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Quantitative Analysis of Flavonoids of *Hylocereus undatus* (Bawanghua)

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Abstract

Bawanghua, the flower of *Hylocereus undatus*, is an edible herb. Quercetin, kaempferol and isorhamnetin glycosides are the major components in Bawanghua. The major flavonoid glycosides in Bawanghua are too identical to be separated in preparative scale which hindered the using of those glycosides as references in routine quality control method. Quantification of the flavonoid aglycones hydrolyzed from their glycosides is alternative quality control approach. The hydrolysis conditions were optimized and an HPLC method was also developed to quantify the hydrolyzed flavonoid aglycones in Bawanghua. The developed method is simple and convenient with good precision and reproducibility, which can be used as a quality control method of Bawanghua.

Keywords: *Hylocereus undatus*; Bawanghua; Flavonoid; Glycoside; Hydrolysis; Quercetin; Kaempferol; Isorhamnetin; HPLC; Quantitative analysis; Sample preparation

Introduction

Bawanghua, the flower of *Hylocereus undatus* (Haw.) Britt. et Rose, is used to relieve cough in South of China [1]. It has been reported that Bawanghua has antioxidant, antibacterial, cholesterol-lowing and wound healing activities [2-6]. Bawanghua is also an edible herbal in South of China. The local residents use it to make Bawanghua soup as a traditional healthcare food. Some products such as Bawanghua candies, Bawanghua health drinks were also in market [7].

As a part of ongoing project on the investigation of phytochemicals and quality control methods of commonly used Chinese herbal medicine in South of China [8-13], we have carried out systematic chemical research on Bawanghua. It has been found that the flavonoid glycosides are the major components in Bawanghua [14-18]. It is well known that flavonoids are antioxidant bioactive components, and also are chemical markers of the quality control of many Chinese herbs rich in flavonoids. Therefore, we intend to choose the flavonoids as the marker compounds for the quantitative analysis of Bawanghua. We have successfully developed a fingerprint method [16] and an HPLC method of simultaneous quantification of major flavonoids [17] for the quality control of the flavonoids in Bawanghua. We also found that quercetin, kaempferol or isorhamnetin (Figure 1) is the aglycone of the major flavonoid glycosides and the structures of the major flavonoid glycosides are too identical to be separate in preparative scale. Some research adopted the hydrolysis method for the quality control of flavonoid glycoside, in which flavonoid glycosides was hydrolyzed into aglycones, and then calculating the total flavonoids content by measuring the content of aglycones [19,20]. In this research, we applied the hydrolysis method using hydrochloric acid, the flavonoid glycosides in Bawanghua were transformed into quercetin, kaempferol and isorhamnetin, and then quantitatively analyzed by HPLC. Different from the hydrolysis method in the literatures [19-20], we integrate the hydrolysis and extraction operation into one step, and optimized the hydrolysis conditions. A rapid and convenient sample preparation method was established.

Experimental

General

The analyses were performed on an Agilent 1200 series HPLC instrument. The hydrolysis and extraction was conducted in a Buchi Syncore Polyvap/Analyst/Reactor (Flawil, Switerland). Methanol was purchased from Merck (Darmstadt, Germany). Ultra pure water was purified by a

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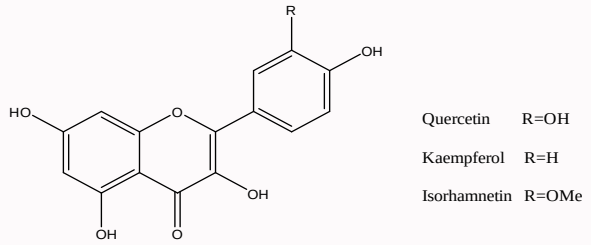

Figure 1: Chemical structures of three flavonoid aglycones.

Milli-Q purification system (Millipore, Bedford, MA, USA).Millex-FG PTFE syringe filters (0.20μm) were purchased from Millipore (Cork, Ireland). Chemical references, quercetin, kaempferol or isorhamnetin, were previously separated by our lab and determined to be more than 98% by peak areas normalization analysis on HPLC.

Bawanghua samples was collected or purchased from different regions in Guangdong and Guangxi provinces and Macao SAR, China, and were identified as the flowers of *Hylocereus undatus* (Haw.) Britt. et Rose by Prof. Zhou Guangxiong from Traditional Chinese Medicine and Natural Medicine Institute of Jinan University. The voucher specimens were deposited in the Institute of Chinese Medical Sciences, University of Macau, and Traditional Chinese Medicine and Natural Medicine Institute of Jinan University.

HPLC analysis

An Agilent SB-C18 column (250mm × 4.6mm, 5μm) (Palo Alto, CA, USA) was used. The column temperature was held at 25°C. The mobile phase consisted of methanol-water (containing 0.4% phosphoric acid, w/v) (54:46, v/v) with a flow rate of 1.0mL/min. The injection volume was 20μl. The chromatogram was monitored at 360nm.

Sample hydrolysis by hydrochloric acid

Bawanghua powder (0.2g) was mixed with 16ml of methanol and 4ml of different concentrations of hydrochloric acid aqueous solution in the tube of Buchi Syncore Polyvap/Analyst/Reactor. Heated the tube at different temperature for various time periods, applied by an orthogonal experiment design method L9 (3^4) (Table 1).

Table 1 listed the schedule of the orthogonal test in which the parameters, hydrochloric acid concentration (A), hydrolysis temperature (B), and hydrolysis duration (C) were selected as the three key factors. Each factor had three levels. Nine experiments with different combinations of the factors and levels would be conducted.

After hydrolysis, the hydrolyte was centrifuged and the supernatant was transferred to a 25ml volumetric flask. Rinse the hydrolyte residue with 4ml of methanol and centrifuged again. The supernatant was transferred to the same volumetric flask, and finally the volume of the solution was made up to the mark with methanol. The solution was filtered through a 0.2μm microporous membrane to obtain the test solution, which was analyzed by the developed HPLC method. Total areas of the three flavonoid aglycones were calculated as an index point to evaluate the hydrolysis results under different factors and levels.

Method validation of HPLC analysis

Methanol stock solutions containing quercetin (0.45mg/mL), kaempferol (0.70mg/mL) and isorhamnetin (0.67mg/mL) were prepared and diluted to appropriate concentrations for the construction of calibration curves. The calibration curves were constructed by plotting the peak areas versus the concentrations of each flavonoid aglycone, respectively.

Table 1: Factors and levels of orthogonal experiment design.

Level	A Temperature (°C)	B Hydrochloric acid concentration (mol·L-1)	C Duration (h)
1	70	4	3
2	80	5	4
3	90	6	5

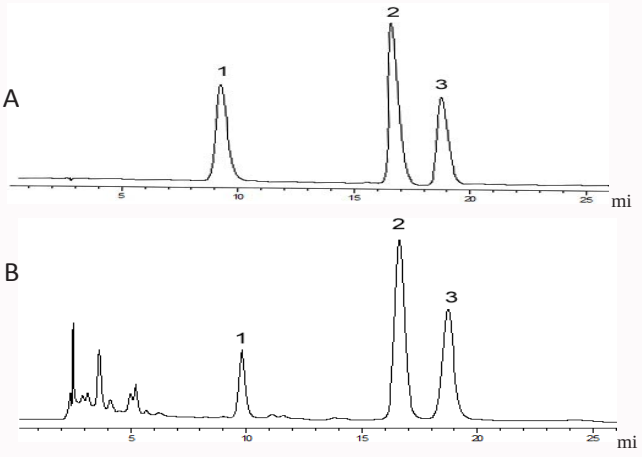

Figure 2: HPLC chromatogram of mixed reference substances (A) and the Bawanghua hydrolyzed sample (B). 1: quercetin, 2: kaempferol, 3: isorhamnetin.

The methanol stock solution containing three analytes was diluted to a series of appropriate concentrations with the same solvent, and an aliquot of the diluted solutions were injected into HPLC for analysis. The limits of detection (LOD) and quantification (LOQ) under the present chromatographic conditions were determined at a signal-to-noise ratio (S/N) of about 3 and 10, respectively, by comparing measured signals from samples with known low concentrations of analyte with those of blank samples.

The precision of the developed method was evaluated by measurements of intra- and inter-day variability. For intra-day repeatability test, the mixed flavonoid aglycones solution was analyzed for six replicates within one day, while for inter-day repeatability test, the mixed flavonoid aglycones solution was analyzed in duplicates for three consecutive days. The relative standard deviation (RSD) was used to evaluate precision.

Accuracy was evaluated with recovery test. The recovery test was performed by adding known amount of individual flavonoid aglycone into a certain amount of sample 14. Three replicates were performed for the test. The mixture was hydrolyzed and analyzed using the method mentioned in Sections 2.2 and 2.3. The recovery was calculated as follows: Recovery (%) = [(amount found-amount original)/amount spiked] × 100%. The accuracy was evaluated by calculating the mean recoveries. The sample 14 was hydrolyzed and extracted as described in Section 2.2. The sample solution was analyzed in duplicates for three consecutive days. The peak areas of the three flavonoid aglycones were recorded, and the RSDs of peak areas at different times were calculated to evaluate the sample stability.

Table 2: Results of orthogonal experiment.

Experiment No.	Factor			Sum of peak area
	A Temperature (°C)	B Hydrochloric acid concentration (mol·L-1)	C Time (h)	
1	70	4	3	1815.6
2	70	5	4	1988.9
3	70	6	5	1821.9
4	80	4	4	1958.2
5	80	5	5	1717.6
6	80	6	3	1958.5
7	90	4	5	1965.6
8	90	5	3	2038.4
9	90	6	4	1409.4
K1	1875.5	1913.1	1942.0	
K2	1878.1	1919.5	1785.5	
K3	1809.0	1729.9	1835.0	
R	69.1	189.6	156.5	

Table 3: Calibration curves and linear ranges of the investigated flavonoid aglycones.

Compounds	Regression equation	R^2	Linear range(μg/mL)
Quercetin	A = 76271C - 108.34	0.99994	2.83-45.33
Kaempferol	A = 76040C - 160.87	0.99995	4.38-70.00
Isorhamnetin	A = 50849C - 126.49	0.99997	4.21-67.33

Sample 14 was used for reproducibility text. Six parallel tests were conducted under the optimal extraction and hydrolysis conditions and the developed HPLC method as described in Sections 2.2 and 2.3.

Results and Discussion

Optimization of HPLC conditions

The flavonoid aglycones, quercetin, kaempferol and isorhamnetin, have been reported in many herbs. It was found that the good separation of the three analytes will be achieved when using methanol - water (containing acid) in a range of 50:50 ~ 60:40 (v/v) as mobile phase [21,22]. Different mobile phase's and elution programs were tested in order to obtain good resolution. Finally, the three flavonoid aglycones were eluted with baseline separation in 20 min on an Agilent SB-C18 column (250mm × 4.6mm, 4μm) by using methanol-water (containing 0.4% phosphoric acid, w/v) (54:46, v/v) (Figure 2). The detailed chromatographic condition is described in section 2.2.

Study on hydrolysis conditions of samples

Acid concentration, hydrolysis duration and temperature are the major factors affecting the hydrolysis of flavonoid glycosides [23]. The experiments using a L9 orthogonal array were implemented to inspect the effects of hydrochloric acid concentration, duration, temperature on hydrolysis of flavonoid glycosides of Bawanghua. The results of the orthogonal experiment are shown in Table 2. The value of R indicated the significance of the factors. The larger the value of R, the more influence the factor had on the hydrolysis of flavonoid glycosides of Bawanghua. The increasing order $R_A < R_C < R_B$ demonstrated that the levels of significance of factors were as follows: hydrolysis temperature<hydrolysis duration <hydrochloric acid concentration. The value R_B was the largest among all these ranges which indicated that the concentration of hydrochloric acid was the most significant factor affecting on the hydrolysis of flavonoid glycosides of Bawanghua. It can be seen from the test results, when the temperature is low, prolonging the hydrolysis time and increasing the hydrochloric acid concentration will improve the hydrolysis efficiency. However, when the temperature is high, prolonging the time and increasing the hydrochloric acid concentration will reduce the hydrolysis efficiency due to that the decomposition of the flavonoid aglycones might occur in such high temperature. It is determined that the optimal acid hydrolysis conditions are A2B2C2: the concentration of hydrochloric acid is 5mol/L, the temperature is 80°C and the hydrolyzing duration for 3 hours. Thus, the optimized hydrolysis took place with 1mol/L (4ml of 5mol/L hydrochloric acid diluted with 16ml of methanol) at 80°C.

Methodological study

The developed method was validated in terms of calibration curve, sensitivity, precision, accuracy, reproducibility and stability.

The linearity, regression and linear ranges of the investigated three flavonoid aglycones were summarized in Table 3. The determined coefficient (R^2> 0.9999) values indicated good correlations between the concentrations and peak areas of investigated flavonoid aglycones within the tested ranges.

The LODs of the three flavonoid aglycones was 0.57, 0.85 and 0.88 ng, respectively, while their LOQs were 2.27, 3.50 and 3.74 ng, respectively, indicating the method is very sensitive for the determination of the three investigated flavonoid aglycones

The RSDs of intra-day precision of the three flavonoid aglycones were 0.36%, 0.37% and 0.37% respectively. The RSDs of intra-day precision of the three flavonoid aglycones was 0.79%, 0.53% and 0.41% respectively. Both RSDs of intra-day and inter-day of all analytes are less than 1.0%, indicating that the method is precise.

In reproducibility test, the RSD (n = 6) of the three flavonoid aglycones were 1.52%, 1.82% and 2.07%, respectively, indicating that the developed method has a good reproducibility.

Six parallel tests using sample 14 were conducted under the optimal extraction and hydrolysis conditions and the developed HPLC method. The RSDs of the content of the three flavonoid aglycones were 1.52%, 1.82% and 2.07%, respectively, indicating that

Table 4: Three flavonoid aglycones content after hydrolysis of fifteen batches Bawanghua samples.

No.	Source	flavonoid aglycones content(mg·g-1)			
		Quercetin	Kaempferol	Isorhamnetin	Total
1	Zhaoqing Qixingyan, Guangdong (wild)	0.34	2.19	0.93	2.92
2	Zhaoqing Jinli, Guangdong (home planting)	0.24	1.52	0.79	2.52
3	Zhaoqing, Guangdong	0.49	1.57	1.76	3.84
4	Shenzhen, Guangdong	0.42	1.78	1.46	3.66
5	Tangxia, Guangdong	0.41	1.31	1.47	3.19
6	Maoming, Guangdong	0.43	1.37	1.46	3.26
7	Guangzhou, Guangdong	0.47	1.81	1.69	3.97
8	Jiangmeng, Guangdong	0.45	1.60	1.54	3.59
9	Huizhou, Guangdong	0.39	1.59	1.40	3.38
10	Shaoguan, Guangdong	0.52	1.60	1.78	3.90
11	Foshan, Guangdong	0.36	1.52	1.02	2.90
12	Heyuan, Guangxi	0.46	1.37	1.48	3.31
13	Hezhou, Guangxi	0.28	1.27	0.82	2.37
14	Nanning, Guangxi	0.51	2.17	2.19	4.87
15	Macau, SAR	0.59	1.95	1.98	4.52

the reproducibility of the method was good.

The results of stability test shows the variation of the three flavonoid aglycones was 3.49%, 1.98% and 3.60% respectively, indicating that the sample solutions were stable at room temperature for 3 days.

The spike recoveries of the three flavonoid aglycones were 96.93%, 105.22% and 100.22% respectively, with RSDs of 1.52%, 1.82% and 1.07% respectively, suggesting the accuracy of the method is acceptable for quality control.

Sample determination

The established method was applied for the quantification of three flavonoid aglycones (quercetin, kaempferol and isohamnetin) in the hydrochloric acid hydrolytes of 15 Bawanghua samples from different locations. The results are shown in Table 4.

Discussion

Flavonoid glycosides are the main component of many kinds of herbs, such as *Ginkgo biloba*. The aglycones of *Ginkgo biloba* flavonoid glycosides are mainly quercetin, kaempferol and isorhamnetin. In the Chinese Pharmacopoeia (2015version), ginkgo leaf is quality controlled by testing three aglycones content through acid hydrolysis, and then converted to total flavonoid glycosides content [24]. For the sample preparation method in Chinese Pharmacopoeia, ginkgo leaf is defatted by chloroform using Soxhlet extraction method, then extracted by methanol, and finally hydrolyzed into aglycones by acid. The sample preparation method including three step processes take 6.5 hours in total [24].

Compared with the determination method of total flavonoid glycosides in *Ginkgo biloba* leaves, this experiment waived the defatted step and combined the extraction and hydrolysissteps by directly adding the methanol and hydrochloric acid solution to the sample powder and hydrolyzing at 80°C for 3 hours. The whole operation not only saves sample preparation time by one half, but also can reduce the error caused by the process of extraction, drying and so on.

Among the fifteen samples, the total content of the three hydrolyzed flavonoid aglycones in thirteen samples is in a range of 2.37 - 4.87mg/g, and most of them are in the range of 2.9-4.0mg/g. It shows that the total content of the three hydrolyzed aglycones can be used as one of the quantitative indicators of quality control. The research shows that this method is simple, convenient, accurate and reproducible, and its result is accurate and reliable, which provides a reference for evaluating the quality of the flower of *Hylocereus undatus* (Bawanghua), medicinal plants in southern China.

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