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Note

Determination of flavonol glycosides in Epimedii Herba by high-performance liquid chromatography

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Epimedii Herba, the dried aerial parts of plants of the genus *Epimedium* (Berberidaceae), is an important crude drug used as a tonic. More than 40 species of the plant grow wild in China, Korea and Japan. It has been reported that icariin[1–4], a flavonol glycoside, is the principle in this crude drug (aerial parts), whereas icariin and epimedoside A–E[5–9] occur contained in the underground parts. Recently, a number of glycosides[10–18] have been also isolated.

For the evaluation of Epimedii Herba, a coulometric method for icariin has been reported[19], but this method is complicated and inaccurate because it involves pre-treatment by preparative thin-layer chromatography. Therefore, a simple method is required to evaluate the quality of this crude drug.

During out studies on Epimedii Herba[20–22], three new flavonol glycosides, 1, 2 and 3 [4′-methoxy-5-hydroxy-8-(3,3-dimethylallyl)flavone 3-glucosyl(1→2)-rhamnoside-7-glucoside, 3-xylosyl(1→2)rhamnoside-7-glucoside and 3-rhamnosyl-(1→2)rhamnoside-7-glucoside] were isolated from *Epimedium koreanum*, together with icariin (Fig. 1). Their contents were comparatively high and these glycosides were considered as indicative compounds for the evaluation.

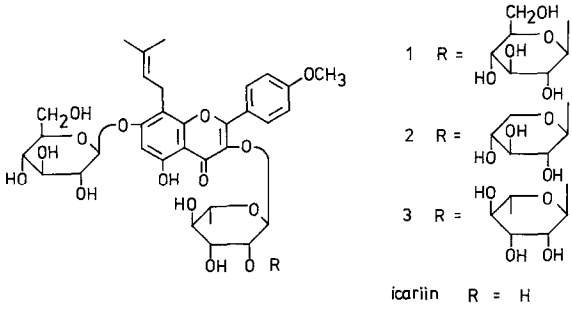

Fig. 1. Structures of four flavonol glycosides. 1 = 4′-methoxy-5-hydroxy-8-(3,3-dimethylallyl)flavone 3-O-β-D-glucopyranosyl (1→2)-α-L-rhamnopyranoside-7-O-β-D-glucopyranoside; 2 = 4′-methoxy-5-hydroxy-8-(3,3-dimethylallyl)flavone 3-O-β-D-xylopyranosyl (1→2)-α-L-rhamnopyranoside-7-O-β-D-glucopyranoside; 3 = 4′-methoxy-5-hydroxy-8-(3,3-dimethylallyl)flavone 3-O-α-L-rhamnopyranosyl (1→2)-α-L-rhamnopyranoside-7-O-β-D-glucopyranoside.

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In this paper, the application of a high-performance liquid chromatographic (HPLC) method to the separation and determination of these four flavonol glycosides, is demonstrated and the analytical results for 20 plant materials are presented.

EXPERIMENTAL

Plant materials

Plant materials were supplied by Showa University, Kyoto College of Pharmacy, Hiroshima University, Hokuriku University and Kyushu University, and were cultivated in our laboratory.

Reagents

Flavonol glycosides, 1, 2, 3 and icariin were isolated from *E. koreanum* and purified by preparative HPLC. The acetonitrile used for the chromatography was of HPLC grade. Deionized water was further purified using a Millipore filter.

Apparatus

A Tosoh Model CCPM liquid chromatograph equipped with a UV-8000 UV spectrophotometer and a stainless-steel column (150 × 4 mm I.D.) packed with chemically bonded ODS silica gel (TSK gel ODS-120A, 5 μm; Tosoh) was used.

Procedure

About 0.5 g of dried, powdered crude drug was weighed accurately, placed in 25 ml of the mobile phase and refluxed on a water-bath at 85°C for 30 min. After cooling, the solution was centrifuged and decanted. The residue was washed twice with 10-ml portions of the mobile phase. The extract and washings were placed in a 50-ml volumetric flask together with 5 ml of internal standard solution (1 mg/ml of benzoin in ethanol) and diluted to 50 ml with the mobile phase. A 10-μl volume of the solution was injected into the HPLC system. The content of each flavonol glycoside was calculated from the ratio of its peak area to the peak area of the internal standard.

HPLC conditions

Water–acetonitrile (73:27) was used as the mobile phase at a flow-rate of 1.0 ml/min. The temperature of the column was maintained at 50°C. The substances eluted were detected with a UV detector at 270 nm.

Calibration graphs and detection limits

Calibration graphs for 1, 2, 3 and icariin were obtained for the concentration ranges 2.5–25.0, 5.0–50.0, 5.0–50.0 and 12.5–125.2 μg/ml, respectively. The corresponding regression equations were as follows: $y = 0.01955x + 0.0008$ ($r = 0.999$); $y = 0.02152x + 0.0022$ ($r = 0.999$); $y = 0.01883x + 0.0102$ ($r = 0.999$) and $y = 0.02823x - 0.0051$ ($r = 0.999$). The detection limits were 2.5, 3.0, 5.0 and 1.3 ng, respectively, at a signal-to-noise ratio of 3:1 for the peak heights.

RESULTS AND DISCUSSION

HPLC conditions

The acetonitrile concentrations of the mobile phase and the column temperature were varied in order to find the optimal elution conditions on chemically bonded ODS silica gel. The acetonitrile concentration was varied from 25 to 29% and 27% was selected for subsequent work, based on the resolution and retention times (Fig. 2). Column temperatures of 30, 40 and 50°C were tried and were found to affect the retention times slightly (Fig. 3); a temperature of 50°C was subsequently adopted.

Extraction solvent for flavonol glycosides

The mobile phase, methanol, methanol–water (50:50), ethanol–water (70:30), ethanol–water (50:50) and water were tried for the extraction of four flavonol glycosides. The temperature of the water-bath and the extraction time were maintained at 85°C and 30 min, respectively. The mobile phase gave the best extraction efficiency (Table I) and was selected for further work.

Determination of flavonol glycosides

The chromatogram of flavonol glycosides in Epimedii Herba is shown in Fig. 4. Table II gives the analytical results for 20 plant materials.

The contents of 1, 2, 3 and icariin varied from 0.05 to 0.98%, from 0.03 to 1.18%, from 0.01 to 0.78% and from 0.02 to 0.76%, respectively, except for *E. sagittatum*. Icariin has been thought to be the main component of Epimedii Herba. However, none of the plant materials analysed in our study contained icariin as the main component.

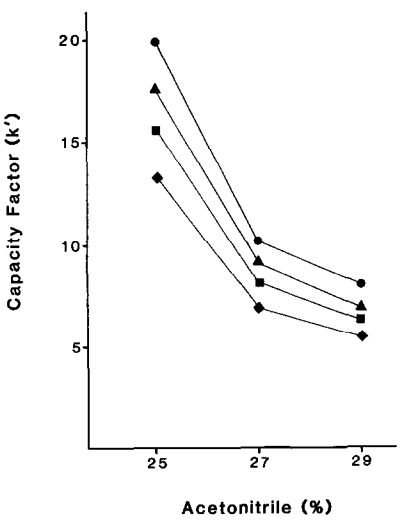

Fig. 2. Effect of acetonitrile concentration on the capacity factors of 1 (◆), 2 (■), 3 (▲) and icariin (●). Mobile phase, water–acetonitrile. Flow-rate, 1.0 ml/min. Column temperature, 50°C.

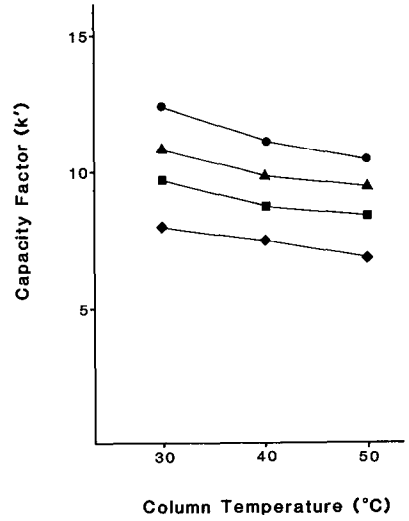

Fig. 3. Effect of column temperature on the capacity factors of 1 (◆), 2 (■), 3 (▲) and icariin (●). Mobile phase, water–acetonitrile (73:27). Flow-rate, 1.0 ml/min.

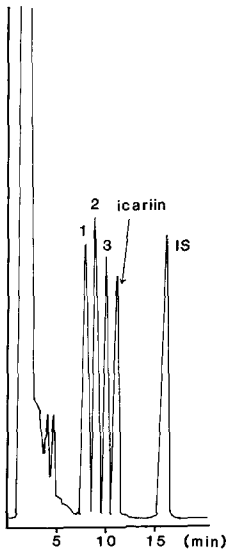

Fig. 4. Chromatogram of Epimedii Herba. Column, TSK gel ODS-120A (5 μm, 150 × 4 mm I.D.). Mobile phase, water–acetonitrile (73:27). Flow-rate, 1.0 ml/min. Column temperature, 50°C.

E. koreanum showed the highest total amount of the four glycosides. These glycosides were not detected in *E. sagittatum*. *E. sagittatum* grows naturally only in China, although the plant materials analysed in this study were all cultivated in Japan. As there are few channels for the importation of *E. sagittatum* from China to Japan, it is concluded that the *E. sagittatum* used in this experiment were grown from the same original plant, which unexpectedly contained few flavonol glycosides.

In conclusion, the proposed HPLC method is simple, rapid and precise, and seems to be useful for the quality control of Epimedii Herba.

TABLE I

EFFECT OF SOLVENTS ON EXTRACTION EFFICIENCY

Solvent	*Extraction (%)*			
	1	*2*	*3*	*Icariin*
Mobile phase	100.0	100.0	100.0	100.0
Methanol	101.1	96.7	94.0	100.5
Methanol–water (50:50)	99.7	99.3	97.9	96.4
Ethanol–water (70:30)	92.6	90.3	89.1	93.5
Ethanol–water (50:50)	99.3	99.0	93.7	100.3
Water	81.4	74.7	75.3	76.8

TABLE II

CONTENTS OF FLAVONOL GLYCOSIDES (%)

Plant material	*1*	*2*	*3*	*Icariin*	*Total (1 + 2 + 3 + icariin)*
E. sagittatum (Sieb. et Zucc.):					
(1) Cultivated in Saitama	Trace	Trace	Trace	Trace	–
(2) Cultivated in Saitama	Trace	Trace	Trace	Trace	–
(3) Cultivated in Kyoto	Trace	Trace	Trace	Trace	–
(4) Cultivated in Yamanashi	Trace	Trace	Trace	Trace	–
(5) Cultivated in Fukuoka	Trace	Trace	Trace	Trace	–
E. sempervirens Nakai:					
(1) Cultivated in Saitama	0.34	0.58	0.25	0.50	1.67
(2) Cultivated in Kyoto	0.05	0.03	0.16	0.05	0.29
(3) Native in Ishikawa	0.11	0.08	0.01	0.02	0.22
(4) Native in Ishikawa	0.08	0.06	0.02	0.01	0.17
E. sempervirens Nakai var. *hypoglaucum* (Makino) Ohwi:					
(1) Cultivated in Hiroshima	0.41	0.40	0.78	0.21	1.80
E. koreanum Nakai:					
(1) Cultivated in Saitama	0.54	0.78	0.46	0.76	2.54
(2) Cultivated in Kyoto	0.71	1.18	0.33	0.53	2.75
E. macranthum Morr. et Decne.:					
(1) Cultivated in Saitama	0.36	0.46	0.68	0.61	2.11
E. diphyllum (Morr. et Decne.) Lodd.:					
(1) Cultivated in Kyoto	0.28	0.27	0.31	0.15	1.01
E. grandiflorum Morr. var. *thunbergianum* (Miq.) Nakai:					
(1) Cultivated in Saitama	0.98	0.60	0.17	0.56	2.31
(2) Cultivated in Saitama	0.66	0.48	0.05	0.15	1.34
(3) Cultivated in Kyoto	0.60	0.64	0.45	0.29	1.98
E. grandiflorum var. *higoense* T. Shimizu:					
(1) Cultivated in Hiroshima	0.31	0.56	0.16	0.49	1.52
E. setosum Koidz.:					
(1) Cultivated in Hiroshima	0.40	0.43	0.65	0.14	1.62
E. setosum Koidz. nm. Sasakii Sugimoto:					
(1) Cultivated in Hiroshima	0.31	0.29	0.38	0.19	1.17

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