Simultaneous Determination of Myricitrin, Hyperin, Quercitroside, and Quercetin in *Folium Rhododendri Micranthi* by RP–HPLC

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Abstract

A reversed-phase high-performance liquid chromatography method was established for the simultaneous determination of four major constituents, namely myricitrin, hyperin, quercitroside, and quercetin in *Folium Rhododendri Micranthi*. The optimal conditions of separation and detection were achieved on a C18 analytical column with a gradient mobile phase consisting of acetonitrile and 1% acetic acid at the flow rate of 1.0 mL/min, and detection wavelength was set at 355 nm. All calibration curves showed good linear regression ($r > 0.9993$) within test ranges. The reproducibility of relative standard deviations was less than 2.3%, and recovery of analytes was greater than 99.4%, respectively. The method was successfully applied to determine the contents of four compounds in *Folium Rhododendri Micranthi*. The results indicated that the contents of myricitrin, hyperin, quercitroside and quercetin in *Folium Rhododendri Micranthi* were 0.166%, 0.303%, 0.299%, and 0.053%, respectively.

Introduction

Folium Rhododendri Micranthi is the branches and leaves of *Rhododendri Micranthi* Turcz., which belongs to the rhododendron section. It mainly has different kinds of flavonoids, multi-saponin, tannin and carbohydrates, and volatial oil. *Folium Rhododendri Micranthi* has been used to cure chronic tracheitis and asthma, dysentery, and puerperal general pain for thousands of years as an important folk medicine in China (1). The total flavonoids from *Folium Rhododendri Micranthi* have the obvious effects of anti-prostatic hyperplasia, anti-inflammatory, and analgesia (2). Several bioactive flavonoids including astragalin, hyperin, kaempferol, and quercetin are found in the leaves (3). But the constituents are different in *Folium Rhododendri Micranthi* in different locations of China. Four main compounds called myricitrin, hyperin, quercitroside, and quercetin were isolated and identified in our lab. The molecular structures of these compounds are shown in Figure 1.

Some related studies showed that hyperin, quercetin, and other flavonoids had a broad range of physiological activities such as analgesia anti-inflammatory, antiallergic, and antioxidant activity for scavenging radicals and inhibition of a variety of enzymes (4–5). In order to control the quality of *Folium Rhododendri Micranthi*, it is necessary to develop a sensitive, selective, dependable, and relative simple assay method to determine the active ingredients in *Folium Rhododendri Micranthi*.

Although quantitative analysis of the medicines had been reported in previous papers (6–7), there was no report on the

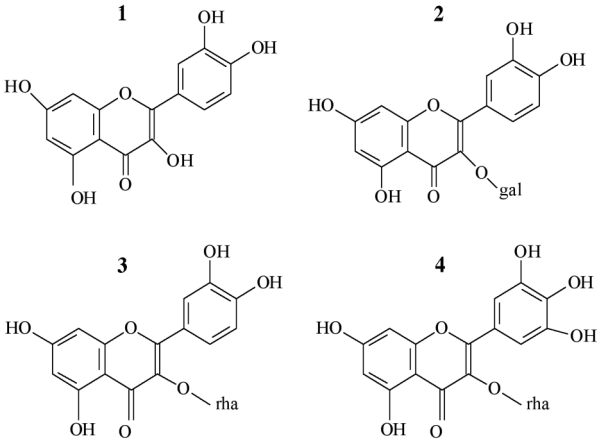

Figure 1 The structure of compounds from *Folium Rhododendri Micranthi*: quercetin, 1; hyperin, 2; quercitroside, 3; and myricitrin, 4.

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simultaneous determination of the four constituents that were mentioned earlier in the plant.

In the present paper, the four constituents from *Folium Rhododendri Micranthi* were simultaneously determined by reversed-phase high-performance liquid chromatography (HPLC). The method could be used to evaluate and compare the quality of the plants in different regions.

Experimental

Chemicals and materials

Hyperin and quercetin standard samples were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The reference standard of myricitrin and quercitroside were extracted and purified from *Folium Rhododendri Micranthi,* which was purchased from a market and identified by Prof. Fengzhi Nie (Department of Pharmacognosy, Hebei Medical University, Shijiazhuang, China) in our laboratory. Their chemical structures were confirmed by ^{1}H and ^{13}C-nuclear magnetic resonance spectroscopy, and the purities were over 98.9% by HPLC analysis. Acetonitrile was of HPLC-grade and was purchased from Tedia (Tedia Company, Inc., Fairfield, OH). Water was purified by double distillation.

HPLC system and conditions

A Waters HPLC system was employed and equipped with a 515 pump and a 2996 UV detector. Compounds were separated on a Diamonsil-C_{18} column (250 × 4.6 mm, i.d., 5 µm, Beijing, China). Solvent A was acetonitrile, and solvent B was 1% acetic acid. For the detection of analytes, the mobile phase consisted of a gradient elution for 0–30 min with 10–20% solvent A, 30–45 min with 20% solvent A, 45–60 min with 20%–30% solvent A, followed by 60–70 min with 30%–10% solvent A at a flow rate of 1 mL/min. The column temperature was kept at 30°C, and the detector wavelength was set at 355 nm.

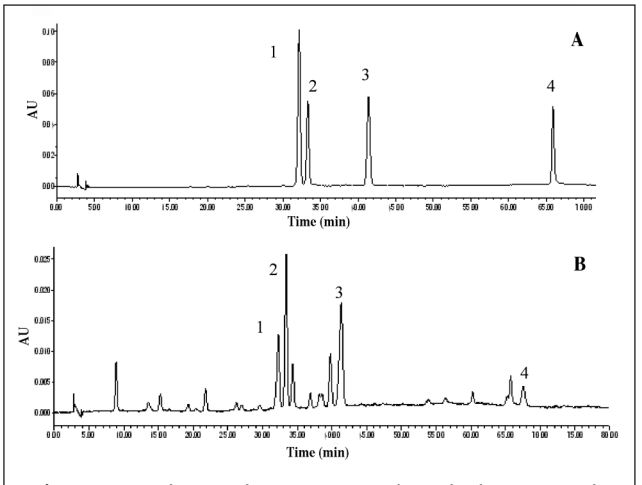

Figure 2 Typical HPLC chromatograms of standard mixture with myricitrin, 1; hyperin, 2; quercitroside, 3; and quercetin, 4 (A); the test solution (B).

Sample preparation

Folium Rhododendri Micranthi was ground into power and accurately weighed. Each weighed sample (~ 0.2 g) was extracted by refluxing with 20 mL of 70% ethanol for 1 h. The extract was cooled to room temperature and filtered. The procedure was repeated three times. Then the filtrates were combined, evaporated to dryness, and then dissolved with methanol into a 50-mL volumetric flask, metered volume. All samples were filtered through a 0.45-µm micropore membrane, and 20 µL of filtrate was injected into the HPLC instrument for analysis.

Validation of the quantitative analysis

For standard solutions, the reference compounds myricitrin (14.75 mg), hyperin (7.71 mg), quercitroside (9.1 mg), and quercetin (5.2 mg) were dissolved in methanol, and then they were diluted with the same solvent for further concentration levels. Six different concentrations were analyzed in triplicate using the same HPLC condition as described previously. Calibration curves of myricitrin, hyperin, quercitroside, and quercetin were generated by plotting the peak area (y) versus the concentration (x) of each compound.

The limit of quantitation (LOQ) was determined as the concentration of the drug that gave a signal-to-noise ratio ~ 10:1. The precision of the developed method was determined by analyzing the same sample six times.

In order to test the repeatability, six sample solutions were prepared. Each solution was injected twice. The contents and the relative standard deviations (RSDs) of myricitrin, hyperin, quercitroside, and quercetin were calculated, respectively.

The accuracy of the method was evaluated from the results of the recovery test. It was carried out by adding the standard solution at three different levels to the powdered samples and assigned six replicates per level. Then the samples were treated according to the sample preparation procedure described earlier. Recovery was obtained by calculating the percent ratio of determined amount to added amount. And bias was also calculated.

The stability of analytes in sample solutions was investigated. It was carried out by determining the analytes in similarly treated sample solutions after storing them at room temperature for different times (0, 2, 4, 6, 8, 12, 24, 36, 48 h).

Results and Discussion

Extraction

In order to achieve quantitative extraction, variables involved in the procedure, such as the extraction method, solvent, and times were investigated. The extraction procedures were carried out by reflux and ultrasonic bath extraction, respectively. It was found that ultrasonic bath extraction could not extract completely, while the reflux procedure could achieve the analysis qualification. Therefore, the method of refluxing extraction proved to be the suitable one. Through the orthogonal experiment, it was shown that all determined constituents in 0.2 g samples were almost completely extracted

three times with 20 mL of 70% ethanol for 1 h, and no compound was detected by HPLC in the fourth extracts.

Validation of the method

Specificity

The chromatograms of samples are shown in Figure 2. Myricitrin, hyperin, quercitroside, and quercetin were well separated from the rest of the substances in the sample and had retention times of ~ 32.4, 33.6, 41.8, and 66.1 min, respectively. No interference peaks were found at the retention times of each analyte.

Because the polarity and solubility of hyperin and quercitroside are so similar, it is difficult to separate them with an isocratic mobile phase in a short time. Thus, gradient elution was developed so as to ensure that all the constituents could be determined in one analysis run. In the beginning, various mixtures of water and methanol were tried as the mobile phase, but separation was not satisfactory. When methanol was replaced by acetonitrile, the situation was greatly improved. However, bad peak shapes of the compositions was still observed. Therefore, acetic acid was added in this study to reduce the ionization of the phenol group and the carboxyl group, so that a satisfactory resolution, as well as satisfactory peak shape, was achieved. The typical chromatograms of samples and

Table I. Results of Regression Analysis on Calibration and Limit of Quantitation for Compounds (*n* = 5)

Compounds	Regression equation	*r*	Linear range (mg/mL)	LOQ (mg/mL)
Myricitrin	$y = 40342x + 18838$	0.9993	0.922–14.75	0.134
Hyperin	$y = 40083x + 10559$	0.9996	1.928–30.84	0.123
Quercitroside	$y = 40950x + 7440$	0.9994	1.144–18.3	0.155
Quercetin	$y = 46752x - 4922.9$	0.9994	0.324–5.190	0.083

Table II. Precision of HPLC Method to Determine Compounds (*n* = 5)

	Myricitrin (µg/mL)	Hyperin (µg/mL)	Quercitrosid (µg/mL)	Quercetin (µg/mL)
Mean	6.623833	12.38483	11.94183	1.971667
SD*	0.051627	0.059968	0.106381	0.045359
RSD† (%)	0.78	0.48	0.89	2.3

* SD = standard deviation
† RSD = relative standard deviation

Table III. Results of Reproducibility Test for Compound 1–4 (*n* = 6)

Run	Myricitrin	Hyperin	Quercitroside	Quercetin
Measured (%)	0.1650	0.2995	0.2970	0.0532
	0.1643	0.3026	0.3082	0.0543
	0.1680	0.3037	0.2985	0.0530
	0.1599	0.3102	0.2969	0.0542
	0.1662	0.3075	0.3003	0.0530
	0.1583	0.2978	0.3051	0.0522
Mean (%)	0.1636	0.3036	0.301	0.0533
RSD (%)	2.3	1.5	1.5	1.5

Table IV. Results of Recovery for Compound 1–4 (*n* = 5)

Compounds	Contained (µg)	Added (µg)	Determined (µg)	Recovery (%)	Mean value (%)
Myricitrin	174.47	177	345.14	98.20	99.75 ± 1.5
	163.44	177	335.95	98.68	
	167.53	177	349.46	101.4	
	171.21	177	352.49	101.2	
	168.74	177	342.97	99.20	
Hyperin	318.45	313	625.13	99.00	99.14 ± 0.68
	298.33	313	603.50	98.72	
	305.79	313	618.68	99.66	
	312.51	313	625.32	99.97	
	308.00	313	610.71	98.34	
Quercitroside	314.25	291.2	602.24	99.47	99.99 ± 0.78
	294.40	291.2	577.28	98.58	
	301.75	291.2	599.29	101.1	
	308.39	291.2	595.33	99.29	
	303.93	291.2	598.46	100.6	
Quercetin	55.70	52	107.17	99.51	100.4 ± 1.8
	52.18	52	106.52	102.3	
	53.49	52	107.8	102.2	
	54.66	52	104.69	98.15	
	53.87	52	105.76	99.90	

Table V. The Results of Stability of the Test Samples

	Myricitrin (µg/mL)	Hyperin (µg/mL)	Quercitrosid (µg/mL)	Quercetin (µg/mL)
Mean	6.57761	12.408	11.82844	1.912889
SD	0.06523	0.318818	0.271495	0.086444
RSD (%)	0.99	2.6	2.3	4.5

Table VI. Contents of Four Compounds in Samples of *Folium Rhododendri Micranthi* (*n* = 5)

	Amount of compounds (%)			
	Myricitrin	Hyperin	Quercitrosid	Quercetin
1	0.165	0.301	0.299	0.054
2	0.166	0.305	0.299	0.054
3	0.161	0.300	0.297	0.052
4	0.166	0.307	0.300	0.053
5	0.171	0.303	0.299	0.051
Mean	0.166	0.303	0.299	0.053
SD	0.003	0.003	0.0011	0.0013
RSD	1.8	0.99	0.37	2.4

mixed standard are shown in Figure 2 from which all target constituents were completely separated within 100 min. In fact, there was an attempt to shorten the analysis time. However, the peaks of hyperin and quercitroside cannot separate from each other if the run time is considerably shorter.

Linearity and Limit of Quantitation

The regression equations, correlation coefficients, and linearity ranges are shown in Table I. The results showed that there was excellent correlation between the ratio of peak area and concentration for each compound within the test ranges.

For each target constituent, the LOQ was determined by serial dilution of standard solution (Table I).

Precision, repeatability, and recovery

The precision was determined by analyzing the same sample solution six times. The RSD values of peak area of each compound were calculated. The results are shown in Table II. The results of reproducibility are shown in Table III. The contents of myricitrin, hyperin, quercitroside, and quercetin were 0.164%, 0.304%, 0.301%, and 0.053%; the RSDs were 2.3%, 1.5%, 1.5%, and 1.5%, respectively.

The results of recovery for all target constituents are listed in Table IV. It could be found that the recoveries were more than 99%, which indicated that the method was sufficient for the determination of the analytes.

Stability

Table V shows that the analytes in the sample solution were stable for two days, with an RSD of less than 5.0%.

Application

The peaks were validated by comparing the retention times and the maximum wavelengths of UV spectra with those of the standards. The contents of the four constituents in samples were calculated, and the results are shown in Table V. We could find that the contents of four analytes in determined sample were: myricitrin, 0.166%; hyperin, 0.303%; quercitrosid, 0.299%; and quercetin, 0.053%, respectively.

Conclusion

This is the first report by using HPLC method to determine four major constituents, namely myricitrin, hyperin, quercitroside, and quercetin in *Folium Rhododendri Micranthi* simultaneously. The method is simple, accurate, and specific and can be used for quality control of *Folium Rhododendri Micranthi* and its preparations. Moreover, it provides an analytical basic for pharmacokinetics and therapeutic basis of *Folium Rhododendri Micranthi*.

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