Simultaneous Determination of Three Flavonoid Aglycones in *Elsholtzia blanda* by Adopting Orthogonal Test and High-Performance Liquid Chromatography

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Abstract

A new HCl hydrolysis/HPLC method, by adopting $L_9(3^4)$ orthogonal test to optimize hydrolysis condition, has been developed for simultaneous determination of three flavonoid aglycones in *Elsholtzia blanda* benth. The HCl concentration, methanol concentration, hydrolysis temperature, hydrolysis time are taking as four inspecting foctors, and the contents of luteolin, apigenin, and 5-hydroxy-6,7-dimethoxyflavone in hydrolytic solution are used as the evaluation indexes. Agilent Zorbax SB-C18 is used as analytical column. The mobile phase is a mixture of methanol–0.2% phosphoric acid (70:30, v/v), and UV detector is set at 350 nm. The flow rate is 1.0 mL/min, the temperature of column is maintained at 30°C. The optimal hydrolysis conditions are 3.0M HCl, 70% methanol, 85°C hydrolytic temperature and 3 h hydrolytic time. Standard curves are linear over the concentration range 8.54–85.4 µg/mL, 1.2–12 µg/mL, 9.2–92 µg/mL, and their average recoveries are 96.8%, 98.0%, and 100.5% for luteolin, apigenin, 5-hydroxy-6, 7-dimethoxyflavone, respectively. Thus, the optimum hydrolysis condition is relatively gentle, and the HPLC method is proved to be simple, accurate, and sensitive, so it will be able be applied to quality control of medicinal plant of *Elsholtzia blanda*.

Introduction

Elsholtzia blanda Benth. belongs to the genus *Elsholtzia* Wild in Labiatae. It is also called si-fang-hao or ji-gan-san in China, which is mainly distributed in south of Yunnan, southeast of Guizhou, west of Guangxi and Sichuan. In folk medicine, it is available for variety of disorders such as hepatitis, dysentery, cold, faucitis, tonsillitis, toothache, acute gastroenteritis, acute and chronicity pyelonephritis (1,2). According to the literature, the main active components of this medicinal plant are volatile oils and flavonoid composition (3,4). The current studies showed that the total flavones from *Elsholtzia blanda* exerted notable effects on myocardial ischemia, and indicated that it was an effective and promising traditional Chinese medicine with both prophylactic and therapeutic properties for the treatment of ischemic heart disease (5,6). Therefore, this medicinal plant has received more and more attention in recent years.

In order to make effective and safe use of *Elsholtzia blanda*, quality control is an important issue for this medicinal plant. The flavonoid components of *Elsholtzia blanda* include luteolin and luteolin-glucosides, apigenin and apigenin-glucoside, 5-hydroxy-6,7-dimethoxyflavone, etc. (3). In previous study, high-performance liquid chromatography (HPLC) methods had been carried out to analyze content of luteolin-glucoside of its extract (7). However, the study demonstrated that luteolin-glucoside was absorbed after hydrolysis to luteolin through the intestinal

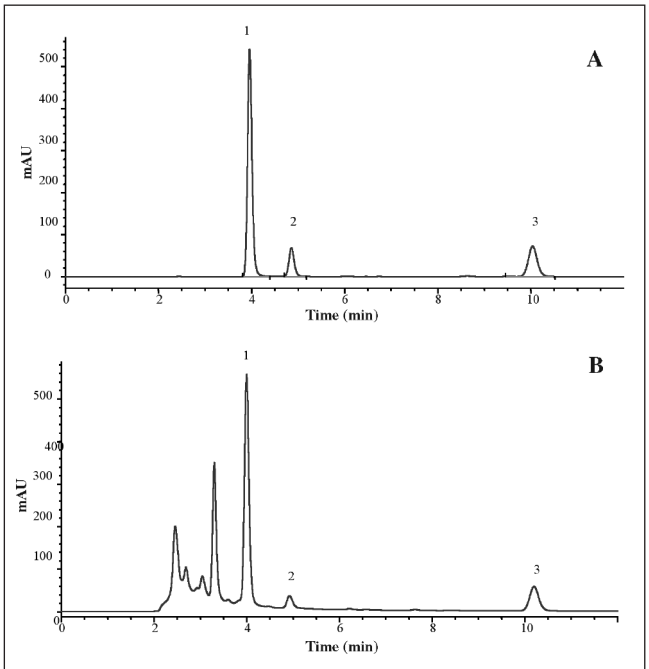

Figure 1. HPLC chromatograms of mixture substance and sample. Column: Agilent Zorbax SB-C18(5 µm, 250 × 4.6 mm i.d.), mobile phase: methanol–0.2% phosphoric acid (70:30, v/v), flow rate: 1.0 mL/min, column temperature: 30°C, wavelength of detection: 350 nm; mixture standard (A), sample (B); luteolin (1), apigenin (2), 5-hydroxy-6,7-dimethoxyflavone (3); sample was operated as sample preparation.

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muscosa, and free luteolin really exerted pharmacological activity on body (8). Futhermore, during past decades, few reports had been found for simultaneous determination of three flavonoid aglycones in *Elsholtzia blanda* by HPLC or any other methods. The purpose of this study was to develop a new HCl hydrolysis/HPLC method for simultaneous determination of three flavonoid aglycones in *Elsholtzia blanda,* and provided a scientific basis for quality control of this medicinal plant.

Experimental

Materials

The medicinal plant of *Elsholtzia blanda* was provided by Jindian Pharmaceutial Company Ltd.. The species of medicinal plant was identified by Professor Li Heng, Kunming Institute of Botany, Chinese Academy of Sciences.

Reagents

Luteolin was purchased from the National Institute for Drugs and Bioproducts Quality Control (Lot 111520–200201) (Beijing, China), apigenin was purchased from Sigma (Lot 064k0653) (St. Louis, MO.), 5-hydroxy-6,7-dimethoxyflavone was obtained from Laboratory of Traditional Chinese Medicine and Natural Drug Research of Zhejiang University (Hangzhou, China). The structural feature was identified by UV, ^{1}H- and ^{13}C–nuclear magnetic resonance spectroscopy. Its purity was more than 98% measured by normatization of the peak area in high-performance liquid chromatography. Methanol (HPLC-grade) was purchased from Ludu Chemical Reagent Factory (Shanghai, China), all other chemicals and solvents were of an analytical grade and obtained from commercial sources.

Table I. The Factors and Levels for the Orthogonal Design

Level	HCl concentration (mol/L)	Methanol concentration (%)	Hydrolytic temperature (°C)	Hydrolytic time (h)
1	1	50	75	1
2	2	60	85	2
3	3	70	95	3

Table II. The Design of Orthogonal Test

Experiment	HCL concentration (mol/L)	Methanol concentration (%)	Hydrolytic temperature (°C)	Hydrolytic time (h)
1	1	50	75	1
2	1	60	85	2
3	1	70	95	3
4	2	50	85	3
5	2	60	95	1
6	2	70	75	2
7	3	50	95	2
8	3	60	75	3
9	3	70	85	1

Chromatographic conditions

The HPLC system was an Agilent 1100 series equipped with an Iso Pump (G1310A), a manual injection (G1328A) with 20 µL of quantitative loop. Data and spectrum were recorded and analyzed by Agilent Chromatograpy Workstation (Agilent Technologies, Palo Alto, CA). Agilent Zorbax SB-C_{18} (5 µm, 250 × 4.6 mm i.d.) (Agilent Technologies) was used as analytical column, the mobile phase was a mixture of methanol–0.2% phosphoric acid (70:30, v/v) and filtered through a 0.45-µm filter and degassed before use. The flow rate was 1.0 mL/min, the temperature of column was maintained at 30°C, and the wavelength of UV detector was set at 350 nm.

Optimum hydrolysis conditions

The degree of flavones hydrolysis directly influenced reliability of determination, so it was necessary to find the optimum hydrolysis conditions. We employed orthogonal test in preliminary experiment. The hydrolysis procedure was optimized by $L_9(3^4)$ orthogonal test design in which HCl concentration, methanol concentration, hydrolytic temperature, and hydrolytic time were considered as four inspecting factors, and each factor was considered from three levels, as shown in Table I. Therefore, considering four factors and three levels, the design involves 9 experiments, which were performed as seen in Table II. All statistical analyses were carried out by using analytical software SPSS 13 for windows.

Sample preparation

At first, the medicinal plant of *Elsholtzia blanda* was divided into six kinds of different samples at picking time (2001–09, 2001–11, 2002–09, 2002–11) and different parts (flower, leaf and stem), then they were dried and ground into powder, respectively. Each sample powder (200 mg) was weighed accurately and hydrolyzed in 30 mL of a mixed solution (3.0M HCl in 70% aqueous methanol) for 3 h at 85°C water bath. After acid hydrolysis, this solution was allowed to cool to room temperature and adjusted to pH 3–4 with 6.0M NaOH solution, then filtered by paper filter and the residue was washed with 5 mL methanol, finally, the solution was diluted to 50 mL volume with mobile phase and filtered through a 0.45 µm filter, the resulting solution was injected into the HPLC system for analysis.

Preparation of standard solution

Standards of luteolin, apigenin, and 5-hydroxy-6,7-dimethoxyflavone were weighed accurately and dissolved with methanol and diluted to 854, 120, 920 µg/mL, respectively. 5 mL of each solution was transferred into a 50 mL volumetric flask and diluted with mobile phase to final volume. The mixture was servered as standard stock solution and stored at 4°C before use. A series of 1, 3, 5, 7, and 10 mL of standard stock solutions were diluted respectively with mobile phase to 10 mL volume, then each solution was injected into the HPLC system for analysis.

Results and Discussion

Results

Chromatograph specificity

The chromatograms showed a good separation and symmetrical peaks for three flavonoid aglycones. Their peaks were confirmed with relative standards, there was no interference appeared at their peak positions in the sample chromatogram. Under the described chromatographic condition, the retention times of three flavonoid aglycones are shown in Figure 1.

Calibration curves

Calibration curves were constructed by analyzing a series of standard solutions for various concentrations. Peak areas (y) of three flavonoid aglycones were measured and plotted against the concentrations (x) of them. The linearity of the calibration curves was over the concentration range from 8.54 to 85.4 µg/mL, 1.2 to 12 µg/mL, 9.2 to 92 µg/mL for luteolin, apigenin, 5-hydroxy-6,7-dimethflavone, respectively. The regression equations of the calibration curves were $y = 94.47x - 4.09$ ($r = 0.9999$, $n = 5$) for luteolin, $y = 98.97x - 1.7$ ($r = 0.9999$, $n = 5$) for apigenin, $y = 23.36x - 0.39$ ($r = 0.9998$, $n = 5$) for 5-hydroxy-6,7-dimethoxyflavone. The limits of detection (LOD) for luteolin, apigenin, and 5-hydroxy-6,7-dimethoxyflavone were 0.35, 0.30, and 0.94 µg/mL at a signal-to-noise ratio of 3, respectively.

Table III. The Results of Orthogonal Test (n = 2)

Experiment	Luteolin (%)	Apigenin (%)	5-hydroxy-6,7-dimethoxyflavone (%)
1	0.716 3	0.051 2	1.079 9
2	1.049 6	0.087 4	1.080 4
3	1.031 1	0.092 3	1.094 9
4	1.310 1	0.119 1	1.081 1
5	1.156 4	0.106 4	1.074 5
6	1.090 6	0.100 5	1.085 6
7	1.380 7	0.117 7	1.074 8
8	1.221 2	0.103 4	1.078 6
9	1.323 6	0.111 4	1.088 4

Optimization results

The results of the orthogonal test was shown in Table III. By the analysis of variance, among the four factors, HCl concentration and hydrolytic temperature had notable influence, while methanol concentration and hydrolytic time were of little importance. By further validation tests, the optimum hydrolysis conditions were confirmed as follows: 3.0M HCl, 70% methanol, 85°C hydrolytic temperature and 3 h hydrolytic time.

Instrumental precision

A standard solution (containing 85.4 µg/mL luteolin, 12 µg/mL apigenin, and 92 µg/mL 5-hydroxy-6,7-dimethoxyflavone) was successively determined six times, peak areas of three flavonoid aglycones were measured, and their relative standard deviations (RSD) were calculated. Their RSDs were 0.5% for luteolin, 1.9% for apigenin, and 0.7% for 5-hydroxy-6,7-dimethoxyflavone (n = 6).

Table IV. Recovery of Three flavonoid aglycones (n = 3)

Component	Amount of sample (µg)	Added amount (µg)	Found amount (µg)	Recovery (%)	Average recovery (%)	RSD (%)
Luteolin	1655	1970	3548	96.1	96.8	0.9
	1671	1640	3252	96.4		
	1634	1310	2915	97.8		
Apigenin	121	144	264	99.3	98.0	1.3
	122	120	238	96.7		
	119	96	213	97.9		
5-H-6,7*	1382	1644	3060	102.1	100.5	1.4
	1391	1370	2752	99.3		
	1365	1096	2463	100.2		

* 5-H-6,7 = 5-Hydroxy-6,7-dimethoxyflavone.

Stability of sample

In order to assess the stability of the sample, a sample solution (2001–11, flower) was operated as the same way (described in sample preparation) and determined in 0, 2, 8, 20, and 24 h, peak areas of three flavonoid aglycones were measured, and their RSDs were calculated. Their RSDs were 0.4% for luteolin, 1.5% for apigenin and 0.4% for 5-hydroxy-6,7-dimethoxyflavone (n = 5). It showed that sample solution was stable for at least 24 h.

Repeatability studies

The repeatability of this method was evaluated by intra-day and inter-day assay variability of sample. Five portions of the same sample (2001–11, leaf) were operated in the same way (described in sample preparation) within a day and in 5 different days. Their peak areas were measured and their contents were obtained by calibration curves constructed, and then their RSDs were calculated. The results showed that the intra-day and inter-day RSDs of this method for luteolin, apigenin, 5-hydroxy-6,7 dimethoxyflavone were 0.5%, 2.6%, 0.5% (n = 5) and 0.9%, 2.6%, 2.9% (n = 5), respectively.

Table V. Contents of Three flavonoid aglycones in *Elsholtzia blanda* (n = 2)

Sample	Picking time	Content of component (%)		
		Luteolin	Apigenin	5-hydroxy-6,7-dimethoxyflavone
Flower	2002–09	1.28	0.12	1.02
Flower	2002–11	1.65	0.12	1.37
Flower	2001–09	1.17	0.11	0.88
Flower	2001–11	1.62	0.12	1.45
Stem	2001–11	0.54	0.09	0.13
Leaf	2001–11	1.60	0.09	1.35

Recovery

Nine portions of same sample (2002–11, flower) were weighed accurately and divided into 3 groups, then the first group was added certain amount of three standards as high levels, the second group was added certain amount of three standards as middle levels, and the third group was added certain amount of three standards as low levels, and these samples were operated as the same way (described in sample preparation). The results showed that the average recovery of this method was 96.8% for luteolin, 98.0% for apigenin, and 100.5% for 5-hydroxy-6,7-dimethoxyflavone (n = 3), respectively (Table IV) .

Determination of samples

Six kinds of different samples were operated in the same way (described in sample preparation). Their peak areas were measured and their contents were obtained by calibration curves constructed, the results of determination of samples are listed in Table V. It was shown that the contents of luteolin and 5-hydroxy-6,7-dimethoxyflavone in the flower are higher in November compared to September, and the content of apigenin had no significant difference. At the same time, it was also observed that their contents in flowers and leaves are higher than that in stems.

Discussion

Mobile phase

In order to achieve good separation of three flavonoid aglycones, we compared three mobile phase systems (methanol–water, methanol–acetic acid, and methanol–phosphoric acid), and found that methanol–0.2% phosphoric acid was most suitable for separation of three flavonoid aglycones. Then by further study, we continually adjusted the proportion of methanol and 0.2% phosphoric acid; in the end, we employed the present mobile phase, as it could achieve a good separation, symmetrical peaks and high column efficiency in a short separation time (11 min).

Sample analysis

As shown in Table IV, the picking time and different parts of the medicinal plant significantly influenced the contents of three flavonoid aglycones. Their contents, indicated mainly in two components (luteolin and 5-hydroxy-6,7-dimethflavone), are higher in November than in September. So the picking time should be in November.

For different parts of the medicinal plant, the contents of luteolin and 5-hydroxy-6,7-dimethflavone in flowers and leaves are the higher than that in stems, while the content of apigenin in flowers is higher than in leaves. So the flowers were the best medicinal part, at the same time, it will be beneficial for remaining the leaves (only picking the flowers) to regenerate resources of this perennial plant. It was just in accordance with folk custom of using this herb and the literature (1,2).

In addition, the result of determination suggested that the contents of three flavonoid aglycones have a certain proportional relationship, that is luteolin: apigenin: 5-hydroxy-6,7-dimethoxyflavone = (13–14): 1: (11–12).

Conclusion

This study has shown that this method developed has good calibration linearity and instrumental precision, satisfactory repeatability and recovery. It can be successfully applied to simultaneous determination of three flavonoid aglycones in *Elsholtzia blanda*, and it also provides a scientific basis for quality control of this medicinal plant.

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