–ESI-MS).

Introduction

Chinese motherwort *(Leonurus japonicus* Houtt.) is a popular traditional herbal medicine known in Chinese as "Yi-Mu-Cao". It belongs to the Labiatae family and commonly found in the mainland of China. People often find a certain therapeutic value associated with this plant. According to the Chinese Pharmacopoeia (1), it has been prescribed for various purposes, such as heart antiarrhythmic (2), sedative (3), antimicrobial (4), anticoagulant (5), antioxidant (6), and antitumoral properties (7), as well as to boost immuno response (8). The major phytochemical molecules present in "Yi-Mu-Cao" were found to be alkaloids (1). Besides, several other type metabolites had also been isolated from this plant that substantiate the recorded activities. For instance, the iridoid glucoside leonurid, isoquercetin, two phenolic glycosides (6), melatonin (9), β-sitostenone (2), and several diterpenes exclusively of the labdane type have been characterized from its aerial parts (10,11). Among them, leonurine (4-guanidino-n-butyl syringate, Figure 1), has long been known as a major alkaloid present in this herbal medicine (1). The reported pharmacological effects of leonurine include the uterotonic action (12), anti-platelet aggregation (13), and vasorelaxant effect (14). Owing to the described bioactivity, leonurine has been used as the diagnostic constitute for the quality control of Chinese motherwort.

Recently, the chromatographic fingerprint technique has been internationally accepted as an efficient tool for the integral quality control of herbal medicines (15). On the other hand, the quality of herbal medicines is highly related to their major constituents in many cases, and thus quantitative analysis of these components is also necessary. Following the general research trend devoted to the development of analytical methods, this current investigation describes a simple ultra-performance liquid chromatography (UPLC) method for both separation and determination of leonurine present in Chinese motherwort. Although the HPLC–UV method had been reported for quantification of leonurine (16), considering the incomparable superiority of UPLC in analysis speed, sensitivity, selectivity, and specificity (17–20), it was predictable that this newly developed technique would become of a great importance in the field of high throughput quality control process. Therefore, a simple, rapid, sensitive, and validated UPLC–DAD-ESI-MS method for both qulitative and quantitative analysis of leonurine is reported. The suggested method has the potentiality for the ease of quality assessment of medicinal plant "Yi-Mu-Cao".

Experimental

Reagents and Chemicals

The reference compound leonurine was purchased from Newdrug Star Pharmaceuticals (Hefei, China). Reverse osmosis Milli-Q water (18 MΩ) (Millipore, Bedford, MA) was used for all solutions and dilutions. Methanol used for UPLC analysis was of

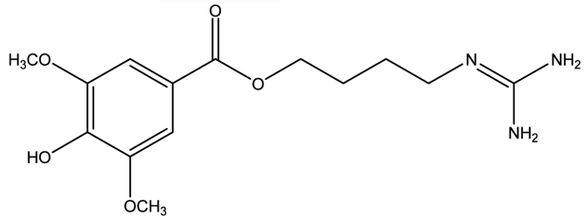

Figure 1. The chemical structure of leonurine.

* Dr. Zhe Chen and Dr. Kang Song both contributed equally to this manuscript. Email: chenzhe_zjtcm@yahoo.cn

chromatographic grade and purchased from Merck (Darmstadt, Germany). The dried leaves of *L. japonicus* were purchased from a local drug store and identified by Professor Xingde Wo (Zhejiang University of Traditional Chinese Medicine, Hangzhou, China). Ammonium formate (analytical grade) was purchased from Huadong Chemicals (Hangzhou, China).

Sample preparation

According to the Chinese Pharmacopoeia (1), the leaves of *L. japonicus* were dried to constant mass at 55°C in a vaccum oven and then pulverized. 100 g of pulverized sample was extracted with 350 mL of 95% ethanol for 2 h under reflux. The extraction procedure was repeated three times. The extracts were combined together and concentrated to dryness. The dried extract was weighed and dissolved in 50 mL 50% methanol. This sample soution was filtered through a 0.22-µm membrane and stored in a refrigerator (4°C) for the subsequent UPLC analysis.

Instrumentation

An Acquity UPLC system (Waters, Milford, MA), equipped with a photodiode array detection (DAD) detector was used for leonurine analysis and quantification under UPLC conditions. Data were processed with Empower 2 software (Waters).

UPLC–ESI-MS peak identification was performed using the described UPLC system coupled with a LCQ DECA plus mass spectrometer equipped with an electrospray interface (Thermo-Finnigan Corp., San Jose, CA). Instrument control and data acquisition were performed using Xcalibur 2.0 software.

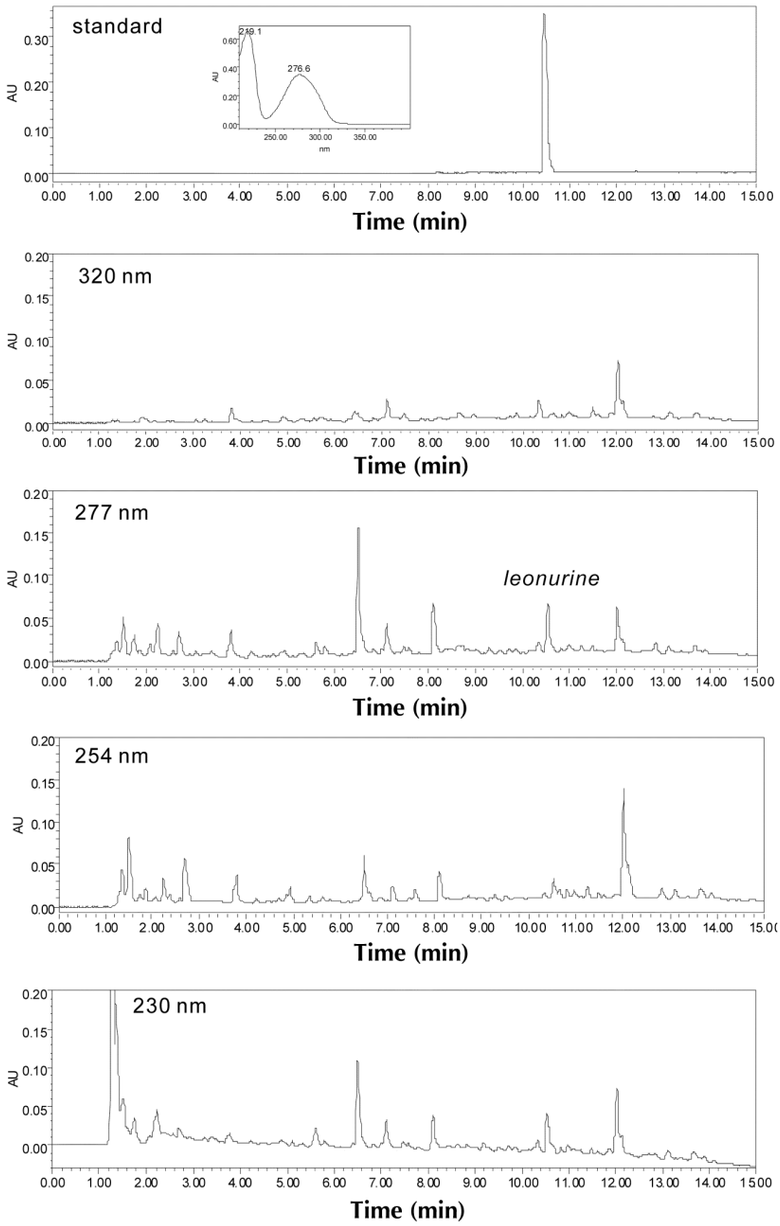

Figure 2. UPLC–DAD chromatograms of standard leonurine and the crude sample at four different wavelength.

Chromatographic conditions

UPLC–DAD quantification was performed on a reversed-phase column Acquity UPLC BEH C18 (100 mm × 2.1 mm) with 1.7 µm spherical porous particles. A linear gradient elution of A (ammonium formate buffer, pH = 4.0) and B (methanol) was performed as follows: 0 min, 2% B; 15 min, 65% B; 17 min 95% B; 20 min, 2% B; v/v. The flow rate was 0.2 mL/min. Column temperature was set at 30°C. Injected volume was 2 µL. The diode-array UV–vis detector (DAD) was used for the detection and the wavelength for quantification was set at 277 nm.

UPLC–ESI-MS analysis was operated in positive ESI mode. The electrospray needle voltage was 4 kV, and the capillary temperature was 250°C. Typical background source pressure was 1.2×10^{-5} Torr as read by an ion gauge. The drying gas was nitrogen. Following ion trapping, ions were mass analyzed and detected in the electron multiplier at 880 V. The LCQ mass analyzer was scanned to 2000 m/z in positive mode to confirm peak identity by observation of the corresponding ionized molecule ([M+H]+). Collision gas for MS–MS experiment is He. Normalized collision energy was 32.5%.

Preparation of standard solution

Stock standard solutions of leonurine were prepared in methanol at a concentration of 0.5 mg/mL, respectively. The appropriate amount of every standard solution was mixed and diluted with methanol as indicated.

Results and Discussion

Optimization of UPLC–DAD Conditions

Chromatographic conditions such as mobile phase gradient, flow rate, column tem-

perature, and injection volume were optimized in order to limit run time while obtaining the best possible peak symmetry and resolution. Considering the complexity of natural extracts that contain an extremely large number of small molecules differing in molecular weight, structural class, and, most important, hydrophobicity, various mixtures of water and methanol in combination with several acids including phosphoric acid, acetic acid, and ammonium formate were tested as a mobile phase to enhance the resolution and eliminate the peak tailing of the target compound. Among them, only ammonium formate effectively improved the peak resolution. As a result, gradient elution of the mobile phases consisting of methanol and ammonium formate buffer solution (pH = 4.0) at a flow rate of 0.2 mL/min was optimzed as UPLC conditions. It can be seen from Figure 2 that good separation and detectability of leonurine in the motherwort sample were obtained with baseline resolved peaks and chromatograms with minimal interference from the herb. Hence, it was relatively easy to estimate the peak area with acceptable accuracy. In addition, the wavelength for detection was tested at 230, 254, 277, and 320 nm and set at 277 nm, where the target peak showed the maximum intensity as measured by a DAD detector as shown in Figure 2. The presence of leonurine was verified by comparing each retention time (10.5 min) and UV spectrum with the reference compound.

Table I. LOD, LOQ, and Characteristic Parameters of Calibration Curves (7 Points)

Analytes	Calibration equation	Correlation coefficients	Calibration range mg/mL	LOD µg/L	LOQ µg /L
leonurine	$Y = 4540000X + 31700$	$R^2 = 0.9995$	0.005~0.5	2.56	8.54

Y, peak area; X, concentration of analyte; detection wavelength is 277 nm.

Compared with the reported HPLC method (16), the proposed UPLC approach greatly saves the separation time. Although the 15 min analysis seemed longer than normal UPLC separation, it is important to note that the present mixture is the crude ethanol extract of the plant, without any sample pretreatment procedure, meaning that it is extremely complex in the molecular weight, polarity, type, etc. Samples like this tend to take more than 30 min in normal HPLC analysis (16). Considering its complexity, the UPLC flow rate was set as 0.2 mL/min and 15 min separation for a single run, which exhibits excellent separation efficiency. In addition, instead of KH_2PO_4 buffer, ammonium formate buffer solution was used in the present study, which is more compatible with the LC–MS analysis.

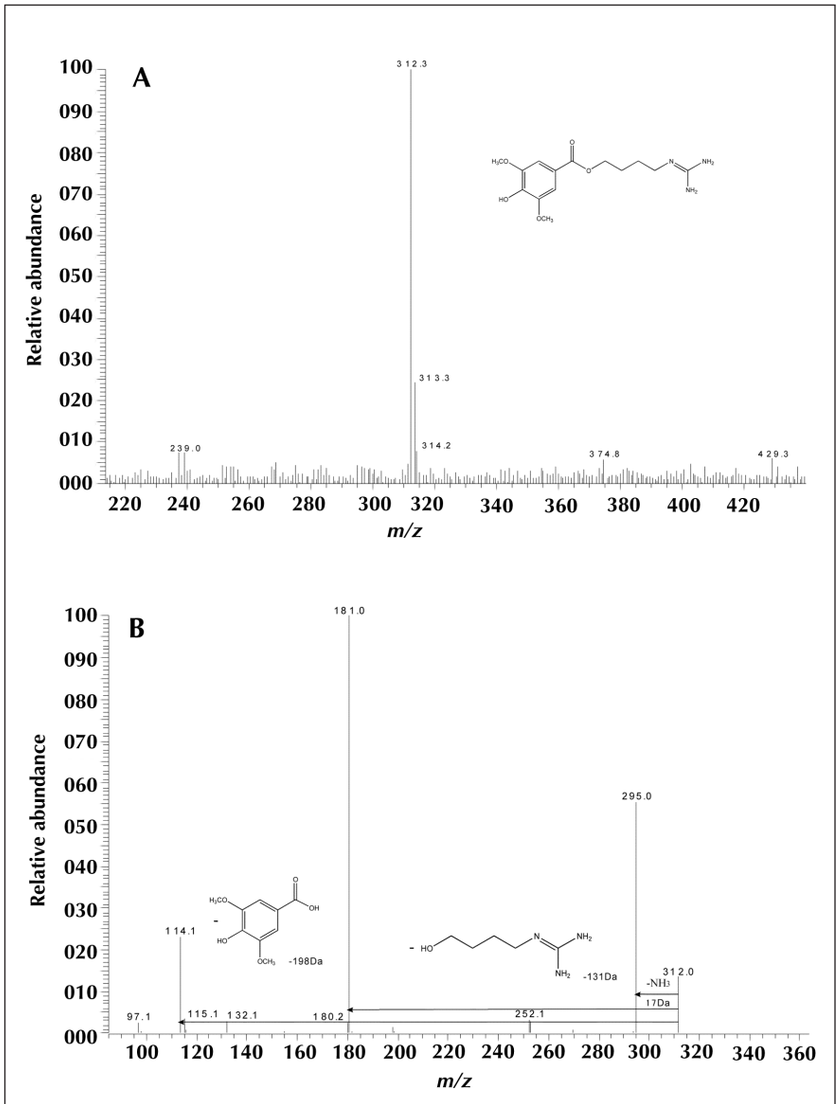

Figure 3. ESI–MS spectrum of target compound: MS spectrum (A) and MS–MS spectrum (B).

UPLC–ESI-MS–MS identification

The compound identification was performed by UPLC–MS experiment in positive mode, and the mass spectrum is shown in Figure 3. The full-scan mass spectrum (Figure 3A) of the target analyte showed the [M+H]+ ion at *m/z* 312, which was consistent with the molecular weight of leonurine. Additionally, in the MS–MS spectrum of [M+H]+ at *m/z* 312 (Figure 3B), the protonated target analyte produced three key fragment ions at *m/z* 259, 181, and 114, which were generated by the neutral losses of 17 Da, 131 Da and 198 Da, respectively. According to the detailed illustration in Figure 3B, it could be verified the target analyte to be leonurine.

Specificity and stability

Specificity was determined by the calculation of peak purity facilitated by DAD. The peak purity was evaluated using DAD and its corresponding computer software, which confirms the singularity of the peak component. The absorption spectrum of a single component remained invariable at each time

Table II. Analytical Results of Precision and Accuracy (*n* = 3)

	Precision				Accuracy		
	Inter-day		Intra-day				
Analyte	Amount (µg/mL)	RSD (%)	Amount (µg/mL)	RSD (%)	Spiked amount µg/mL	Recovery (%)	RSD (%)
% Leonurine	20	1.16	20	0.25	5.0	101.5	4.98
	10	2.37	10	0.66	10.0	103.6	3.04
	5	4.35	5	0.85	50.0	104.4	2.32

point in one peak, which supported specificity of the target peak. Moreover, the stability of the standard and sample solutions was also determined by monitoring the peak area and migration time of standard mixture solutions and sample solutions over a period of 1 week. The results showed that the migration time and peak area of each analyte remained almost unchanged, and that no significant degradation is observed within the given period, indicating the solutions are stable for at least 1 week without the results being affected.

Linearity

A series of standard samples (5.0, 10.0, 50.0, 100, 150, 250.0, and 500.0 µg/mL) containing leonurine were prepared to study the relationships between the peak area and the concentrations of the alkaloid under selected conditions. The results showed that the peak area was linearly related to the amount of leonurine for a range of 5.0–500.0 µg/mL. The results of these linearity studies are presented in Table I.

Limits of detection and limits of quantitation

The limits of detection (LOD) and quantitation (LOQ) of leonurine were determined by using signal to noise approach as defined in International Conference on Harmonization (ICH) guideline (21). The increasingly dilute solution of each drug was injected into the chromatograph and signal to noise ratio was calculated for the target compound at each concentration. The results are shown in Table I.

Precision and accuracy

The precision test was carried out by the intra- and inter-day variability for leonurine. The intra-day variability was assayed at three concentrations on the same day and inter-day variability at three concentrations on three sequential days (1, 3, 5 days). As listed in Table II, the RSD of intra-day and inter-day variability was less than 5.0%, which demonstrated good precision of this method. The accuracy of the method set up in this study was determined by the method of standard addition. The mean recoveries of leonurine from motherwort sample were evaluated by spiking three different levels of motherwort (5.0, 10.0, and 50.0 µg/mL) to the sample in replicates of three. The measured concentrations were compared with the theoretical concentrations to calculate the recovery rates. The experimental results showed that the average recovery of the target alkaloid was 103.2%.

Determination of leonurine in Chinese motherwort

The concent of leonurine in Chinese motherwort was defined as follows: extraction amounts (mg/g) = mass of leonurine in extracte solution (mg)/mass of sample (g). The mass of leonurine in extraction solution was determined by the proposed UPLC–DAD method. In present work, the extraction procedure was repeated three times and the average contents of leonurine was 0.15 mg/g (RSD = 5.2%), which was consistent with the existed HPLC method (16).

Conclusion

In conclusion, a rapid and reliable UPLC–DAD-ESI-MS method for separation and determination of the major active alkaloid present in herbal medicine *L. japonicus*, leonurine, has been developed and validated. The extracted amount of leonurine is 0.15 mg/g with the recovery of 103.2%. The method fulfilled all the requirements to be identified as a reliable and feasible method, showing good specificity, precision, linearity, and accuracy data. Therefore, this established method is useful for the quality control of Chinese motherwort.

Acknowledgements

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