Simultaneous determination of eight main flavonoids in *Flos lonicerae* by high performance liquid chromatography

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HPLC法同时测定金银花中 8种黄酮的含量

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摘要: 目的 建立同时测定金银花不同种(忍冬、山银花、红腺忍冬、灰毡毛忍冬、细毡毛忍冬)中芦丁、金丝桃苷、木犀草素 7-*O*-β-*D*半乳糖苷、忍冬苷、苜蓿苷、金圣草素-7-*O*新橙皮糖苷、苜蓿素-7-*O*新橙皮糖苷、槲皮素 8种黄酮的 HPLC分析方法。方法 色谱柱为 Aglient Zorbax 80A, Extend-C_{18} (250 mm × 4.6 mm ID, 5 μm)。梯度洗脱,流动相 A为 1.2% THF, 0.5% HAc; B为甲醇-乙腈(40∶60)。流速 1.0 mL·min^{-1},检测波长 355 nm。结果 线性范围内 8个黄酮化合物的标准曲线呈良好的线性关系,平均加样回收率均在 97% - 102%。所有化合物的精密度和重复性的 RSD均 <3%,定量限(*S*/*N* =10)低于 6.0 ng。结论 该分析方法简单、灵敏、准确、重复性好,可同时分析金银花中的黄酮类成分。

关键词: HPLC; 黄酮; 金银花; 定量分析

Flos loniceme (Chinese name Jinyinhua), derived from the dried flower buds of *Lonicem* species (Caprifoliaceae), is a commonly used traditional Chinese medicinal (TCM) herb with activities of removing heat and toxic substance for the treatment of acute fever and epidemic disease in TCM practice[1]. Extensive phytochemical and pharmacological studies show that chlorogenic acid and its analogues, flavonoids, iridoid glucosides, and triterpenoidal saponins in *Flos loniceme* have biological activities contributing to the traditional efficacy of *Flos loniceme*. For instance, some flavonoids have been clarified to possess activities of anti-inflammation[2].

An HPLC method has previously been described for the quantification of the organic acids (including chlorogenic acid) in *Flos loniceme*[3]. In the present paper, an HPLC method was developed for the simultaneous analysis of eight flavonoids, namely rutin, hyperoside, luteolin-7-*O*-β-*D*-galactoside (LG), lonicerin, tricin-7-*O*-β-*D*-glucopyranoside (TGP), chrysoeirol-7-*O*-neohesperidoside (CN), tricin-7-*O*-neohesperidoside (TN) and quercetin in *Flos loniceme*.

Materials and methods

Chemicals and plant materials The HPLC grade methanol was purchased from Hanbang Science & Technology (Nanjing, China), acetonitrile from Merck (Darmstadt, Germany) and the deionized water from Robust (Guangzhou, China). Other solvents

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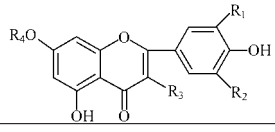
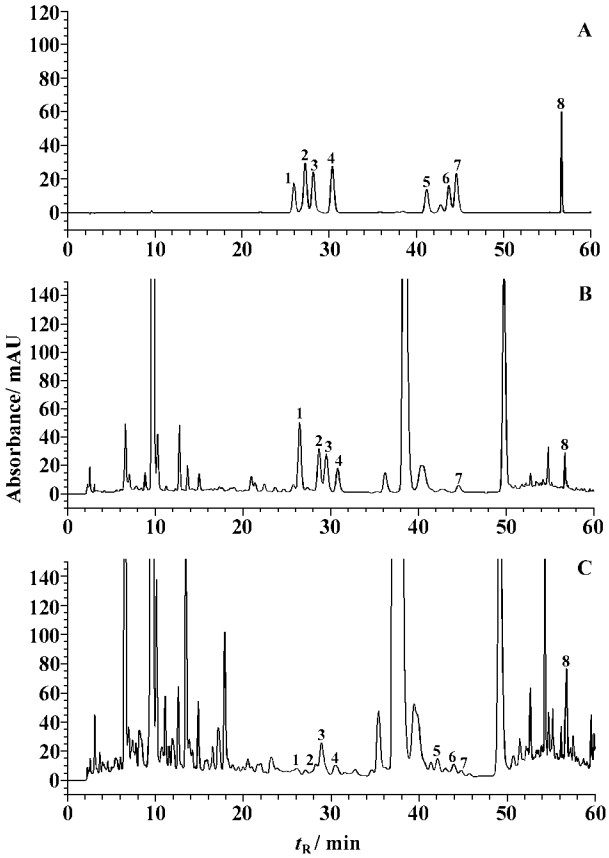
were of analytical grade, and were purchased from Nanjing Chemical Factory (Nanjing, China). The eight reference flavonoids were isolated and purified from *Flos loniceme* in our laboratory. Their structures were elucidated by comparing their chemical and spectroscopic (IR, UV, ^{1}H NMR, ^{13}C NMR) data with those in references[4,5] (Table 1). Standards **1 - 8** have purities above 98% except standard **5**. This is evident in Figure 1.

Table 1　Structures of eight flavonoids



	R_1	R_2	R_3	R_4
Rutin	OH	H	Glc(6-1)rha	H
Hyperoside	OH	H	Gal	H
LG	OH	H	H	Gal
Lonicerin	OH	H	H	Glc(6-1)rha
TGP	OMe	OMe	H	Glc
CN	OMe	H	H	Glc(2-1)rha
TN	OMe	OMe	H	Glc(2-1)rha
Quercetin	OH	H	OH	H



1: Rutin; 2: Hyperoside; 3: LG; 4: Lonicerin; 5: TGP; 6: CN; 7: TN; 8: Quercetin. A: Mixed standards; B: *L. japonica*; C: *L. confusa*

Figure 1　HPLC chromatograms of mixed standards and methanol extracts of different *Flos loniceme* samples

Five *Flos loniceme* samples derived from *L. japonica* (Fengqiu, Henan Province), *L. macranthoieds* (Longhui, Hunan Province), *L. similes* (Longhui, Hunan Province), *L. hypoglauca* (Jiujiang, Jiangxi Province) and *L. confusa* (Xupu, Hunan Province) respectively, were collected in China and were authenticated by Dr. LI Ping. The voucher specimens were deposited at the Department of Pharmacognosy, China Pharmaceutical University.

Apparatus and conditions　An Agilent 1100 series HPLC system was used. Chromatography was carried out on an Agilent Zorbax 80A, Extend-C_{18} column (250 mm × 4.6 mm ID, 5 μm). The column temperature was set at 30 ℃. The gradient elution profile was used to obtain good resolution for all analytes. Optimum separation was obtained using a linear gradient elution of the mobile phase A: B starting from 10% to 20% B (0 - 12 min), then isocratic at 20% B (12 - 45 min), and 20% to 58% B (45 - 60 min). A: deionized water containing 1.2% tetrahydrofuran and 0.5% HAc; B: mixture of 40% methanol and 60% acetonitrile. The flow rate was 1.0 mL·min^{-1}. The detection wavelength was set at 355 nm.

Results

1　Calibration curves

Methanol stock solution containing eight flavonoids —— rutin (51.5 μg·mL^{-1}), hyperoside (48.0 μg·mL^{-1}), LG (53.5 μg·mL^{-1}), lonicerin (51.5 μg·mL^{-1}), TGP (41.5 μg·mL^{-1}), CN (41.2 μg·mL^{-1}), TN (59.7 μg·mL^{-1}) and quercetin (22.3 μg·mL^{-1}) was prepared. 0.1, 0.5, 1, 5, 10 and 15 μL of the stock solution were accurately injected for analysis. Each volume was analyzed in triplicate. It was found that the calibration curves of all eight flavonoids were linear with the regression coefficients of 0.999 7 - 1.000 0 in the testing ranges (Table 2). The limits of quantification (S/N = 10) were less than 6.0 ng for all analytes.

2　Precision, repeatability and recovery

The precision was determined by analyzing the same mixed standard solution for three times. The relative standard deviation (RSD) values of peak area of each compound were calculated. The RSDs (n = 5) were as follows: rutin 1.19%, hyperoside 0.38%, LG 1.96%, lonicerin 1.72%, TGP 1.37%, CN 0.32%, TN 0.27% and quercetin 1.67%.

In order to test the repeatability, five sample

solutions of *L. japonica* were prepared. Each solution was injected twice. The contents of rutin, hyperoside, LG, lonicerin, TN and quercetin were calculated and the RSDs were 1.5%, 2.4%, 1.2%, 0.8%, 0.5% and 2.2%, respectively.

Table 2 Calibration curves for eight flavonoids concerned

Analyte	Calibration curve	*r*	Linear range /ng	LOQ /ng
Rutin	$Y=1\ 494.5X-0.251\ 8$	0.999 8	7.3 - 772.5	5.2
Hyperoside	$Y=3\ 000.4X-0.431\ 2$	0.999 9	7.2 - 720.0	3.6
LG	$Y=2\ 199.0X-0.765\ 1$	0.999 9	5.4 - 802.5	5.4
Lonicerin	$Y=2\ 609.4X+0.154\ 1$	1.000 0	5.2 - 772.5	5.2
TGP	$Y=1\ 758.7X-0.498\ 8$	0.999 9	4.2 - 623.1	4.4
CN	$Y=1\ 918.8X-1.476\ 1$	0.999 9	4.1 - 618.5	4.5
TN	$Y=2\ 105.3X-1.927\ 7$	0.999 7	5.9 - 889.1	5.9
Quercetin	$Y=4\ 029.1X-0.819\ 1$	1.000 0	2.2 - 333.8	1.1

Y: Peak area count; *X*: Contents of reference compound

Recovery experiment was carried out to evaluate the accuracy of the method. Known quantities of eight flavonoids were added to the five samples of *L. japonica* powder (0.5 g). Each solution was injected twice. The content of each flavonoid was determined by the corresponding calibration curve, while the content of each spiked flavonoid was calculated by subtracting the detected amount of the corresponding flavonoid present in the original sample powder from the total content. Consequently, the average recoveries of the eight compounds were all between 97% - 102% (Table 3).

3 Analysis of eight flavonoids in the *Flos lonicerae* samples

A set amount of powders of *Flos Loniceme* samples (1.0 g) were refluxed with petroleum ether in Soxhlet extractor at 85 ℃ for 3 h to defat, then exhaustively extracted with methanol in Soxhlet extractor at 90 ℃ for 6 h. The methanol extracts were evaporated to dryness, and the residue was dissolved in methanol and transferred to volumetric flasks and diluted to volume with methanol. The solutions were filtered through a 0.45 μm Millipore filter, 5 μL of the filtrates was injected into HPLC for analysis.

Optimized chromatographic conditions were achieved after several trials with methanol, acetonitrile, tetrahydrofuran, acetic acid and water in different proportions as mobile phase. As shown in Figure 1 (A), all eight reference flavonoids **1 - 8** were eluted with highly symmetrical peaks under the optimized chromatographic conditions. The representative chromatograms of different *Flos loniceme* samples were shown in Figure 1 (B) - (C). It was found that there were differences among the overall HPLC profiles of *L. japonica* and *L. confusa*.

Table 3 Recoveries of eight flavonoids ($n=5$, $\bar{x}\pm s$)

Compound	Contained /μg	Added /μg	Found /μg	Recovery /%
Rutin	473 ±9	453.1	917 ±10	98.2 ±2.3
Hyperoside	49.0 ±1.3	47.3	95 ±3	97.4 ±1.8
LG	200 ±4	198.2	396 ±5	99.1 ±0.9
Lonicerin	101.5 ±2.3	98.5	200.5 ±2.6	100.5 ±1.2
TGP	b	87.6	87.2 ±1.3	99.6 ±0.7
CN	b	84.3	83.1 ±2.7	100.8 ±1.1
TN	10.6 ±1.2	13.6	23.9 ±1.1	98.7 ±1.5
Quercetin	45.3 ±2.0	42.1	87.8 ±2.4	101.2 ±2.1

b: Undetectable

Different contents of eight flavonoids analyzed in five *Flos loniceme* samples were also found (Table 4). For example, rutin was found as the main flavonoid determined in *L. japonica* and *L. confusa*, but as minor compound in *L. hypoglauca*, or even undetectable in *L. similis*. On the other hand, *L. japonica* contains relatively high levels ($>100\ \mu g\cdot g^{-1}$) of rutin, LG and lonicerin, while *L. confusa* was found to contain relatively high levels of only rutin and luteolin-7-*O*-β-*D*-galactoside, and *L. hypoglauca*, *L. macmnthoieds* and *L. similes* contain only luteolin-7-*O*-β-*D*-galactoside as major flavonoid concerned.

Table 4 Contents of flavonoids in different *Flos lonicerae* samples ($\mu g\cdot g^{-1}$, $n=3$)

Flavonoid	*L. japonica*	*L. confusa*	*L. hypoglauca*	*L. macmnthoieds*	*L. similis*
Rutin	951 ±8	111 ±8	a	81 ±3	b
Hyperoside	88 ±5	44 ±4	14.5 ±0.5	26.1 ±1.2	12.7 ±1.1
LG	408 ±10	116 ±6	106 ±7	116 ±10	165 ±6
Lonicerin	205 ±6	20.9 ±1.8	b	46.9 ±2.3	25.1 ±1.5
TGP	b	65 ±5	65 ±5	44.6 ±2.3	66 ±3
CN	b	93.5 ±1.8	b	b	b
TN	21.2 ±2.5	60 ±4	c	b	b
Quercetin	93.1 ±1.7	59.9 ±1.5	26.9 ±2.2	c	c

a: Trace; b: Undetectable; c: Not detected

Discussion

In conclusion, a simple, sensitive, precise and reproducible HPLC method was developed for simultaneous analysis of flavonoids in *Flos loniceme*. In addition, this method can be used as a strategy to evaluate the quality of *Flos loniceme* derived from the flower buds of *L. japonica*, *L. hypoglauca*, *L. confusa*, *L. macranthoieds* and *L. similis*.

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