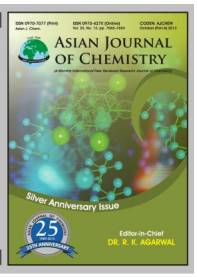
Asian Journal of Chemistry; Vol. 25, No. 16 (2013), 9321-9324

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2013.15500



Development and Validation of High-Performance Liquid Chromatography for Simultaneous Determination of Seven Bioactive Compounds in *Flos Lonicerae japonicae*

HAO CAI[1,2,3,*], GANG CAO[2,3], SUCAI LUO[2], XIAO LIU[1,3], ZHIWEI XU[2] and BAOCHANG CAI[1,2,3]

[1]College of Pharmacy, Nanjing University of Chinese Medicine, Nanjing, P.R. China
[2]Research Center of TCM Processing Technology, Zhejiang Chinese Medical University, Hangzhou, P.R. China
[3]National First-Class Key Discipline for Science of Chinese Materia Medica, Nanjing University of Chinese Medicine, Nanjing, P.R. China

*Corresponding author: Tel/Fax: +86 25 86798281; E-mail: haocai_98@126.com

(*Received*: 18 March 2013; *Accepted*: 23 September 2013) AJC-14169

A selective high-performance liquid chromatographic method for simultaneous determination of seven bioactive compounds in *Flos Lonicerae japonicae* was developed and validated. In this paper, the seven components were separated on a Waters Symmetry C_{18} column (250 mm × 4.6 mm, 5 μm) and detected by a diode array detector. The mobile phase was composed of (A) aqueous formic acid (0.5 %, v/v) and (B) acetonitrile using a gradient elution. Analytes were performed at 30 ºC with a flow rate of 1 mL min^{-1} and the detection wavelength was set at 238 nm. All calibration curves showed good linear regression ($r^2 \geq 0.9991$) within tested ranges. Overall intra-day and inter-day variations (RSDs) were less than 2.13 % and the average recoveries of seven analytes varied from 97.41 to 103.10 % with RSDs ranging from 0.87 to 2.81 %. The proposed method would be sensitive enough and reliable for comprehensive quality control of *Flos Lonicerae japonicae* and its related processed drugs or preparations.

Key Words: *Flos Lonicerae japonicae*, Bioactive compounds, Quantitative analysis, Quality control and evaluation.

INTRODUCTION

Flos Lonicerae japonicae, named as Jinyinhua in Chinese, is derived from the dried flower buds of *Lonicera japonica* Thunb. and is one of the oldest and most frequently used Chinese medicinal herbs for oriental medicine in China[1]. In the last decades, *Flos Lonicerae japonicae* has been extensively investigated in phytochemistry and the results showed that organic acids, flavonoids, essential oils, iridoid glycosides, saponins, triterpenes and inorganic elements are the main active components in *Flos Lonicerae japonicae*[2-5]. Pharmacological studies and clinical practices have demonstrated that *Flos Lonicerae japonicae* possesses various biological and pharmacological activities, including antiinflammatory, antiviral, antibacterial, antioxidant, antipyretic, hypoglycemic, immunological enhancement, hepato-protective effects and inhibition of the formation of biofilm involved in the antivirus process and cholagogue activity for increasing secretion of bile[6-11]. These reports indicate that these potentially bioactive compounds may be responsible for the various biological activities of *Flos Lonicerae japonicae*. In this study, A selective high-performance liquid chromatography method for simultaneous determination of seven bioactive compounds in *Flos Lonicerae japonicae* was developed and validated. The proposed method would be reliable for comprehensive quality control of *Flos Lonicerae japonicae* and its related processed drugs or preparations.

EXPERIMENTAL

Flos Lonicerae japonicae samples were collected from seven suppliers in Henan, Hunan, Shandong, Shanxi, Zhejiang, Anhui and Hebei provinces of China and identified by Prof. Jianwei Chen in Nanjing University of Chinese Medicine. Reference compounds of chlorogenic acid, caffeic acid and rutin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) and reference compounds of isoquercitrin, galuteolin, isochlorogenic acid A and sweroside were purchased from the Shanghai Yousi Bio-technology Co., Ltd. (Shanghai, China). The purity of each reference compound was greater than 98 % determined by HPLC analysis. The chemical structures of these seven compounds are shown in Fig. 1. HPLC grade acetonitrile from TEDIA Company Inc. (Fair-field, USA) and all chemical reagents with high grade were obtained from others. Ultrapure water was prepared using Milli-Q SP system (Millipore, Bedford, MA, USA).

Chromatographic conditions: Analyses were performed using Varian 920-LC HPLC system, including Prostar 240 quatpump, Prostar 410 automatic sampler, Prostar

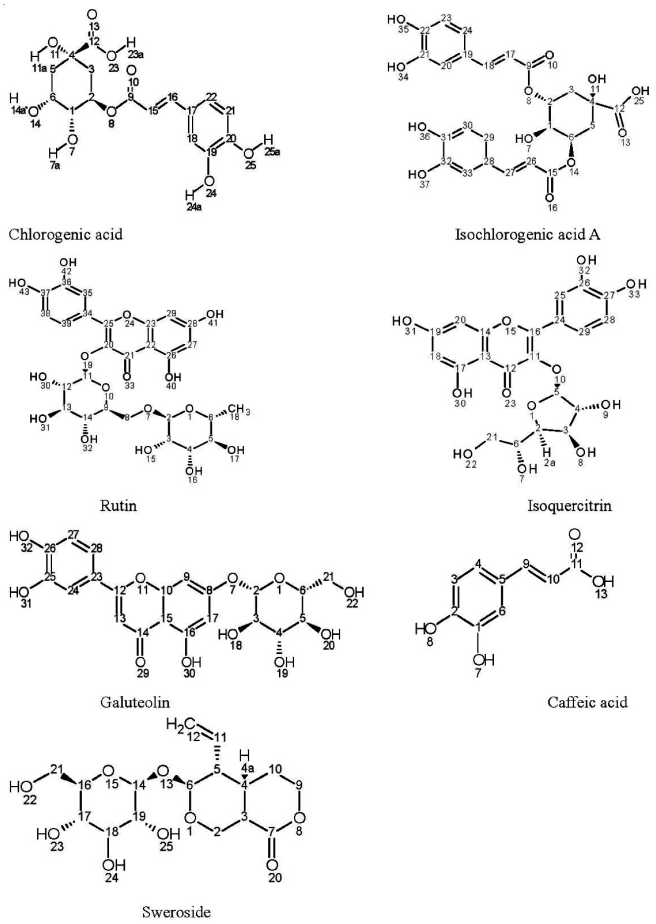


Fig. 1. Chemical structures of seven active compounds in *Flos Lonicerae japonicae*

335 DAD, Galaxie Chemstation station (USA Varian). The detection wavelength was set at 238 nm. A Waters Symmetry C_{18} column (250 mm × 4.6 mm, 5 μm) was used with a flow rate of 1 mL min^{-1}. The injection volume was 10 μL and the column temperature was maintained at 30 ºC. The mobile phase was composed of (A) aqueous formic acid (0.5 %, v/v) and (B) acetonitrile using a gradient elution of 95-88 % A at 0-25 min, 88-88 % A at 25-30 min, 88-82 % A at 30-42 min, 82-80 % A at 42-70 min.

Preparation of sample solutions: The powder of *Flos Lonicerae japonicae* sample, was precisely weighed (1 g) and extracted with 50 mL of 50 % ethanol in an ultrasonic bath for 1 h and cooled at room temperature, then 50 % ethanol was added to compensate for the lost weight. The sample solution was centrifuged for 10 min and the supernatant was filtered through a 0.45 μm filter membrane before subjecting 10 μL to the HPLC system for analysis.

Preparation of standard solutions: The reference compounds of chlorogenic acid, caffeic acid, rutin, isoquercitrin, galuteolin, isochlorogenic acid A and sweroside were dried and accurately weighed and then dissolved with methanol to produce each stock standard solution, respectively. A mixed stock standard solution of reference compounds, containing the concentrations of chlorogenic acid (2.89 mg mL^{-1}), caffeic acid (2.76 mg mL^{-1}), rutin (1.016 mg mL^{-1}), isoquercitrin (2.35 mg mL^{-1}), galuteolin (2.10 mg mL^{-1}), isochlorogenic acid A (12.7 mg mL^{-1}) and sweroside (3.07 mg mL^{-1}), was finally prepared. The working standard solutions were prepared by diluting the mixed stock standard solution with methanol to give seven different concentrations for calibration curves and were filtered through the 0.45 μm filter membranes prior to injection. All calibration curves were constructed from peak areas of the reference compounds versus their concentrations and all solutions were stored in a refrigerator under 4 ºC before use.

RESULTS AND DISCUSSION

Calibration curves: Linear regression analysis for seven reference compounds was performed by external standard method. A series of the working standard solutions containing seven reference compounds in seven different concentrations were freshly prepared with methanol, respectively and an aliquot (10 μL) of each working standard solution was subjected to HPLC analysis with three replicates for determining linear ranges of seven analytes. The regression equations were calculated in the form of Y = aX + b, where Y and X were the concentration of each reference compound and value of the peak area, respectively. The calibration curves of all analytes showed good linearity ($r^2 \geq 0.9991$). The results were given in Table-1.

Precision, repeatability and stability: The injection precision was determined by replicated injection of the same working standard solution containing seven reference compounds six times in 1 day. The relative standard deviations (RSDs) of the peak areas of seven reference compounds were all lower than 2 %.

The intra- and inter-day precisions of the developed method were determined by analyzing the working standard solutions containing seven reference compounds at low, medium and high concentrations with six replicates in a single day and on three consecutive days, respectively. The RSDs of the peak areas of seven reference compounds in overall intra- and inter-day variations were less than 2.13 %.

To further evaluate the repeatability of the developed assay, six independent sample solutions prepared from the same

TABLE-1
STANDARD CURVE EQUATIONS OF SEVEN REFERENCE COMPOUNDS

Reference compound	Regression equation	r^2	Linear range (mg mL^{-1})
Chlorogenic acid	Y = 13729X + 170.16	0.9995	1.0119-0.0316
Caffeic acid	Y = 30446X + 78.219	0.9997	0.276-0.00862
Sweroside	Y = 11739X + 54.674	0.9993	0.5219-0.0163
Rutin	Y = 9851X + 8.3494	0.9994	0.0508-0.00158
Isoquercitrin	Y = 13175X + 0.987	0.9998	0.03525-0.0001
Galuteolin	Y = 9425.4X + 0.9363	0.9994	0.021-0.0006563
Isochlorogenic acid A	Y = 14207X + 341.8	0.9991	1.016-0.03175

source of *Flos Lonicerae japonicae* were analyzed. The contents of seven analytes in *Flos Lonicerae japonicae* were calculated from the corresponding calibration curves. The RSDs of the contents of seven analytes were all less than 2.45 %. The stability of the solution was assessed by analyzing one of the above *Flos Lonicerae japonicae* sample solutions at 0, 2, 4, 8, 10, 12 and 24 h at room temperature. The RSDs of the contents of seven analytes were all less than 2.47 %. The results suggested that the sample solution was stable and feasible to be analyzed within 1 day.

Accuracy: Recovery tests were used to evaluate the accuracy of the developed method. Six powders of an appropriate amount (0.5 g) from one source of *Flos Lonicerae japonicae* were accurately weighed, respectively and each spiked with the known amount (equivalent to the concentration contained in 0.5 g of the sample powder) of each reference compound. The resultant samples were then extracted and analyzed with the above described method of preparation of sample solutions. The average recoveries of seven analytes varied from 97.41 to 103.10 % with RSDs ranging from 0.87 to 2.81 %, which demonstrated the reliability and accuracy for the measurement of these bioactive compounds.

Sample analysis: The developed quantitative analysis method was applied to the simultaneous determination of seven bioactive compounds in seven batches of *Flos Lonicerae japonicae* samples collected from different provinces in China. Each sample was analyzed in triplicate and the peaks in chromatograms were identified by comparing the retention times and UV spectra with those of the reference compounds. Fig. 2 showed the typical separations of the mixture of the reference compounds and the extract of *Flos Lonicerae japonicae* sample obtained under above given optimized HPLC conditions. The contents were calculated and summarized in Table-2. The quantitative analysis results revealed that there were significant differences existed in individual and total contents of seven bioactive compounds from different suppliers, which might be due to the differences of growing conditions (soils and climates), postharvest processing, manufacturing or storage in each region. Therefore, it is necessary to find and establish the good agricultural practice bases which are suitable for the cultivation of *Flos Lonicerae japonicae* and the maintenance of its main active components.

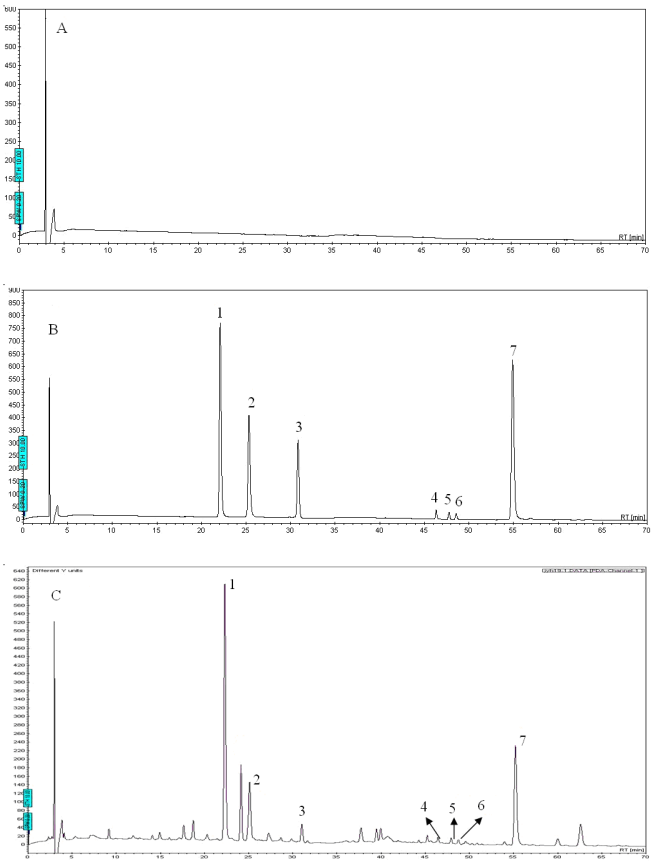


Fig. 2. Representative chromatograms of simultaneous determination of seven constituents in *Flos Lonicerae japonicae* (A. Blank solution; B. Mixed solution of reference compounds: 1 chlorogenic acid, 2 caffeic acid, 3 sweroside, 4 rutin, 5 isoquercitrin, 6 galuteolin, 7 isochlorogenic acid A; C. Sample solution)

Conclusion

Compared with the reported analysis methods of *Flos Lonicerae japonicae*, this novel and newly established HPLC method provided much higher specificity, sensitivity, precision and accuracy for simultaneous quantification of seven bioactive compounds in *Flos Lonicerae japonicae* and can be used for intrinsic quality control of *Flos Lonicerae japonicae* from different sources and suppliers in China. The present study can also be further applied for comprehensive quality evaluation of *Flos Lonicerae japonicae* and its related herbal products in Chinese Pharmacopoeia.

ACKNOWLEDGEMENTS

The authors are grateful for the financial supports of the National Natural Science Foundation of China (No. 81173546, No. 30940093 and No. 81202918), the Natural Science Foundation of Jiangsu Province, China (No. BK2009495), the International Science and Technology Cooperation Project of Jiangsu Province, China (No. BZ2011053), the Project of Science Technology Department of Zhejiang Province, China (No. 2012D60SA1C0065 and No. 2012D60SA1C0066), the Open Project of National First-Class Key Discipline for Science of

TABLE-2
RESULTS OF DETERMINATION OF SEVEN CONSTITUENTS IN *Flos Lonicerae japonicae* (mg g^{-1})

Source	Chlorogenic acid	Caffeic acid	Sweroside	Rutin	Isoquercitrin	Galuteolin	Isochlorogenic acid A
Henan	21.88	2.11	2.96	0.00	0.27	0.09	13.78
Hunan	44.31	3.58	4.66	0.00	0.46	0.16	48.82
Shandong	21.59	9.39	8.81	2.44	0.44	0.81	46.43
Shanxi	12.57	4.79	7.45	0.93	0.21	0.26	45.05
Zhejiang	31.35	6.04	21.25	0.94	0.42	0.39	51.13
Anhui	47.47	13.41	27.05	2.20	0.29	0.82	46.17
Hebei	29.77	2.59	12.43	1.69	0.48	0.49	43.05

Chinese Materia Medica, Nanjing University of Chinese Medicine (No. 2011ZYX2-006 and No. 2011ZYX2-001), the Chinese Medicine Research Program of Zhejiang Province, China (No. 2008ZA002), the Science Foundation of Zhejiang Chinese Medical University (No. 2011ZY25 and No. 7211093), the Project of Science and Technology for Chinese Medicine of Zhejiang Province, China (No. 2013KYB183) and the Fund of Zhejiang Modernization of Traditional Chinese Medicine Item (No. [2008]436 and No. [2012]680).

REFERENCES

1. X. Chen, Y. Xia, Y. Lu and J. Liang, *J. Pharm. Biomed. Anal.*, **54**, 406 (2011).
2. X.G. Chen, L.H. Hu, X.Y. Su, L. Kong, M.L. Ye and H.F. Zou, *J. Pharm. Biomed. Anal.*, **40**, 559 (2006).
3. L.W. Qi, C.Y. Chen and P. Li, *Rapid Commun. Mass Spectrom.*, **23**, 3227 (2009).
4. Z.M. Qian, X.D. Wen, H.J. Li, Y. Liu, S.J. Qin and P. Li, *Biol. Pharm. Bull.*, **31**, 126 (2008).
5. M.T. Ren, J. Chen, Y. Song, L.S. Sheng, P. Li and L.W. Qi, *J. Pharm. Biomed. Anal.*, **48**, 1351 (2008).
6. Z.P. Ji, Y.Y. Zhu, W.P. Ni and X.D. Wu, *Chin. J. Med. Guide*, **11**, 82 (2009).
7. X. Shang, H. Pan, M. Li, X. Miao and H. Ding, *J. Ethnopharmacol.*, **138**, 1 (2011).
8. N. Ohno, E. Yoshigai, T. Okuyama, Y. Yamamoto, T. Okumura, K. Sato, Y. Ikeya and M. Nishizawa, *HOAJ Biol.*, **1**, 1 (2012).
9. O.H. Kang, J.G. Choi, J.H. Lee and D.Y. Kwon, *Molecules*, **15**, 385 (2010).
10. D. Tang, H.J. Li, J. Chen, C.W. Guo and P. Li, *J. Sep. Sci.*, **31**, 3519 (2008).
11. J.K. Jeong and J.S. Kim, *J. Med. Plants Res.*, **6**, 47 (2012).