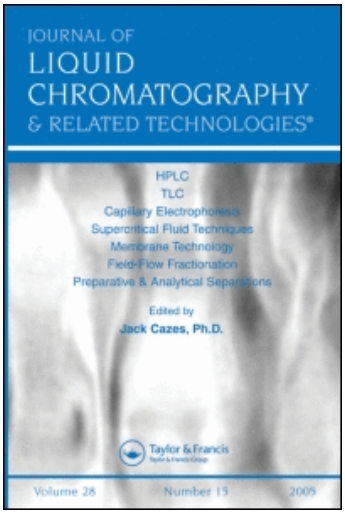

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Duo-Long Di[a]; Juan Chen[a]; Yan-Ping Shi[ab]
[a] State Key Laboratory for Natural Medicine of Gansu Province, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou, P.R. China [b] State Key Laboratory of Applied Organic Chemistry, Lanzhou University, Lanzhou, P.R. China

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Determination of Phenylpropanoid Glycosides in Chinese Herbal Extracts from *Pedicularis* Species by HPLC

Duo-Long Di,[1] Juan Chen,[1] and Yan-Ping Shi[1,2,*]

[1]State Key Laboratory for Natural Medicine of Gansu Province, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou, P.R. China
[2]State Key Laboratory of Applied Organic Chemistry, Lanzhou University, Lanzhou, P.R. China

ABSTRACT

A simple, rapid, and accurate high performance liquid chromatographic (HPLC) technique coupled with photodiode array (PDA) detection was developed for the simultaneous determination of three phenylpropanoid glycosides (PPGs), echinocoside (ECH), verbascoside (VER), pedicularioside A (PED-A) in extracts of *Pedicularis* species. The optimized method was achieved for the separation and detection of selected constituents, using acetonitrile–water–acetic acid (20 : 80 : 1, v/v/v) as the mobile phase and 331 nm as the detection wavelength. The proposed

*Correspondence: Yan-Ping Shi, State Key Laboratory for Natural Medicine of Gansu Province, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou 730000, P.R. China; E-mail: shiyp@lzu.edu.cn.

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method was employed to analyze the three different herbal medicines of *Pedicularis* species, named *Pedicularis longiflora, P. longiflora* var *tubiformis*, and *P. kansuensis.* The results demonstrate the differentiation of the contents of the three PPGs in the three herbal medicines.

Key Words: *Pedicularis*; Phenylpropanoid glycosides; Echinocoside; Verbascoside; Pedicularioside-A; High performance liquid chromatography.

INTRODUCTION

Pedicularis is considered to be the largest genus of the Scrophulariaceae family, and one of the largest angiosperm genera with at least 600–800 species, primarily occurring in the mountain and alpine zones of the northern hemisphere. Over 352 species are distributed in China,[1] and many species (about 50 species) have been listed in certain medicinal dictionaries and widely used as traditional Chinese medicine for a long time, owing to their therapeutic effects on cardiac-tonic of collapse, exhaustion, spontaneous sweating, seminal emission, and senility, invigorating circulation of blood, aiding digestion, increasing vitality, and relieving uneasiness of body and mind.[2] They are usually called "native-ginseng" by local inhabitants living in the northwestern part of China. *Pedicularis* species contain a number of effective constituents, in which phenylpropanoid glycosides (PPGs) have been paid considerable attention. PPGs extracted from other species have been reported to have antibacterial,[3] antiviral,[4] antitumor cells,[5,6] antiplatelet aggregation[7] activity, and to inhibit formation of leutriene B_4.[8] It is indicated from the present studies that PPGs isolated from *Pedicularis* also possessed antioxidative and chelating activity[9] and retardation of muscle fatigue[10] and fast repair of DNA damage.[11,12] As PPGs make important contributions to the biological activity of *Pedicularis*, the standardization of *Pedicularis* may be based on the contents of PPGs. The three PPGs, echinocoside (ECH), pedicularioside A (PED-A), and verbascoside (VER) have been isolated from the *Pedicularis* species. So, they were used as indices of quality control for the three *Pedicularis* species, *P. longiflora, P. longiflora* var *tubiform*, and *P. kansuensis* in this paper.

Analytical methods of PPGs described in the literatures are mainly based on thin-layer chromatography (TLC),[13] liquid chromatography (LC),[14] and LC–mass spectrometry (LC–MS).[15,16] Recently, LC–MS served as a valuable technique for providing molecular mass and structural information of compounds and has received extensive attention, but the instrument is expensive and not available for most laboratories. Despite its limited information to

elucidate molecular structure, the high performance liquid chromatography (HPLC)-UV method is still the most popular technique with its rapidity, simplicity, and convenience.[17] Although several HPLC-UV methods have been reported for analyzing PPGs in some plants, there are no reports on the separation and determination of three PPGs (ECH, PED-A, and VER) in the *Pedicularis* species to the best of our knowledge. This paper described first a simple, rapid, and accurate HPLC method coupled with a photodiode array (PDA)-detector, for the analysis of ECH, PED-A, and VER in the three *Pedicularis* species, i.e., *P. longiflora, P. loniflora* var *tubiformis*, and *P. kansuensis.* Chromatographic condition, method validation, sample preparation, and quantitation were targeted in the current research.

EXPERIMENTAL

Chemicals

Reference standards, ECH, PED-A, and VER (structures shown in Fig. 1), were the kind gifts from Prof. Zhong-Jian Jia of National Laboratory of Applied Organic Chemistry, Department of Chemistry of Lanzhou University, China.

P. longiflora, P. longiflora var *tubiformis*, and *P. kansuensis* were collected in Qinghai province in July 2002, and identified by Prof. Ji Ma of Faculty of Pharmacy, First Military Medical University of PLA, Gangzhou, China.

Acetonitrile was of chromatographic grade and purchased from Institute for the Chemical Engineering of Huaiyin Plastic Product Factory (Jiangsu, China). Distilled and deionized water was used for the preparation of all samples and solutions. Other chemicals were of analytical grade and purchased from Tianjing Chemical Reagent Corporation (Tianjing, China).

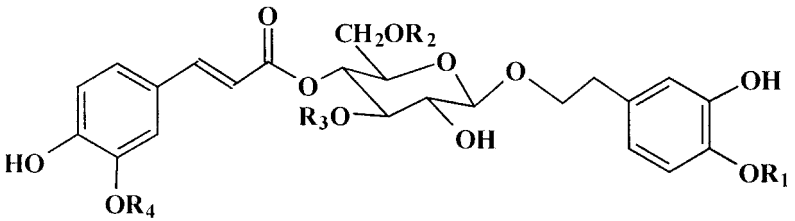

Figure 1. The structures of PPG. ECH: $R_1 = R_4 = H$, R_2 = glucosyl, R_3 = rhamnosyl. VER: $R_1 = R_2 = R_4 = H$, R_3 = rhamnosyl. PED-A: $R_1 = R_4 = H$, R_2 = apiosyl, R_3 = rhamnosyl.

Apparatus and Chromatography

The HPLC system (Milford, MA) consisted of a Waters quaternary pump (Model Delta 600E), a PDA detector (Model 2996), and a manual injector. The chromatographic data were recorded and processed with Waters Millennium[32] software. The chromatographic separation of analytes was performed on a Kromasil C_{18} column (5 μm, 4.6 × 250 mm^2 I.D.) (Dalian Institute of Chemical Physics, Chinese Academy of Sciences) (Dalian, China). The mobile phase consisted of acetonitrile–water–acetic acid (20 : 80 : 1, v/v/v) at a flow rate of 0.8 mL/min. Helium (He) was used for degassing the mobile phase. The temperature of the column during analysis was maintained at 25°C. The injection volume was 10 μL.

Standard Solutions Preparation

Reference standards, ECH, PED-A, and VER (each 2.0 mg), were accurately weighed and transferred into a 2-mL-volumetric flask and dissolved in acetonitrile–water (1 : 1, v/v) to make stock solutions. The stock solutions were stored at 4°C and brought to room temperature before use. Calibration standard working solutions were freshly prepared by appropriate dilution of the stock solutions.

Sample Solution Preparation

The influence of extraction time on the extraction efficiency of the plant was evaluated. After being air-dried and crushed into powder, 0.5 g of *P. loniflora* var *tubiformis* was accurately weighed and extracted with 25 mL of methanol in an ultrasonicator for 30 min. The extract was filtered and evaporated to dryness, and the residue was dissolved by acetonitrile–water (1 : 1, v/v) and transferred to a 25-mL-volumetric flask and made up to volume with dissolving solution (the resulting resolution marked as I). A second 0.5 g of the accurately weighed *P. loniflora* var *tubiformis* was extracted twice with methanol as described above, then the extracts were mixed and the following procedure was the same as that of the above procedure (marked as II). A third and fourth 0.5 g of the plant were extracted three and four times, respectively, then followed by the same procedure as that of the second procedure (marked as III, IV). The concentrations of the major constituents in the resulting solutions (I, II, III, IV) were calculated based on the equations for the calibration curves, and the extraction efficiency was compared.

The influence of extraction solvents on the extraction efficiency of the plant was also investigated. Pure and aqueous methanol (80%, 50%) were

employed as extraction solutions in this study, and the other conditions were the same as that of the above second procedure (the resulting solution marked as II, V, VI).

RESULTS AND DISCUSSION

Optimization of Chromatographic Conditions

It was reported that most HPLC methods monitored PPGs at 280 nm,[14,18] with other wavelengths used.[19] In the present study, the UV spectra of all PPGs reviewed by PAD demonstrate that a much higher sensitivity is obtained at 331 nm, which is in accordance with the report.[19] So, 331 nm was selected as detection wavelength in the subsequent studies.

In one of the early reports[15] on the HPLC method of PPG compounds, different mixtures of acetic acid, water, and methanol were used to separate members of several classes of PPGs, and the effects of organic modifier on selectivity were deduced. Since then, numerous mobile phases have been employed with different modifiers (usually methanol, acetonitrile, or tetrahydrofuran), acids (acetic or phosphonic acid), and salts (ammonium phosphate). In the present study, effects of the proportion of acetonitrile and acetic acid on the separation were tested for achieving an optimum condition.

Various proportions of acetonitrile, ranging from 80% (v/v) to 20% (v/v), were tested for separating the PPGs. It is shown, in Fig. 2, that the

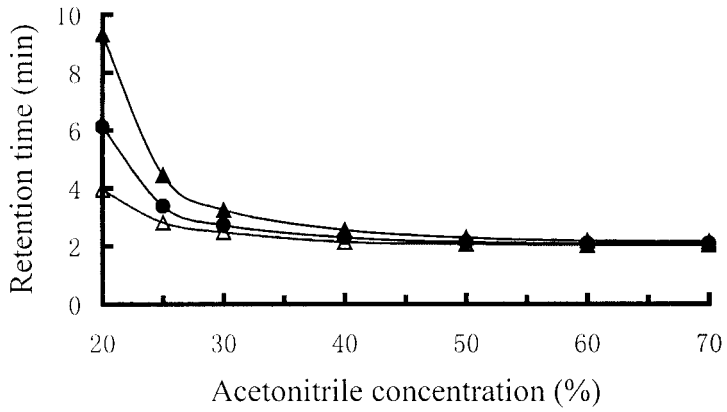

Figure 2. Effect of acetonitrile concentration on the retention times of ECH (△), PED-A (●), and VER (▲).

retention times of ECH, PED-A, and VER increased, and the separation of three PPGs was improved with decreasing acetonitrile concentration. When the proportion of acetonitrile decreased to 20% (v/v), three PPGs could be completely separated, but have broad peaks. In the literature,[18] owing to the phenolic feature of PPG compounds, elution was made with an acidified eluent, which allowed for a satisfactory separation. In our study, to improve the peak shape (restrain the peak tailing), 1% acetic acid was added as a mobile phase modifier to inhibit the dissociation of the phenolic hydroxyl group of PPGs. The resolution increased greatly because of the improvement of the peak shape. So acetonitrile–water–acetic acid (20 : 80 : 1, v/v/v) was used as the mobile phase to achieve the best separation, efficiency, and resolution for the analytes.

Linearity

The calibration was based on the five duplicate analyses of calibration working solutions, at seven concentration levels, for ECH (0.03–10 μg), PED-A (0.03–10 μg), and VER (0.006–10 μg). The regression equations and their correlation coefficients were calculated as follows:

ECH: $y = 1.54 \times 10^{-6}x - 0.0058, \quad r = 0.9998$

PED-A: $y = 1.06 \times 10^{-6}x - 0.1760, \quad r = 0.9991$

VER: $y = 7.06 \times 10^{-7}x - 0.0207, \quad r = 0.9999$

The detection limits for ECH, PED-A, and VER were 1.7×10^{-3} μg, 2.2×10^{-3} μg, and 2.0×10^{-3} μg, respectively, at a signal-to-noise ratio of 3.

Method Validation

The reproducibility of the procedure was obtained from the relative standard deviation (RSD) of retention time and peak area calculated for five replicate injections. The values were 0.51% and 3.44% for ECH, 0.72% and 1.68% for PED-A, 1.04% and 0.74% for VER.

The stability of the assay was evaluated by intra-day variability. The standard solution was analyzed on five consecutive days, and the RSDs of both retention times and peak areas of the three PPGs were all less than 5%.

The accuracy of the method was confirmed by analyzing the mixture prepared, by adding suitable amounts of standard mixture to the plants with known contents of these target compounds. The average recoveries ($n = 5$)

Table 1. Contents of the three PPGs in *P. longiflora* var *tubiform* under different extraction conditions ($mg\,g^{-1}$).

	Content[a] (%)		
Resulting extract	ECH	PED-A	VER
I (once, methanol)	1.78	2.56	16.26
II (twice, methanol)	2.14	3.03	20.17
III (three times, methanol)	2.14	3.05	20.24
IV (four times, methanol)	2.17	3.07	20.29
V (twice, 80% methanol)	1.72	2.41	14.78
VI (twice, 50% methanol)	1.19	1.76	11.09

[a]Values are means of triplicate determinations.

of ECH, PED-A, and VER were 98.1%, 97.1%, and 97.1% with a RSD of 4.86%, 3.77%, and 2.96%, respectively.

Evaluation of Extraction Efficiency

The concentrations of the three PPGs in the six resulting extracts (I, II, III, IV, V, VI) are shown in Table 1. After sonication two times, the contents of the three constituents almost did not increase with increasing extraction time, and more than 98.6% of the three constituents could be extracted, which indicates that the major constituents could be extracted completely after sonication twice. So, extraction time was chosen as twice. The contents of the three PPGs using pure methanol as the extraction solvent are higher than those extracted by 80% and 50% methanol (Table 2). This difference implies that pure methanol has a better solubility and extraction power than the other aqueous methanol. So, methanol was chosen as the extraction solvent. The

Table 2. Contents of desired PPGs in the three different *Pedicularis* species.

	Content[a] (%)		
Pedicularis species	ECH	PED-A	VER
P. longiflora var *tubiform*	0.21	0.30	2.02
P. longiflora	0.068	0.14	1.68
P. kansuensis	0.021	0.032	0.90

[a]Values are means of triplicate determinations.

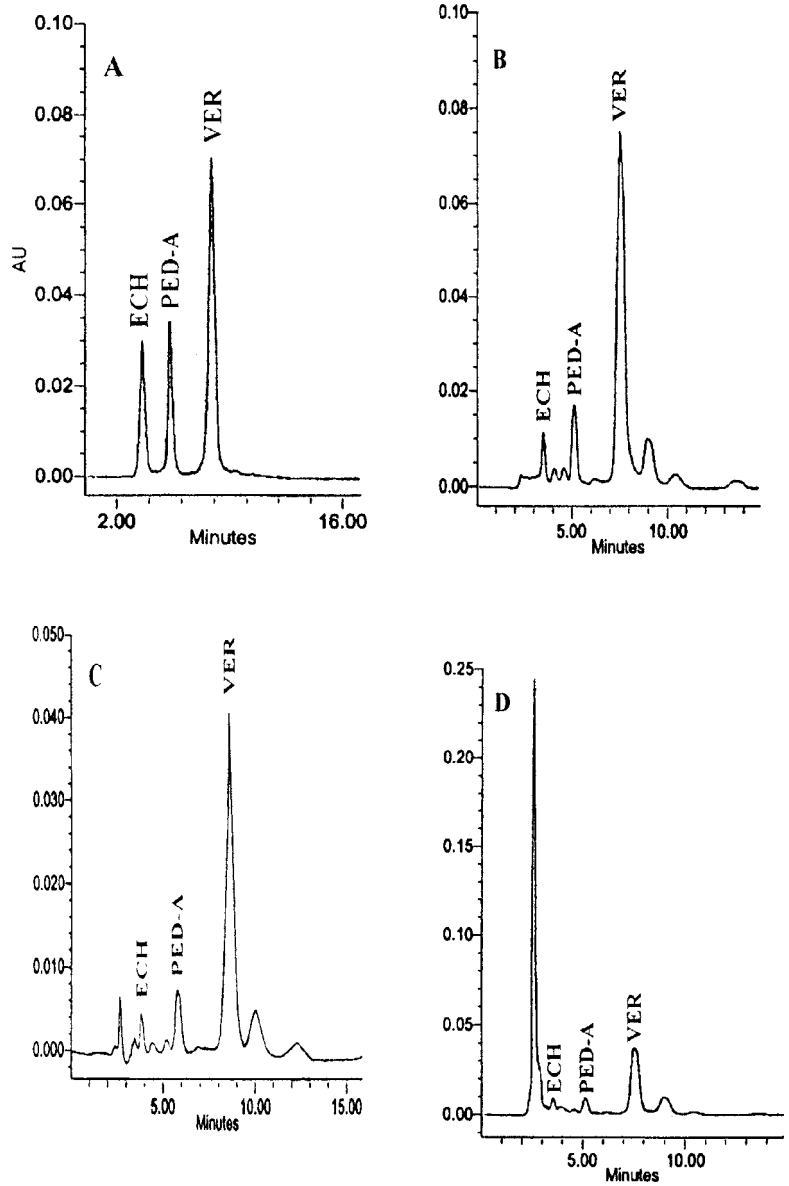

Figure 3. Chromatograms of the mixture of PPGs: ECH, PED-A, and VER (A), and the methanol extracts from plant samples of *P. longiflora* var *tubiform* (B), *P. longiflora* (C), and *P. kansuensis* (D). Separation condition: acetonitrile–water–acetic acid (20 : 80 : 1, v/v/v) and detection wavelength: 331 nm.

result of the recovery test also demonstrated that the extraction method was adequate and appropriate for the analysis.

Quantitation of the Three *Pedicularis* Species

The optimum method was applied to the analysis of the three PPGs in methanol extracts from the three *Pedicularis* species. The desired compounds from herbal plants were identified by comparing both the retention times and the UV spectra of PPGs with those of the reference standards. The analytes were further confirmed by spiking standards in actual samples. The peak purity of the component was checked using the PDA-detector and Waters Millennium[32] software. Figure 3 illustrates the typical chromatograms of the PPGs mixture, along with the methanol extracts from *P. longiflora, P. longiflora* var *tubiform*, and *P. kansuensis.* The PPGs and the other unknown compounds were well resolved within 10 min under the optimum condition described above. The results obtained (Table 1) showed the common presence and the comparisons of the contents of the three PPGs, i.e., ECH, PED-A, and VER in the three plant species. The contents of three PPGs in *P. longiflora* var *tubiform* were all much higher than those in *P. longiflora* and *P. kansuensis.*

CONCLUSIONS

The present study successfully develops a HPLC method for the simultaneous determination of the three PPGs (ECH, PED-A, and VER) in traditional Chinese herbal medicine of *Pedicularis* species. Compared with other existing methods, the proposed methodology has the major advantages of fastness and simplicity, only requiring an organic liquid extraction in an ultrasonicator, and an isocratic HPLC-UV detection. The proposed method promises to be applicable in the quality control of traditional Chinese medicine. In addition, it is important to mention that the preliminary work indicates that the proposed method is applicable for different *Pedicularis* species. Whether it can be applied to other species or other genera containing PPGs is an interesting topic to further investigate.

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