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Simultaneous determination of 24 constituents in Cortex Lycii using high-performance liquid chromatography–triple quadrupole mass spectrometry

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ABSTRACT

A fast high-performance liquid chromatography (HPLC) coupled with electrospray ionization (ESI) tandem mass spectrometry method was developed to determine 24 components including 11 phenolic compounds, 9 phenolic amides, and 4 cyclic peptides in Cortex Lycii. The analytes were quantified by a triple quadrupole instrument in multiple reaction monitoring (MRM) mode. The fragmentation patterns of phenolic amides and cyclic peptides using ESI and collision-induced dissociation (CID) techniques are reported. This assay method was validated with respect to linearity ($r^2 > 0.9920$), precision, repeatability, and accuracy (recovery rate between 93.0 and 105.9% with RSD < 4.4%). The analytical results of 28 batches of Cortex Lycii indicated that cyclic peptides and phenolic amides were not only the abundant constituents, but also the characteristic components for Cortex Lycii to distinguish from the adulterants. Principle component analysis (PCA) was used to discriminate samples from different geographical regions of China, and cyclic peptides were considered to be the chemical markers responsible for the classification. The systematic and integrated assessment of Cortex Lycii provides sufficient evidence for the establishment of the quality standard.

1. Introduction

Cortex Lycii, the root bark of *Lycium chinense* Mill. or *Lycium barbarum* L., is official in Chinese Pharmacopeia (2010 version) [1]. It has been widely used in eastern countries as an antipyretic and for the treatment of pneumonia, night-sweats, cough, hematemesis, inflammation, and diabetes mellitus. Studies on pharmacology and clinical practice have demonstrated that Cortex Lycii possesses the functions of lowering blood glucose, blood pressure, and lipid levels [2–4].

There are very few reports regarding the quantification of the active components of this commonly used traditional medicine including determination of scopoletin content by HPLC [5] and assay of seven ingredients by capillary zone electrophoresis with amperometric detection method [6]. In the monograph of Chinese Pharmacopeia (2010 version), only TLC method is used to identify the authenticity of Cortex Lycii by comparing with the crude drug reference, and no content determination item is recorded, which is not sufficient for the quality evaluation and standardization of this herbal medicine. On the other hand, an adulterant named "white Cortex Lycii" is popular on the herbal medicine market because of its similar appearance with the certified products and the lower price. In view of the current situation, it is necessary to develop a rapid and reliable method to assay the bioactive components for the quality control of Cortex Lycii.

The curative effects of TCMs are integrative action derived from a group of bioactive components, and TCMs have typical characteristic of synergistic effect of multiple components on multiple target sites [7–9]. Therefore, in the process of modernization and globalization of TCMs, development of effective methods to quantify as many bioactive components as possible for their overall quality control has become a rational strategy [10,11]. The multiple-type compounds in Cortex Lycii include alkaloids, cyclic peptides, flavonoids, coumarins, terpenoids, organic acids, and their derivatives [12–18]. In the present study, three types of compounds including 11 phenolic compounds (consisting of 6 phenolic acids, 3 coumarins, and 2 flavonoids), 9 phenolic amides, and 4 cyclic peptides were quantitatively determined as the chemical markers. These analytes were selected on the basis of previous phytochemical study [18] and pharmacological literature surveys. Phenolic compounds exert a wide range of effects, such as antioxidant, anti-cancer, and so on [19,20]. Phenolic amides

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possess anti-inflammatory, anti-cancer, anti-fungal, and platelet anti-aggregatory functions, while cyclic peptides exert anti-ACE and -renin properties which may be related to the hypotensive activity [13,14,21,22]. Therefore, the 24 analytes were chosen in the quantitative analysis.

Since the constituents in Cortex Lycii are extremely complex and some in trace amounts, it is almost impossible to simultaneously determine multi-components by using HPLC-UV method. Fast HPLC separation with detection by ESI-MS/MS using a triple quadrupole instrument in MRM mode was employed and excellent sensitivity and selectivity were obtained. The validated method was applied to evaluate the quality of 28 samples from different geographical regions, and the results were further analyzed by PCA to provide more information about the chemical difference in each sample. To the best of our knowledge, this is the most comprehensive report on the quantitative analysis of Cortex Lycii.

2. Experimental

2.1. Chemicals, reagents, and materials

24 constituents, including scopolin (**1**), dihydro-*N*-caffeoyltyramine (**2**), isoscopoletin (**3**), fraxidin (**4**), *trans*-*N*-caffeoyltyramine (**5**), (*E*)-2-(4,5-dihydroxy-2-{3-[(4-hydroxyphenethyl)-amino]-3-oxopropyl}phenyl)-3-(4-hydroxy-3,5-dimethoxyphenyl)-*N*-(4-acetamidobutyl)acrylamide (**6**), (1,2-*trans*)-N^3-(4-acetamidobutyl)-1-(3,4-dihydroxyphenyl)-7-hydroxy-N^2-(4-hydroxyphenethyl)-6,8-dimethoxy-1,2-dihydro-naphthalene-2,3-dicarboxamide (**7**), (*E*)-2-(4,5-dihydroxy-2-{3-[(4-hydroxyphenethyl)-amino]-3-oxopropyl}phenyl)-3-(4-hydroxy-3-methoxyphenyl)-*N*-(4-acetamidobutyl)acrylamide (**8**), *trans*-*N*-*p*-hydroxycinnamoyl-tyramine (**9**), lyciumin A (**10**), *trans*-*N*-isoferuloyltyramine (**11**), (*E*)-2-(4,5-dihydroxy-2-{3-[(4-hydroxyphenethyl)amino]-3-oxopropyl}phenyl)-3-(4-hydroxy-3,5-dimethoxyphenyl)-*N*-(4-hydroxyphenethyl)acrylamide (**12**), 7-hydroxy-1-(3,4-dihydroxy)-N^2,N^3-bis(4-hydroxyphenethyl)-6,8-dimethoxy-1,2-dihydronaphthalene-2,3-dicarboxamide (**13**), lyciumin B (**14**), lyciumin D (**15**), lyciumin C (**16**), apigenin (**17**), acacetin (**18**), dihydrocaffeic acid (**19**), *p*-hydroxybenzcic acid (**20**), caffeic acid (**21**), *p*-coumaric acid (**22**), ferulic acid (**23**), and 4-methoxysalicylic acid (**24**) were isolated from the root bark of *L. chinense* in our laboratory. These compounds (Fig. 1) were identified using ESI-MS^n, ^{1}H and ^{13}C NMR techniques, and by comparing their experimental and reported spectroscopic data [15,18]. The purity of all constituents exceeded 97% by HPLC analysis.

Acetonitrile and formic acid were of HPLC grade (Burdick & Jackson, Honeywell International, Inc., USA). The methanol used for extraction was supplied by Sinopharm Chemical Reagent Co. Ltd., China. HPLC grade water was prepared using a Milli-Q water purification system (Millipore, MA, USA). Cortex Lycii samples collected in present study were of the root bark of *L. chinense* from Shanxi (SX), Gansu (GS), Ningxia (NX), Qinghai (QH), Henan (HN), and Anhui (AH) provinces in China. The adulterants of "white Cortex Lycii" were from SX and Shaanxi (SaX) provinces. The material origin was identified by associate professor Jin-Gui Shen (Shanghai Institute of Materia Medica, Chinese Academy of Sciences).

2.2. Instrumentation and conditions

Chromatographic analysis was performed on an Agilent Rapid Resolution HPLC system, 1200 series (Agilent, Germany) equipped with a binary pump, micro degasser, an auto plate-sampler, and thermostatic column compartment. Separation was performed on an Agilent Eclipse plus C_{18} (2.1 mm × 50 mm, 1.8 μm) column with an in-line filter in front of the column. The mobile phase was composed of 0.1% formic acid (A) and acetonitrile (B), with a gradient elution as follows: 0 min, 18% (B); 10 min, 45% (B); 10 min, 90% (B); 15 min, 90% (B) when detected in the positive ion mode and 0 min, 12% (B); 8 min, 40% (B); 10 min, 90% (B); 15 min, 90% (B) in negative ion mode. The column temperature was set at 40 °C. The flow rate was 0.3 ml/min.

Mass spectrometry was performed using an Agilent 6410 triple quadrupole mass spectrometer equipped with an electrospray ionization source (ESI). The MS conditions were as follows: drying gas temperature, 300 °C; drying gas flow, 8 l/min; nebulizer pressure, 35 psi; corona current, 10 nA; and capillary voltage, 4000 V. MRM mode was applied for quantitative analysis using precursor/product ion information. Data acquisition was performed with MassHunter Workstation (Agilent Technologies, USA).

2.3. Preparation of standard solutions

Each accurately weighted standard was dissolved in methanol to give the stock solutions. Working standard solutions containing 18 and 6 reference standards respectively detected in positive and negative ion mode were prepared by diluting the stock solutions with acetonitrile–water (50:50, v/v) solvent to a series of proper concentrations. The solutions were stored at 4 °C.

2.4. Preparation of samples

The dried powder of Cortex Lycii (0.5 g) was accurately weighed and ultrasonicated with 25 ml 80% methanol for 30 min. The mixture was filtered and the obtained residue was extracted again with the same method. Respective supernatants were combined and concentrated in a rotary evaporator at 40 °C under reduced pressure. The obtained extract was diluted with acetonitrile–water (50:50, v/v) to 5 ml. Prior to use all samples were filtered through a 0.22 μm membrane filter. Two microliter of each filtrate was injected into the HPLC instrument for analysis.

2.5. Method validation

The calibration curves (the peak area versus the concentration of each analyte) were established by injecting each working solution twice. Limit of detection (LOD; S/N>3), limit of quantification (LOQ; S/N>10), precision, repeatability, and recovery were studied respectively as described below:

The precision of the developed method was determined by the intra- and inter-day variations. For intra-day variability test, a sample solution prepared as the method described in Section 2.4 was analyzed for six replicates within one day, while for inter-day variability test, the sample was examined in duplicates for consecutive three days. The relative standard deviation (RSD) for peak area was calculated as the measure of precision.

To confirm the repeatability, five replicates of the same samples were extracted and analyzed. The RSDs were used to evaluate the method repeatability.

Recovery was determined by analyzing spiked samples. A known amount of the standards (low, medium, and high concentrations) were added into a certain amount of samples (0.25 g), and then extracted and analyzed with the same procedures. Three replicate extractives at each level were used to calculate the extraction recovery rates for evaluating the method accuracy.

2.6. PCA based on the data obtained from quantitative determination

PCA was carried out based on the contents of 24 analytes in 28 samples, using SIMCA-P+ 13.0 Software.