College of Pharmaceutical Sciences[1], Obstetric and Gynecology Hospital[2], Zhejiang University, Hangzhou, P.R. China

Analysis of luteolin in *Elsholtzia blanda* Benth. by RP-HPLC

F. M. WANG[1,2], T. W. YAO[1], S. ZENG[1]

Received October 25, 2004, accepted November 1, 2004

Prof. Su Zeng, College of Pharmaceutical Sciences, Zhejiang University, 353 Yan'an Road, Hangzhou 310031, P.R. China
zengsu@zjuem.zju.edu.cn

Pharmazie 60: 648–649 (2005)

The total luteolin content in *Elsholtzia blanda* Benth. extracts (EBBE) was determined using reversed phase HPLC. C_{18} was used as the packing material and 0.01 M phosphate buffer (pH 2)-tetrahydrofuran-isopropanol (70 : 30 : 5) as the mobile phase with detection wavelength 360 nm. The recovery of the method was 96.4% ~ 101.8%, and the assay was linear at concentrations from 5 to 200 μg/ml ($r = 0.9999$). The results indicated that the content of luteolin in EBBE extracted under different conditions varied significantly. This method can be used to optimize the extraction procedure and determine the content of luteolin in *Elsholtzia blanda* Benth. extracts.

1. Introduction

Elsholtzia blanda Benth. is a traditional Chinese medicinal herb growing abundantly in the southwest and southeast of Yunnan Province, the southeast of Guizhou Province, the west of Guangxi Province, and the Sichuan Provinces of China. *Elsholtzia blanda* Benth. was used for treating nephritis, hepatitis, dysentery, pharyngritis, acute enteritis of standing the blending, etc (Lu et al. 2001). Zhang et al isolated and identified several principal flavonoids from the inflorescence of *Elsholtzia blanda* Benth., such as luteolin-7-glucoside, lutelin-3′-glucoside, and luteolin-7-galactoside. All of these are transformed into luteolin after hydrolysis (Zhang et al. 1999). Luteolin-glucosides are the main biologically active constituents in EBBE and their content would be an important index for quality control of EBBE. However, as yet there has been no publication about the determination of luteolin in EBBE by chromatography. In this paper, a simple, rapid and accurate RP-HPLC method for determination of the total luteolin in EBBE is presented. The method has been applied to evaluate the quality of EBBE and optimize the extraction procedures for EBBE.

2. Investigations, results and discussion

The reference standard luteolin solution was diluted with the mobile phase to seven different concentrations. An aliquot of 20 μl of the solution was injected into the HPLC system, and the peak area (y) of luteolin was measured and plotted against the concentration (x) of the luteolin. The linearity of the method was in the range of 5 ~ 200 μg/ml. The regression equation of the calibration curve was $y = 79727x + 68446$ ($r = 0.9999$).

The studies of luteolin recovery were carried out by adding the standards at three different concentrations to the EBBE (concentration known), which was hydrolyzed as described under Sample preparation. The total concentration of luteolin was obtained from the calibration curve and the recovery (R) of the method was calculated by the formula:

$$R\% = \frac{C_T - C_b}{C_{std}} \times 100\% \qquad (1)$$

in which C_T is the total concentration of luteolin measured, C_b is the concentration of luteolin before spiking with the reference standard of luteolin, and C_{std} is the concentration of the reference standard of luteolin used to spike. The results (Table 1) show the average recovery of the luteolin was $98.2 \pm 5.53\%$ ($n = 15$).

The precision test was carried out with three different concentrations of luteolin, 5 μg/ml, 10 μg/ml, and 100 μg/ml. Intra-assay variability was determined by analyzing five samples within one day and inter-assay variability was determined by analyzing samples on five separate days. The relative standard deviations (RSD) were calculated, and the results showed that the RSD, within- and between-day were 4.2% and 5.1%, respectively (Table 2).

The limit of detection (LOD) of the assay was measured as 50 ng/ml for luteolin based on $S/N = 3$. The limit of

Table 1: Recovery of assay of luteolin in EBBE

Conc. spiked (μg/ml)	Recovery ($\bar{x} \pm s\%$, $n = 5$)
5.0	105.3 ± 7.8
10.0	97.2 ± 3.3
100.0	92.1 ± 5.5

Table 2: Precision of assay of luteolin in EBBE

Conc. spiked (μg/ml) luteolin	Average relative standard deviation (%, $n = 5$)	
	Within-day	Between-day
5.0	6.20	6.51
10.0	3.56	5.10
100.0	2.82	3.84

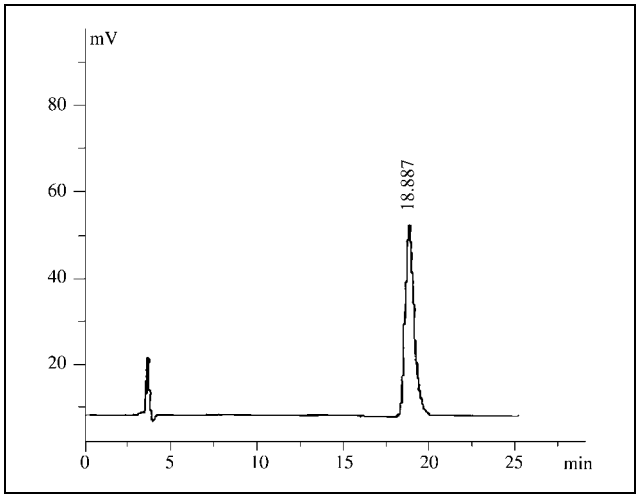

Fig. 1: Chromatogram of the reference standard of luteolin

quantitation (LOQ) calculated from the calibration curve was 4.86 ± 0.30 μg/ml (n = 5) with RSD 6.2% for luteolin.

The EBBE accurately weighed was analyzed according to the procedure described above. The contents of luteolin in the samples were calculated and are presented as the mean values of three repeated injections.

The amount of flavonoids in *Elsholtzia blanda* Benth. extracts is usually expressed as luteolin-glucoside content. The total concentration of luteolin-glucosides in the EBBE was calculated by the formula:

$$C_G\% = C_L\% \times W_G/W_L \quad (2)$$

in which C_G is the total content of luteolin-glucosides in the EBBE before hydrolysis, C_L is the content of luteolin measured after hydrolysis of the extract. W_G is the molecular weight of luteolin-glucoside and W_L is the molecular weight of luteolin. The data showed that the luteolin-glucosides contents in these samples were quite different with different procedures.

The peak diffusion was reduced with tetrahydrofuran and isopropanol (30 : 5, v/v), and the best peak performance was obtained using pH 2 phosphate buffer in the mobile phase. Decreasing the acidity of the mobile phase resulted in a tailed peak.

Different concentrations of HCl were added to the same concentration of *Elsholtzia blanda* Benth. extract, and the samples were then hydrolyzed at different temperatures for different times. The maximum amount of luteolin was recovered after heating at 85 °C for 1 h with 25% HCl, so acid hydrolysis was carried out by adding 1 ml 25% HCl to 1 ml of sample (in 95% ethanol) and heating at 85 °C for 1 h (Fig. 3).

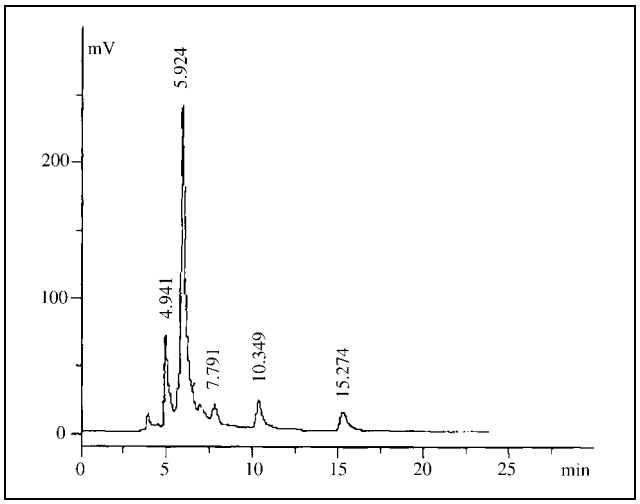

Fig. 2: Chromatogram of *Elsholtzia blanda* Benth. extracts before hydrolysis

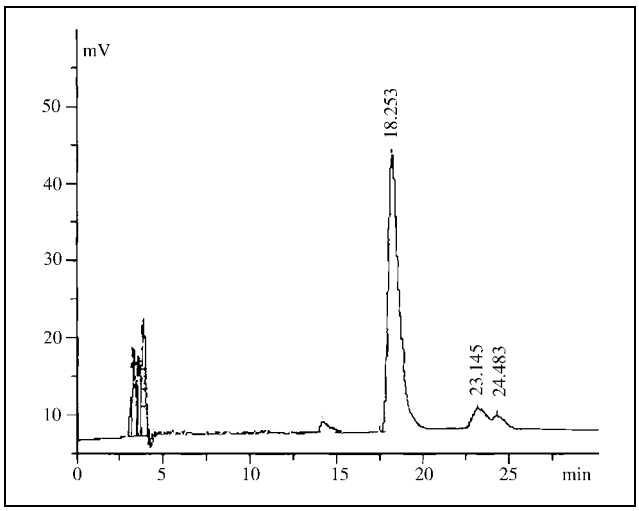

Fig. 3: Chromatogram of *Elsholtzia blanda* Benth. extracts after hydrolysis

3. Experimental

3.1. Materials

Lutelin standard was purchased from Sigma Chemical Company (St. Louis, MO, USA). Tetrahydrofuran, isopropanol and phosphoric acid were of analytical grade. The water used was of HPLC quality purified in a Milli-Q water purification system (Millipore, MA, USA). The solvents were filtered through 0.45 μm membranes and degassed in an ultrasonic bath before use. The *Elsholtzia blanda* Benth. extracts were presented by the Department of Natural Products and Traditional Chinese Medicine, College of Pharmaceutical Sciences, Zhejiang University.

3.2. HPLC apparatus and chromatographic conditions

The modular HPLC equipment comprised an LC-10AT vp with SPD-10A vp (Shimadzu, Japan) and N2000 chromatographic data system (Zhejiang University, China). A Platinum EPS C_{18} 100A (5 μm, 250 mm × 4.6 mm I.D.) was used as the analytical column with a guard column (5 μm, 10 mm × 4.6 mm I.D. packed with YWG-$C_{18}H_{37}$). A mixture of phosphate buffer (pH 2)-tetrahydrofuran-isopropanol (7 : 30 : 5, v/v) was used as the mobile phase. The flow rate of the mobile phase was 0.7 ml/min. The wavelength of the UV detector was set at 360 nm and the injection volume for analysis of samples was 20 μl.

3.3. Reference standard solution

The accurately weighed reference luteolin was dissolved in methanol and diluted to 1.0 mg/ml. The standard solutions were stable for at least 2 months when stored at −20 °C.

3.4. Sample preparation

The accurately weighed EBBE was dissolved in 95% ethanol and 25% HCl (v/v, 1 : 1) and hydrolyzed for 1 h at 85 °C, the hydrolyzed sample was filtered through a membrane filter (0.45 μm pore size), the initial filtrate was discarded, the clear filtrate was diluted with the mobile phase to the appropriate concentration, and an aliquot of 20 μl of the resulting solution was injected into the HPLC system.

Acknowledgements: This work was supported by the Zhejiang Natural Science Foundation of China (No. 302682) and University Disciplinary Foundation of China (No. 20040335013).

References

Lu JS, Shen T, Guo Z (2001) The chemical constitutents of *Elsholtzia blanda*. Acta Bot Sin 43: 545–550.

Zhang WL, Jiang DU, Zou YM (1999) Isolation and identification of flavonions in Elsholtzia blanda Benth. China J Chin Mat Med 24: 96–98.