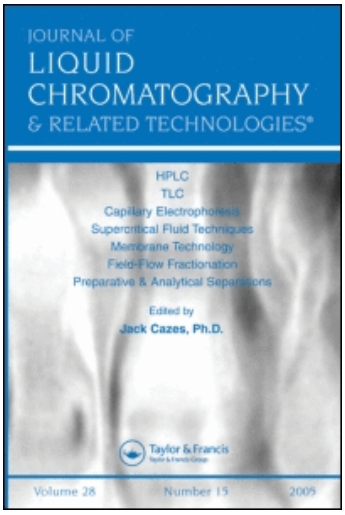

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Hong-Xia Li[a]; Ming-Yu Ding[a]; Kun Lv[a]; Yang Wei[a]; Jian-Yuan Yu[a]
[a] Tsinghua University, Beijing, China

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IDENTIFICATION AND DETERMINATION OF THE ACTIVE COMPOUNDS IN *GASTRODIA ELATA BLUME* BY HPLC

Hong-Xia Li, Ming-Yu Ding,* Kun Lv, Yang Wei, and Jian-Yuan Yu

Tsinghua University,
Beijing, 100084, China

ABSTRACT

A simple, sensitive, and specific high-performance liquid chromatography method has been developed for the simultaneous quantification of the three major biologically active ingredients, gastrodin (GA), 4-hydroxybenzyl alcohol (HA), and 4-hydroxybenzaldehyde (HD), in *Gastrodia elata Blume* for the first time. The HPLC assay was performed on a reversed phase ODS column by using methanol-water-isopropyl alcohol (35:55:10, v:v:v) as mobile phase with a flow rate of 0.4mL/min. The detection wavelength was set at 270 nm. Regression equations revealed linear relationships (correlation coefficients: 0.9997–0.9999) between the peak area of each constituent (GA, HA, HD) and its concentration.

The relative standard deviations of the retention times of three constituents range between 0.2~0.5%. The recoveries for the three constituents ranged between 96.6~98.5%. The GA, HA, HD contents measured 12.0 mg/g (2.53% RSD), 2.1 mg/g (2.04% RSD), and 0.13 mg/g (2.63% RSD), respectively, in the ethanol extracts of *Gastrodia elata Blume*.

INTRODUCTION

Gastrodia elata Blume (Orchidaceae) is a saprophyte widespread in Chinese Province Yun-nan, Si-chuan, Shan-xi, Gui-zhou, etc. Its steamed and dried roots of the plant are used as a folk medicine under the Chinese name: Tianma.[1] It is a commonly used Chinese herbal drug which was considered to have many beneficial properties. It is said to aid in expelling all kinds of toxins from the body, to enhance strength and virility, and to improve the circulation and the memory. It is prescribed for rheumatism, neuralgia, paralysis, lumbago, headaches, and other neuralgic and nervous affections.[2–5]

Phytochemical studies of this plant have revealed the presence of 4-hydroxybenzyl alcohol (HA), 4-hydroxybenzaldehyde (HD), 4-(β-D-glucopyranosyloxy) benzyl alcohol (GA), vanillyl alcohol, 4-hydroxybenzyl methyl ether, bis (4-hydroxybenzal) ether, daucosterol, β–sitosterol, citric acid, palmitic acid, and sucrose.[6–8] The former three (HA, HD, GA) were reported as the main active constituents of this plant.

Several methods for determination of GA or HA have been reported, i.e., spectrophotometry,[9] thin-layler chromatography (TLC),[10] high performance liquid chromatography (HPLC),[11–12] capillary electrophophoresis (CE).[13] However, most of these methods seem to be unsuitable for quantitative determination of these compounds, owing to either poor resolution or low sensitivity.

Furthermore, the known methods require tedious pretreatment of extracts before analysis, leading to a possible source of error. The present paper using RP-HPLC combined with diode array detector separated and quantified GA, HA, and HD in *Gastrodia elata Blume.* It was the first time to provide a HPLC method for the simultaneous determination of GA, HA, and HD, which was a simple, reliable, and rapid method, avoiding the time-consuming and costly isolation.

EXPERIMENTAL

Reagents

4-Hydroxybenzyl alcohol (HA) and 4-hydroxybenzaldehyde (HD) were of analytical grade, purchased from Sigma; 4-(β-D-glucopyranosyloxy) benzyl alcohol (GA) was of analytical grade, purchased from Chinese Medicine Control Institute. Methanol was of HPLC grade; other chemicals were of analytical grade.

Gastrodia elata Blume (crude medicinal material) was collected from Chengdu, Si Chuan Province.

Sample Preparation

10.0 g crude medicinal material of *Gastrodia elata Blume* was extracted with 150 mL 75% ethanol by refluxing on a water bath at 80°C for 1hr, then was placed in an ultrasonic bath for 20 minutes. Extraction was repeated three times. The extracts were filtered with Whatman No1 filter paper and the ethanol was removed with a rotary evaporator at 40°C under vacuum.

The residue was diluted to proper concentration with methanol. The clarified solution of sample was filtered through a 0.45 μm membrane filter before HPLC analysis.

Preparation of the Ether Extract and Butanol Extract

The ethanol extracts dissolved in water were extracted with petroleum ether to remove esters. Then the aqueous solution was successively extracted with ethyl ether and butanol three times, respectively. Organic solvents were removed with a rotary evaporator at 40°C under vacuum to obtain the ethyl ether extracts and butanol extracts. The ethyl ether extract and butanol extract were dissolved with methanol and filtered through a 0.45 μm filter for HPLC analysis.

High Performance Liquid Chromatography (HPLC)

An HP 1100 Chromatograph, consisted of a quaternary-pump, a diode array detector (with a wavelength range from 190–900 nm, sampling interval is <1 nm) and a HP Chemstation data analysis system, is employed. A ODS column Zorbax SB-C18 (250 x 4.5 mm, I.D., 5 μm) was used for separation.

Methanol-water-isopropyl alcohol (35:55:10, v/v/v) was used as the mobile phase with a flow rate of 0.4 mL/min. The mobile phase was filtered by a Millipore vacuum filter system equipped with a 0.45 μm filter before use. The detector was set at 270 nm and the column temperature was 30°C. The inject volume of samples was 20 μL.

RESULTS AND DISCUSSION

Optimization of Separation of HA, HD and GA

A normal-phase column (Zorbax Rx-Sil, 4.6 mm ID ×25 cm, 5 μm) was used to separate the mixture of the standard HA, HD, and GA, using hexane–ethyl acetate-methanol (30:10:10, v/v) as the mobile phase at a flow rate

of 0.8 mL/min, detected at 270 nm. The compounds can not be baseline-separated (shown in Fig. 1). Changing the constitutes and concentration of the mobile phase, the separation was still not satisfactory. Because the normal-phase separation using organic solvent as mobile phase will cost more than the reversed-phase separation, and the organic mobile phase results in more serious pollution, we selected the reversed phase for separation.

The separation was achieved on a reversed phase column ODS Zorbax SB-C18. Methanol-water-isopropyl alcohol (35:55:10, v/v/v) was used as the mobile phase with a flow rate of 0.4 mL/min. In the present paper, we also tested the methanol-phosphate buffer system as the mobile phase. The chromatographic peaks were baseline-separated, while the peak symmetry was poor. Changing the concentration of the phosphate and the ratio of methanol or adding acetic acid in the mobile phase, the peak symmetry was not improved. The reason probably was that the solutes were adsorbed on the stationary phase strongly.

The Identification of GA, HA, and GA in *Gastrodia elata Blume*

The ethanol extracts dissolved in methanol was analyzed without further purification. Baseline separation was achieved within 10 min for three com-

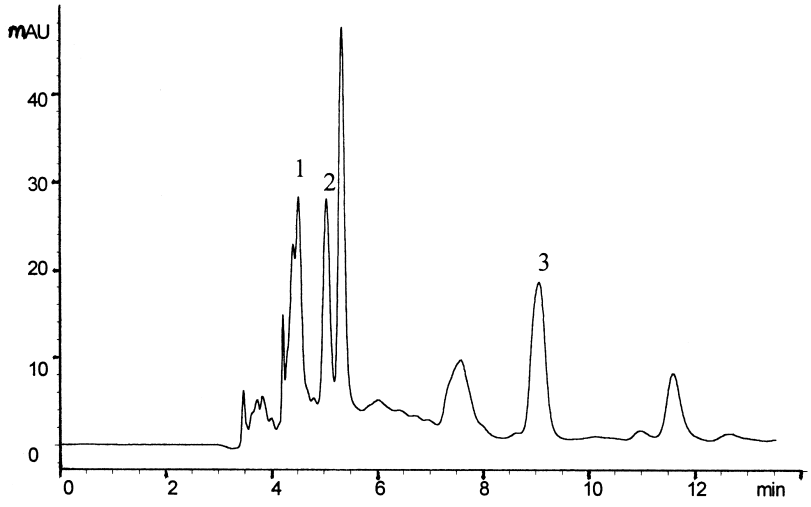


Figure 1. Chromatogram of the ethanol extracts of *Gastrodia elata Blume* separated. Column: normal-phase column Zorbax Rx-Sil, (4.6 mm ID ×25 cm, 5 μm); mobile phase: hexane–ethyl acetate-methanol (30:10:10, v/v); flow rate: 0.8 mL/min; detector: DAD, 270 nm; Peak: 1- HD, 2- HA, 3- GA.

pounds (Fig. 2a). Comparing the retention time of the extracts and the standards (Fig. 2b), peak 1, 2, and 3 were corresponding to GA, HA, and HD, respectively. Changing the constitutes and concentration of the mobile phase, the retention time was still corresponding well.

In order to further identify HA, HD, GA, the sample was treated by ethyl ether and butanol to prepare the ethyl ether extract and butanol extracts (shown as experimental). The chromatograms and UV spectra of these two fractions were shown in Fig. 3 and Fig. 4. From these figures, it can be seen that the ethyl ether extract mainly contains HA, HD, the butanol extract mainly contains GA, which can be identified by comparing the retention time and UV spectrum of the sample and the authentic compounds.

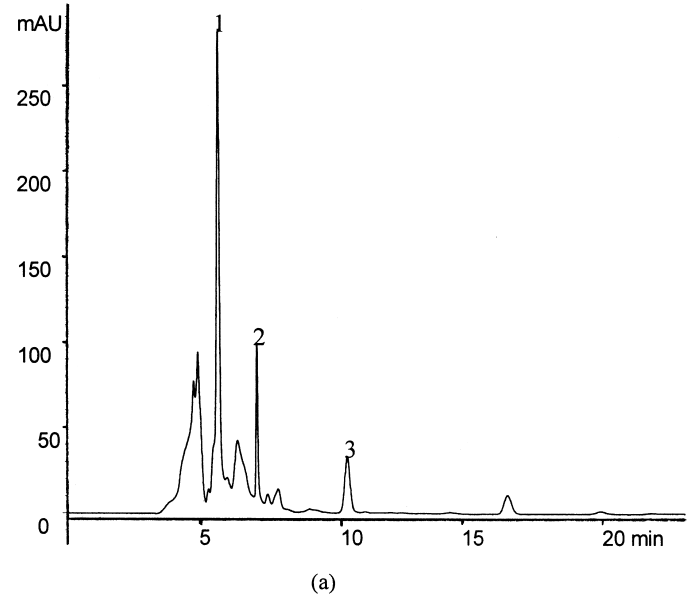
By comparing the retention time and UV spectrum of the extracts and the standard, GA, HA, and HD in *Gastrodia elata Blume* were identified. The retention time of GA, HA, and HD was 5.7 min, 7.4 min, and 10.8 min, respectively, with the relative standard deviations 0.5%, 0.15%, and 0.15%, respectively.

The Quantitative Analysis of GA, HA, and GA in *Gastrodia elata Blume*

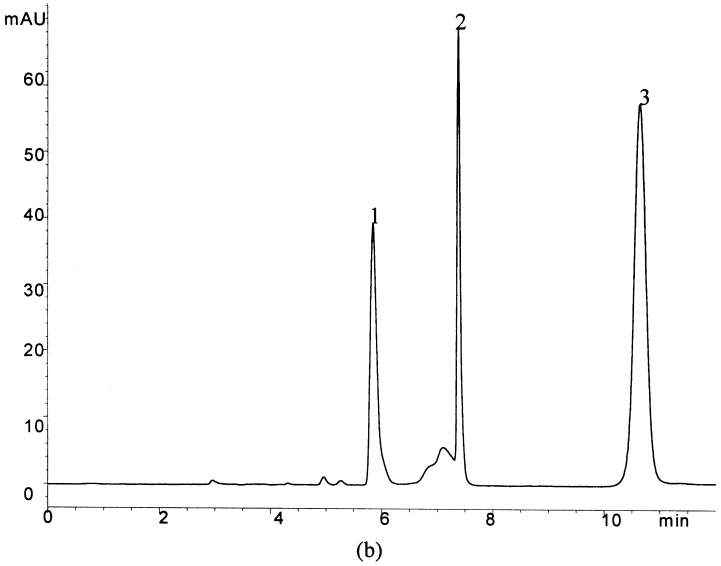
The contents of GA, HA, and HD in *Gastrodia elata Blume* were quantified from the corresponding peak area, using linear equations. For this purpose, standard solutions of pure samples of GA, HA, and HD, in variable concentrations, were run three times. The average peak area was calculated and plotted, and linear regression analysis was performed. The linear relationship between the concentration of three compounds and the corresponding peak areas was found in the concentration range of 8.5–890 μg/mL for GA, 10–500 μg/mL for HA, 6.4–400 μg/mL for HD.

The linear ranges of the calibration curves of the peak areas for the analytes was over two orders of magnitudes with a correlation coefficient of 0.9997–0.9999. The linear range of the peak height calibration curves was narrower than that of the peak areas.

Chromatographic precision, expressed as relative standard deviation (RSD), was calculated by injection of five replicates of the central point of the calibration curve. The detection limit was calculated by the formula: 3S.D/b, where S.D is the standard deviation, calculated by injecting 5 replicates of the lowest concentration solution of the calibration curve, and b is the value of the calibration curve slope. This formula is one of the possible algorithms for calculating the detection limit defined as the analyte amount that gives a signal-to-noise ratio:3. The limits of detection of GA, HA, and HD were 10, 5, and 0.5 ng/mL (20 μL injection), respectively. This is, in principle, sufficient for analyte recognition.



(a)



(b)

Figure 2. Chromatograms of the ethanol extracts of *Gastrodia elata Blume* (a) and the standards of GA, HA and HD (b). Column: ODS Zorbax SB-C18 (250×4.5 mm, I.D., 5 μm); mobile phase: methanol-water-isopropyl alcohol (35:55:10, v/v/v); flow rate: 0.4 mL/min; peak: 1-GA, 2- HA, 3- HD.

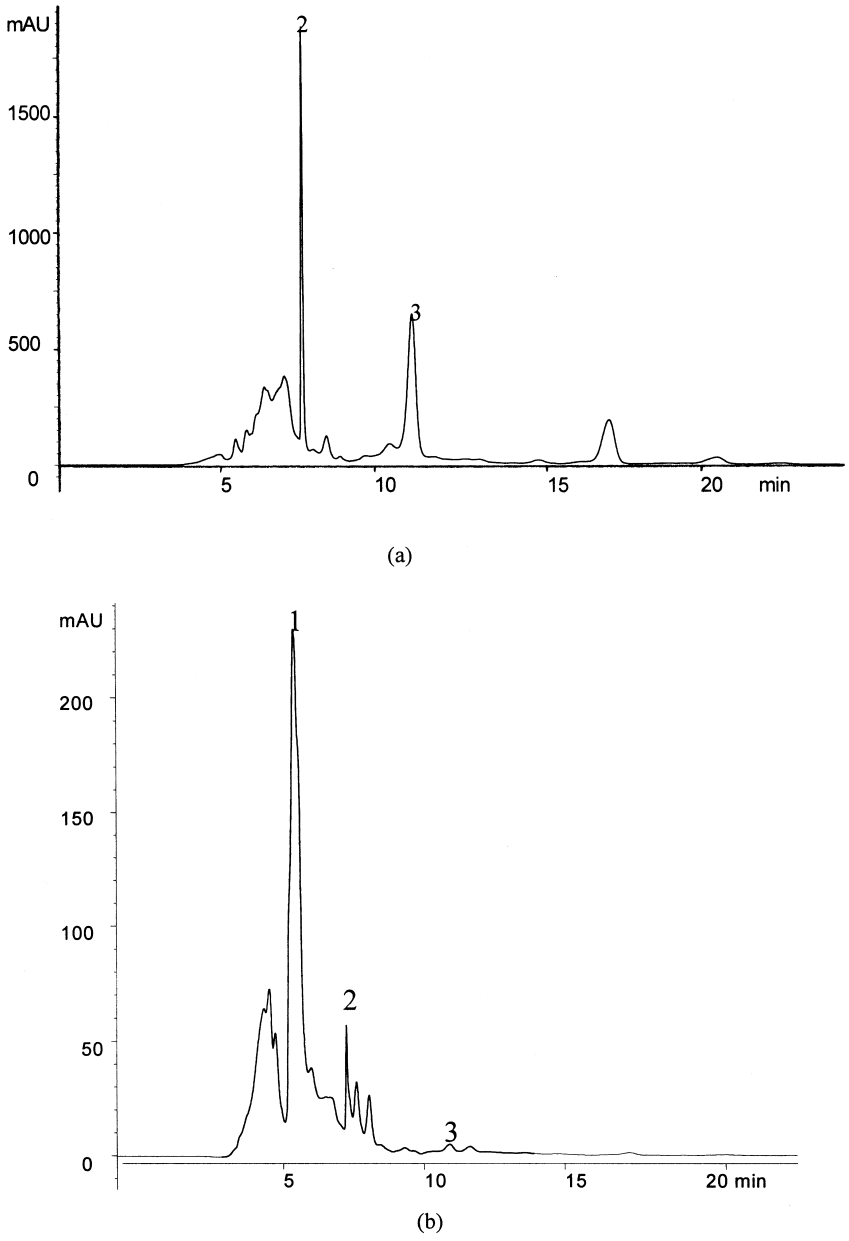


Figure 3. Chromatograms of the ethyl ether extracts (a) and butanol extracts (b). Separation conditions were the same as the Fig. 2; peaks:1- GA, 2- HA, 3-HD.

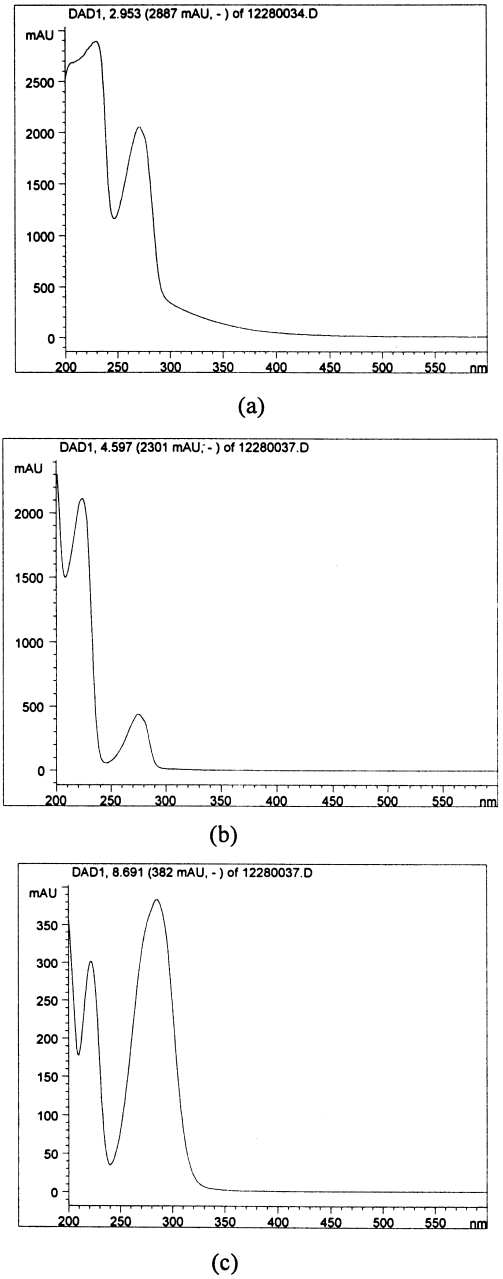


Figure 4. UV spectra of the peak 1 (a), peak 2 (b), and peak 3 (c) in Fig. 2a.

Table 1. Calibration Curves of the Standards

Analyte	Calibration Curve	Calibration Coefficient	Linear Range (μg/mL)	Detection Limit (ng/mL) (S/N=3)
GA	y=54.20+8.24x	0.9997	8.5–890	10
HA	y=5.73+11.40x	0.9999	10–500	5
HD	y=6.60+155.24x	0.9999	6.4–400	0.5

Calibration curves of the standards (GA, HA, and HD), linear range and the detection limit were shown in Table 1.

The sample was filtered through a 0.45 μm membrane filter and then injected into an HPLC. The quantitative results were shown as Table 2. Suitable amounts of standard GA, HA, and HD were added to the sample of known compound content, and the whole was analyzed by the procedure stated above. Recovery was expressed for each component, as the mean percentage ratio between the measured amounts and the actual ones. The average recoveries of GA, HA, and HD were 98.5%, 96.7%, and 96.6%, respectively. The reproducibility of the total procedure was tested using the sample of *Gastrodia elata Blume* extracts.

The relative standard deviations (RSD) were 2.53, 2.04, and 2.63% (n=5) for GA, HA, and HD, respectively. The results showed that the method had good recoveries and sensibility and could be readily utilized as a quality control method for *Gastrodia elata Blume.*

CONCLUSIONS

GA, HA, and HD can be extracted by ethanol aqueous solution accompanied by ultrasonic extraction.

RP-HPLC accompanied by diode array detection provides a more powerful tool for compound identification.

Table 2. The Contents of GA, HA, and HD in *G. elata Blume*

Analyte	Content (g/100 g)	RSD (%) (n = 5)	Recovery (%)
GA	1.2	2.53	98.5
HA	0.21	2.04	96.7
HD	0.013	2.63	96.6

The paper provides an easy, economic, rapid, and reliable method to determine the active constituents in *Gastrodia elata Blume*, avoiding the time-consuming, costly pretreatment. It appeared to be a suitable method for the analysis of Chinese herbal preparations, especially, for large numbers of samples and for quantity control in pharmaceutical plants.

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