Journal of Chromatographic Science, Vol. 45, April 2007

Simultaneous Determination of Seven Flavonoids in *Potentilla multifida* by HPLC

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Abstract

A reversed-phase high-performance liquid chromatographic method is described for the simultaneous determination of seven flavonoids in *Potentilla multifida*: hyperin, quercetin-3-*O*-β-D-glucopyranoside, luteolin-7-*O*-β-D-glucuronide, apigenin-7-*O*-β-D-glucuronide, quercetin, tribuloside, and apigenin. The method involves the use of a Hypersil octadecylsilyl silica (ODS) analytical column (125Å, 5 µm, 4.6 × 250 mm) at 25°C with the mixture of acetonitrile and aqueous H_3PO_4 as the mobile phase and detection at 254 nm. The recovery of the method is 95.4–104.8%, and linearity (*r* > 0.9998) is obtained for all the flavonoids. The results indicate that the flavonoid content of *P. multifida* varied significantly from locality to locality.

Introduction

The whole plant of *Potentilla multifida* (Rosaceae) has been used in north China to treat various diseases, including hepatitis, enterobiasis, functional uterine hemorrhage, traumatic hemorrhage, and, in recent years, it has been used to treat type 2 diabetes (1,2). Thirty compounds were isolated in our previous research (2–5). It was found that the major components in this plant were flavonoids. The pharmacological research showed that the 95% ethanol and water extracts of the whole plant of *P. multifida* has antidiabetic activities. Flavonoids have been reported to have antidiabetic activities by stimulating the exertion of insulin, improving glucose consumption in outer tissues, aldose reductase inhibitory activity, and α-glycosidase inhibitory activity (6,7). There was no report on the analysis of flavonoids from the previous plant. In this paper, an accurate high-performance liquid chromatographic (HPLC) method for the quality control of flavonoids from *P. multifida* was developed. The contents of the flavonoids in the whole plant collected from six different areas of China have been determined.

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Experimental

Apparatus

The HPLC was performed using a Jasco (Tokyo, Japan) HPLC system, including a Jasco MD-1510 multiwavelength detector, a PU-1580 intelligent HPLC pump, a 7725i injector (Jasco, Tokyo, Japan), and a Jassco HSS-2000 Monitor-system. A Hypersil octadecylsilyl silica (ODS) analytical column from Thermo Electron (Waltham, MA) (125Å, 5 µm, 4.6 × 250 mm) was used. The column temperature was at 25°C, a UV detector wavelength was kept at 254 nm, and the flow rate was 1.0 mL/min.

Materials and reagents

The plant materials of *P. multifida* were purchased from different areas of north China. All materials were sorted and identified by Professor Hubiao Chen with voucher specimens (DNM2002-01-DNM2002-06) deposited in the Herbarium of School of Pharmaceutical Sciences at the Peking University Health Science Center (Beijing, China). Acetonitrile (HPLC grade) was purchased from Thermo Fisher. Ultra-pure distilled water was used. All organic solvents used were of analytical grade.

Standards

All seven flavonoids as reference standards: hyperin (1), quercetin-3-*O*-β-D-glucopyranoside (2), luteolin-7-*O*-β-D-glucuronide (3), apigenin-7-*O*-β-D-glucuronide (4), quercetin (5), tribuloside (6), and apigenin (7), were isolated in our previous chemical investigation of *P. multifida* L. Their structures (Figure 1) were characterized by spectroscopic methods and reported in our previous paper (3). The purity of the seven standards was greater than 98%, as suggested by the purity analysis of reverse phase-HPLC.

Mobile phase

The mobile phase consisted of solvent A (acetonitrile) and solvent B (0.1 % aqueous phosphoric acid). The elution profile was: 0–18 min, 83% B; 18–22 min, 83–80% B; 22–34 min, 80–76% B; and 34–60 min, 76–70% B. All gradient steps were

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linear. Acetonitrile and water were previously filtered under a vacuum through 0.45-µm nylon filters before injection into the HPLC apparatus.

Preparation of standard solution

To prepare the standard solution, an accurately weighed amount of the seven standards (9.95, 10.12, 12.66, 14.03, 10.01, 10.56, and 2.22 mg, respectively) were dissolved in 60% methanol (10, 10, 5, 5, 10, 10, and 10 mL, respectively) for the stock solution.

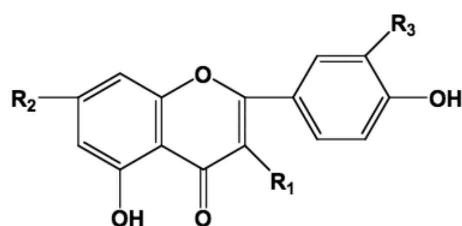
Preparation of sample solution

The air-dried whole plants of *P. multifida* (1 g) collected in different areas were separately pulverized and extracted with 50% MeOH (150 mL) in an ultrasonic water bath at room temperature for 45 min. Each extract was filtered. The filtrate was concentrated to dryness, which was dissolved in 50% MeOH (10 mL). The afford solution was filtered through a 0.45-µm syringe filter prior to HPLC.

Results and Discussion

Optimization of chromatographic conditions

The detection wavelength was chosen at 254 nm based on the best baseline separation of the seven flavonoids. Several solvent systems based on the various mixtures of acetonitrile or methanol with different acids (acetic acid and phosphoric acid) in different concentrations and ratios were tested in order to achieve optimal separation in a relatively short time. With respect to the separation efficiency and sensitivity, better results were achieved with acetonitrile instead of methanol. The use of 0.1% phosphoric acid in the solvent system could reduce the ionization of the phenol group, then give the more symmetrical peak and a better separation for flavonoids. The gradient eluting system was tested to shorten the analysis time within 60 min because the seven standards contained aglycones and glycosides. Therefore, the mobile phase was acetonitrile–0.1% phosphoric acid (0–18 min, 83% B; 18–22 min, 83–80% B; 22–34 min, 80–76% B; 34–60 min, 76–70% B). All gradient steps were linear.



		R_1	R_2	R_3
1	Hyperin	*O*-β-*D*-gal*p*	OH	OH
2	Quercetin-3-*O*-β-D-glucopyranoside	*O*-β-*D*-glc*p*	OH	OH
3	Luteolin-7-*O*-β-D-glucuronide	H	O-gluA	OH
4	Apigenin-7-*O*-β-D-glucuronide	H	O-gluA	H
5	Quercetin	OH	OH	OH
6	Tribuloside	*O*-β-*D*-(6-*O*-*p*-coumaroyl)-glc*p*	OH	H
7	Apigenin	H	OH	H

Figure 1. The chemical structures of seven flavonoids.

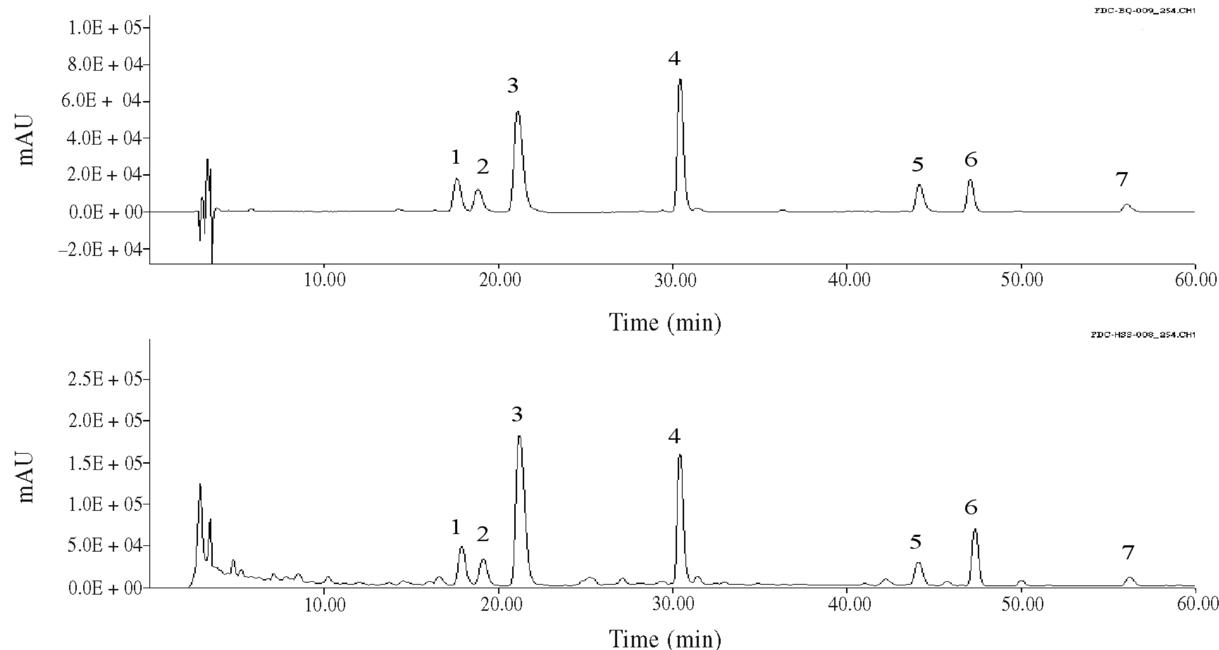


Figure 2. HPLC chromatogram of standard of the flavonoids 1–7 (A) and extract of *P. multifida*, λ = 254 nm (B).

Optimization of extraction conditions

Firstly, ethanol, methanol, and water were used to investigate the effect of solvents on the extraction of the seven flavonoids from the plant. It was found that methanol could extract the seven flavonoids in high yields. Given that five of the seven detected flavonoids were glycosides, methanol and aqueous methanol with different ratio were compared. Aqueous methanol (50%, v/v) was found to give the hightest yields. Compared with 50% (v/v) aqueous methanol, ethanol and methanol could not extract the flavonoid glycosides efficiently, and water could not extract the flavonoid aglycones efficiently. Additionally, the sample solution prepared by 50% (v/v) aqueous methanol had less lipid–soluble pigments. Secondly, the effect of the extraction method was studied. Ultrasonication (30 min) was proven to give a more efficient extraction than reflux (2 h) and infusing (12 h). Furthermore, the different ultrasonication time (30, 45, and 60 min) and different volume of 50% (v/v) aqueous methanol (40, 100, 150, and 200 mL) on the content of the seven flavonoids were investigated. It was found that 45 min of ultrasonication with 150 mL of 50% (v/v) aqueous methanol was sufficient to extract all flavonoids.

Calibration and linearity

An external standard method was used for quantitative determinations. The retention times of the standards 1–7 were 17.26, 18.25, 22.00, 30.00, 43.00, 48.00, and 56.00 min, respectively (See Figure 2A). In order to check the linearity of the relationship between the peak area and the concentration, the standard solution was prepared and diluted with 50% methanol. Each of seven different concentrations (1.2–460 µg/mL for 1, 1.0–370 µg/mL for 2, 7.0–2560 µg/mL for 3, 6.0–2530 µg/mL for 4, 0.5–500 µg/mL for 5, 1.3–500 µg/mL for 6, and 0.3–120 µg/mL for 7) of standard solutions were in-jected four times into the HPLC system. A typical HPLC chromatogram of the standard mixture is shown in Figure 2. The calibration graphs were obtained by plotting the peak area against the concentration of the standards. In the simultaneous determination, the calibration graphs were found to be linear in the mentioned concentrations (the correlation coefficients are shown in Table I). All the compounds showed good linearity ($r \geq 0.9998$) and obeyed Beer's law in the concentration ranges investigated (Table I). The limits of detection (LOD) of selected compounds (1–7), determined with the UV detector at the operative wavelength of 254 nm, ranged from 28 (6) to 231 ng/mL (4). Calculation of the detection limits for the compounds studied were based on a signal-to-noise ration of 3 throughout this work. The limits of quantitation (LOQ) ranged from 93 (6) to 572 ng/mL (4) (Table I). The results obtained for the LOQ confirmed that the proposed HPLC method was sufficiently sensitive for the simultaneous determination of the flavonoids (1–7).

Table I. Analytical Characteristics of the Calibration Graphs

Compound	Range* (µg/mL)	Slope (*b*)	Intercept (*a*)	*r*†	LOD‡ (ng/mL)	LOQ§ (ng/mL)
1	1.2–460	4.0×10^7	–121542	0.9998	89	295
2	1.0–370	3.0×10^7	–77702	0.9998	52	173
3	7.0–2560	2.0×10^7	–438115	0.9998	173	572
4	6.0–2530	2.0×10^7	–412292	0.9998	231	763
5	0.5–500	5.0×10^7	–85530	0.9999	48	157
6	1.3–500	3.0×10^7	–128671	0.9999	28	93
7	0.3–120	4.0×10^7	–25356	0.9998	34	111

* No. of calibration points $n = 6$ (each injected in four times).
† Correlation coefficients of the regression equation $y = a + bx$, where x is the compound concentration (µg/mL) and y is the peak area.
‡ LOD has a signal-to-noise ratio of 3:1.
§ LOQ is the lowest concentration of the linear range of calibration curve, with a signal-to-noise ratio of 10:1.

Table II. Recovery and Precision of the Method ($n = 6$)

Standard	Amount added (mg)	Recovery (%)		Precision RSD (%)	
		Mean	RSD	Intraday	Interday
1	0.1668	95.4	2.3	1.8	2.0
2	0.1630	99.8	2.7	2.6	2.0
3	1.4211	104.8	2.1	1.2	1.0
4	0.8290	95.6	2.7	0.8	1.5
5	0.0975	96.4	2.1	1.2	2.4
6	0.2725	101.9	2.3	0.2	2.8
7	0.0524	98.5	2.4	1.5	2.2

Table III. Analysis of Flavonoids in Whole Plant ($n = 3$)

No.	Collection place	Collection date	Amount of compounds (mg/g)						
			1	2	3	4	5	6	7
1	Jining, Inner Mongolia	Jan. 2002	0.39	0.34	2.79	1.84	0.23	0.52	0.10
2	Harbin, Heilongjiang	Jun. 2004	1.18	0.50	3.33	2.19	0.08	0.45	0.03
3	Shenyang, Liaoning	Aug. 2004	0.31	1.57	0.33	0.44	0.04	0.41	n.d.*
4	Changchun, Jilin	Mar. 2002	0.09	1.19	0.93	0.83	0.06	0.37	0.03
5	Shijiazhuang, Hebei	Aug. 2004	0.09	0.55	0.43	0.30	0.03	0.50	n.d.
6	Xian, Shanxi	Aug. 2004	0.46	0.70	2.50	1.70	0.08	0.25	0.04

* n.d.= not detectable.

Precision and reproducibility

To assess the precision of the method, the sample solution was injected six times within 24 h and over a 3-day period analysis. The coefficient variations of intra- and inter-day studied were both less than 3.0% (Table II). The reproducibility of the method was performed by adding a known amount of the standard compounds to the powdered plant material of *P. multifida* (0.5 g), and then undergoing analysis according to the method. The results of the recoveries of 1–7 ranged from 95.6% to 104.8%. The relative standard deviations (RSD) of recoveries of the seven flavonoids ranged between 2.1% and 2.7% (Table II).

Analysis of the plant material

When the sample solutions were analyzed in the same method, the peaks were identified by comparison of the retention time with those corresponding to authentic samples purified from *P. multifida* (Figure 2B). The contents of the seven flavonoids in a whole plant of *P. multifida* growing in different locations were analyzed, demonstrating that all of these flavonoids in the plants were strikingly dependent on the locality (Table III). Climatic, seasonal, and geochemical factors were presumably own to the contents of flavonoids in this medicinal plant.

Conclusion

This method is simple and sensitive, and the LODs and LOQs are low enough to analyze the seven flavonoids in *P. multifida.* The method is suited for rapid and routine analysis.

Acknowledgments

The authors thank the National Natural Science Foundation of China (Grant No. 20432030) for financial support of this work. This work was also supported by the program for Changjiang Scholar and Innovative Team in University (Grant No. 85-2-063-112). The authors are grateful to Professor H.B. Chen for the identification of the plant materials.

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Manuscript received May 16, 2006;
revision received January 4, 2007.