Simultaneous determination of four bioactive compounds in *Glechoma longituba* extracts by high performance liquid chromatography

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ABSTRACT

Background: *Glechoma longituba*, one of the long-practiced traditional Chinese medicines in history, is still commonly used nowadays in oriental countries. Previous study indicates that phenolic acid and flavonoids have considerable bioactivities, thus, chlorogenic acid, caffeic acid, apigenin-7-O-glucoside and rosmarinic acid were chosen as the marker components for the simultaneous determination to evaluate the intrinsic quality of *G. longituba*, and related high performance liquid chromatographic method was urgent to established. **Materials and Methods:** A HPLC method was established for simultaneous determination of four major active components in *G. longituba*, three batches which collected from different suppliers in Zhejiang, Anhui and Jiangsu Provinces were determined. **Results:** The contents of chlorogenic acid, caffeic acid, apigenin-7-O-glucoside and rosmarinic acid in *G. longituba* samples analyzed were 0.00225-0.00234%, 0.0238-0.0242%, 0.00271-0.00313%, 0.830-0.896%, respectively. **Conclusion**: The developed method can be applied to the intrinsic quality control of *G. longituba*.

Key words: *Glechoma longituba*, High performance liquid chromatography, quality control, simultaneous determination

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INTRODUCTION

Glechoma longituba, the dried aerial part of *G. longituba* (Nakai) Kupr (Labiatae), is distributed widely in Asia, Europe and America. It has been used for centuries in traditional Chinese medicine for the treatment of cholelithiasis, urolithiasis, dropsy and various ailments, asthma, bronchitis, colds and inflammation.[1-3] Previous research on the chemical constituents, including phenolic acid, flavonoids, triterpenoids and essential oil, has been isolated from the herb and identified.[4-5] Pharmacological studies have indicated that phenolic acid such as caffeic acid, chlorogenic acid and rosmarinic acid have significant antioxidant bioactivities, while flavonoids are famous for their antioxidant and antiradiation abilities.[6-9] Hence, chlorogenic acid, caffeic acid, rosmarinic acid and apigenin-7-O-glucoside were chosen as marker components for intrinsic quality assessment of *G. longituba*. Three batches of *G. longituba* from Zhejiang, Anhui, Jiangsu Provinces were collected. An efficient and reliable approach for quality evaluation of *G. longituba* has been established based on high performance liquid chromatography, which can also be further applied for the determination of phenolic acid and flavonoids in its related processed drugs or preparations.

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EXPERIMENTAL METHOD

Materials and reagents

Three batches of *G. longituba* were collected from different suppliers in Zhejiang, Anhui, Jiangsu Provinces and identified by Prof. Zhang Yun, in Zhejiang Chinese Medical University. Reference standards of caffeic acid and chlorogenic acid were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Rosmarinic acid was purchased from Shanghai Tongtian Biotechnology Co. Ltd. (Shanghai, China). Apigenin-7-O-glucoside was purchased from Shanghai Yongheng Biotechnology Co. Ltd. (Shanghai, China). The purity for each standard compound was greater

than 98% by HPLC analysis. The structures of these four compounds were shown in [Figure 1]. All reagents with high grade were obtained from others. Milli-Q water (Millipore, Bedford, MA, USA) was used throughout the study.

Preparation of sample solutions

The powder of *G. longituba* samples (1.0 g) was accurately weighed and dissolved in 10 ml of 80% methanol solution and then refluxed for 60 min and cooled at room temperature; methanol was added to compensate for the lost weight. The sample solution was centrifuged (× 4000 g) for 15 min and the supernatant was filtered through a 0.45-μm membrane filter, before subjecting 10 μL to HPLC analysis.

Preparation of standard solutions and calibration curve

The standards of chlorogenic acid, caffeic acid, rosmarinic acid and apigenin-7-O-glucoside were dried and accurately weighed and then dissolved with methanol to produce stock standard solutions. A mixed stock solution of standards, containing chlorogenic acid (8.8 mg/mL), caffeic acid (1.64 mg/mL), rosmarinic acid (4.94 mg/mL), and apigenin-7-O-glucoside (5.8 mg/mL) was finally prepared. The calibration curves were established based on seven concentrations of each standard by diluting the stock solutions with methanol in appropriate quantities. All calibration curves were constructed from peak areas of the reference standards versus their concentrations. The standard stock and working solutions were all prepared in calibrated flasks and stored at 4°C. The solutions were filtered through a 0.45-μm membrane prior to injection.

Chromatographic conditions

The chromatographic analyses were carried out on an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA, USA) with diode array detector. An Agilent Zorbax Extend C_{18} Column (250 mm × 4.6 mm, 5 μm) was used with a flow rate of 1.0 mL/min for the sample. The injection volume was 10 μL and the column temperature was maintained at 30°C. Mobile phase was consisted of acetonitrile (A) and aqueous phosphoric acid (0.2%, v/v) (B) with gradient elution: 0~15 min (2:98), 15~18 min (14:86), 19~30 min (20:80), 31~69 min (30:70), 70~80 min (40:60), 81~89 min (50:50). The optimized detection wavelength was 325 nm.

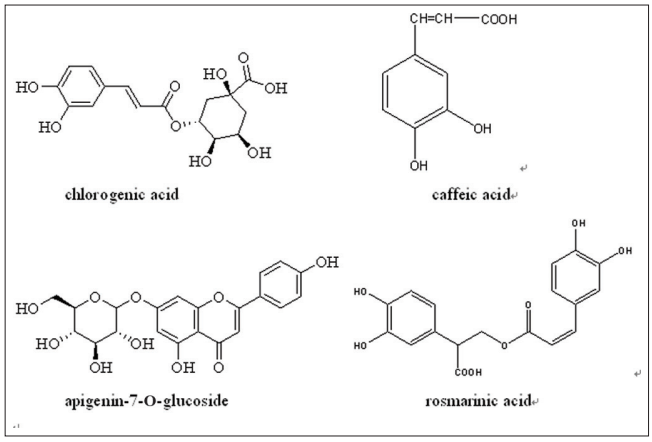

Figure 1: The chemical structures of four active components in *G. longituba*

RESULTS AND DISCUSSION

Optimization of HPLC chromatographic conditions

The aim of this study was to develop a HPLC method using DAD detection for simultaneous determination of chlorogenic acid, caffeic acid, rosmarinic acid and apigenin-7-O-glucoside. HPLC conditions including mobile phase, gradient elution procedure, detection wavelength, flow rate of the mobile phase and column temperature were optimized, respectively, to achieve good separation of marker components. The mobile phase composition and gradient program were first investigated. Several elution systems were trialled, the phosphoric acid, acetic acid as well as formic acid were employed for the study of condition optimization with acetonitrile as the organic phase. The results suggest that the elution system composed of 0.2% phosphoric acid with acetonitrile was selected as the most appropriate eluent.

The choice of detection wavelength is also crucial for developing a reliable determination method for accurate quantitative analysis of these four bioactivity ingredients in the herb. The UV spectra of the compounds were obtained at 210, 230, 280, 300 and 325 nm by diode array detector under the chromatographic conditions as described in chromatographic system. The optimal detection wavelength in the HPLC analysis was to be 325 nm, with marker compounds sensitively detected in the analysis. Typical separation of a standard mixture in different wavelength (A); reference standards (B) and *G. longituba* extracts (C) were shown in [Figure 2] under the optimized HPLC conditions.

Method validation

The HPLC method was validated by defining the linearity, limits of quantification and detection, repeatability, precision, stability and recovery.

Linearity, limits of quantification and detection

Series of standard solutions of four compounds were freshly prepared in methanol respectively, and were used to determine linear range of the analytes. The results of the standard calibration curves of regression equation and linearity were summarized in [Table 1]. Good linear relationships and correlation coefficients ($r > 0.9995$) were achieved. The limit of detection (LOD) and limit of quantification (LOQ) values of individual compounds

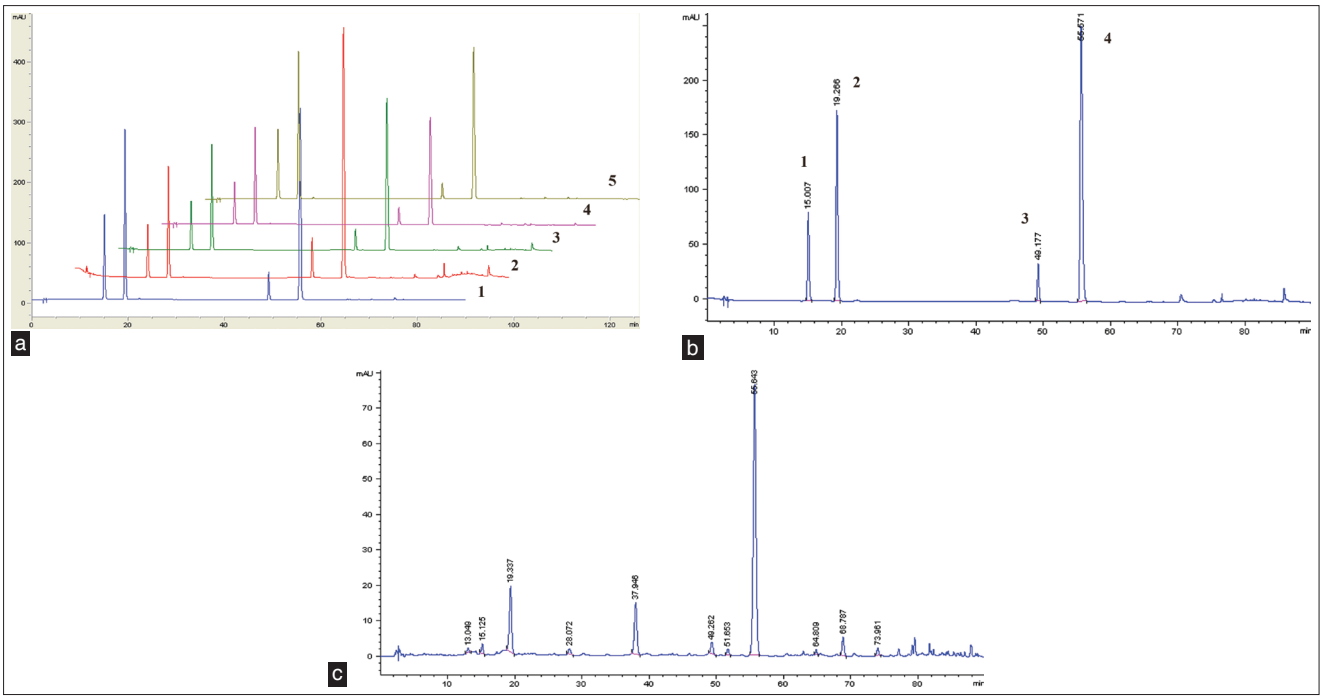

Figure 2: (a) Typical chromatograms of reference standards in different detection wavelengths: (1) 325 nm, (2) 210 nm, (3) 230 nm, (4) 280 nm, (5) 300 nm; (b) Typical chromatograms of reference standards in optimized wavelength: (1) chlorogenic acid; (2) caffeic acid; (3) apigenin-7-O-glucoside; (4) rosmarinic acid; (c) Typical chromatograms of *G. longituba* samples

Table 1: Calibration plots, LOD and LOQ of the four compounds in *G. longituba* extract

Compound	Calibration equation	r	Linearity range (mg/ml)	LOD (μg/ml)	LOQ (μg/ml)
Chlorogenic acid	Y = 23.46X + 0.98	0.9999	1.64 ~ 164.00	1.2	6.0
Caffeic acid	Y = 48.61X + 41.59	0.9998	0.176 ~ 880.00	1.3	4.2
Apigenin-7-O- glucoside	Y = 3.15X - 2.39	0.9999	0.174 ~ 580.00	1.4	5.1
Rosmarinic acid	Y = 3.02X - 26.43	0.9999	370.50 ~ 2470.00	1.8	6.5

listed in [Table 1] clearly indicated that the analytical method was acceptable with excellent sensitivity.

Precision, repeatability and stability

Intraday and interday variability were investigated by determining each marker compound at one concentration level in six replicates during a single day and by replicating the experiments on three consecutive days, variations were expressed by relative standard deviations (RSD) and remained <1.74% for all the marker compounds. Six independently prepared sample solutions with the same amount were calculated for evaluation of repeatability, RSD were less than 2.89%. The processes of the treatments were in parallel. One of the samples was injected into the instruments at 0 h, 2 h, 4 h, 6 h, 8 h, 12 h and 24 h to evaluate the stability of the solution. RSD values of the peak area were less than 1.39% for all the compounds studied. These results suggested that it was feasible to analyze samples within 1 day.

Accuracy

Recovery tests were used to evaluate the accuracy of the method by analysis of spiked samples at one certain concentration level (approximately equivalent to 1.0 time to the concentration contained in the sample). The extracted solution was analyzed by the proposed HPLC method. Quantity of each compound was subsequently obtained by use of the corresponding calibration plots. The recoveries of analytes varied from 99.77% to 100.89% and RSD values were in the range of 0.20-1.75%, which demonstrated the reliability and accuracy for the measurement of these constituents.

Sample analysis

The developed method was applied to the simultaneous determination of chlorogenic acid, caffeic acid, apigenin-7-O-glucoside and rosmarinic acid in three batches of *G. longituba* samples obtained in different provinces in China. Each sample was determined in triplicate, and the peaks in chromatograms were identified by comparing the retention times and UV spectra with those of the standards. The contents were calculated and summarized ($n = 3$) in [Table 2]. According to the quantitative analysis results, we noticed that the total contents of four compounds varied in the same

Table 2: Concentrations (%) of the four compounds in three batches of *G. longituba*

Batch	Content (n = 3)			
	Chlorogenic acid	Caffeic acid	Apigenin-7-O-glucoside	Rosmarinic acid
Zhejiang	0.00225	0.0242	0.00271	0.840
Anhui	0.00225	0.0242	0.00273	0.830
Jiangsu	0.00234	0.0238	0.00313	0.896

type of samples from different provinces, which might be due to the differences of growing condition and climate in each region. Thus it is necessary to control the main active components in *G. longituba* by good agricultural practice (GAP) and the norm of Chinese medicinal materials processing.

CONCLUSIONS

This novel HPLC method for simultaneous quantitative analysis of four active components was established and validated for quality evaluation of *G. longituba*. Chlorogenic acid, caffeic acid, apigenin-7-O-glucoside and rosmarinic acid were considered as the marker components to evaluate the intrinsic quality of *G. longituba* methanol extract, and three batches of samples which collected from Zhejiang, Anhui and Jiangsu Provinces were analyzed. The newly developed method has achieved desired linearity, precision and accuracy, and has been elucidated to be a simple, sensitive, accurate and reliable procedure for quality control of *G. longituba*, which can also be further applied for the quantification of phenolic acids and flavonoids in its related processed drugs or preparations.

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