TẠP CHÍ KHOA HỌC
TRƯỜNG ĐẠI HỌC QUY NHƠN

Xác định hàm lượng verbascosid trong củ Địa hoàng 19 bằng phương pháp sắc ký lỏng hiệu năng cao

Phạm Thanh Loan*

Viện Nghiên cứu Ứng dụng và Phát triển, Trường Đại học Hùng Vương, Phú Thọ, Việt Nam

Ngày nhận bài: 04/10/2022; Ngày sửa bài: 24/11/2022;
Ngày nhận đăng: 02/12/2022; Ngày xuất bản: 28/02/2023

TÓM TẮT

Nghiên cứu nhằm xây dựng quy trình định lượng verbascosid trong củ của giống Địa hoàng 19 bằng sắc ký lỏng hiệu năng cao (HPLC) để phục vụ công tác đánh giá chất lượng dược liệu. Kết quả đã lựa chọn được điều kiện sắc ký phù hợp là sử dụng cột gemini C18 (250 × 4,6 mm, 5 µm), detector UV 334 nm, pha động acetonitril - acid phosphoric 0,1%, tốc độ dòng 0,8 mL/phút. Diện tích pic và nồng độ verbascosid có tương quan tuyến tính chặt (r = 0,9997), dạng hàm Y = 2349X + 7259,6. Quy trình có độ đúng, độ lặp lại tốt với RSD < 2%. Quy trình này được áp dụng để định lượng verbascosid trong củ của giống Địa hoàng 19 trồng tại 2 tỉnh Vĩnh Phúc, Phú Thọ cho kết quả lần lượt là 0,027% và 0,028%.

Từ khóa: *Verbascosid, HPLC, định lượng, Địa hoàng 19.*

**Tác giả liên hệ chính.*
Email: Loandhhv@gmail.com

QUY NHON UNIVERSITY
JOURNAL OF SCIENCE

Determination of verbascoside in the root of *Rehmannia glutinosa* varieties 19 by high performance liquid chromatography

Thanh Loan Pham*

Institute of Applied Research and Development, Hung Vuong University, Vietnam

Received: 04/10/2022; Revised: 24/11/2022;
Accepted: 02/12/2022; Published: 28/02/2023

ABSTRACT

This study aimed to validate a procedure for the quantification of verbascoside in the root of *Rehmannia glutinosa* varieties 19 by high performance liquid chromatography (HPLC) to serve the assessment of medicinal quality. The results have selected suitable chromatographic conditions, using the gemini C18 column (250 × 4.6 mm, 5 µm), detector at 334 nm, mobile phase acetonitrile - phosphoric acid 0.1%, and the flow rate at 0.8 mL/min. Peak area and verbascoside concentration are strongly correlated (r = 0.9997), Y = 2349X + 7259.6. The procedure has good accuracy and repeatability with RSD < 2%. This procedure was applied to quantify verbascoside in the root of *Rehmannia glutinosa* varieties 19, which is grown in Vinh Phuc, Phu Tho provinces, and the results determination of verbascoside were 0.027% and 0.028%, respectively.

Keywords: *Verbascoside, HPLC, quantification, Rehmannia glutinosa varieties 19.*

1. INTRODUCTION

Most of the *Rehmannia glutinosa* materials used in Vietnam are imported from China, and the samples assigned to evaluate the quality of this medicinal plant are made on imported samples. *Rehmannia glutinosa varieties 19* has been recognized as a new variety and circulated in Vietnam from August, 2020.[1] Currently, there is no published evaluation of its quality in Vietnam. The active ingredient verbascoside is an important chemical component in the root of the *Rehmannia*, which is regulated by the Vietnam Pharmacopoeia V as a marker to test the quality of medicinal herbs.[2] Verbascoside has strong biological activities such as antibacterial, anti-inflammatory, and re-epithelialization[3-5] and has diuretic, antioxidant, wound healing, cell autoimmunity, and protective effects on the nervous system.[3,6,7] Active ingredient verbascoside is being researched and developed by pharmaceutical companies for medicinal ingredients and health foods. The research results contribute to the assessment of this variety of quality grown in Vietnam.

2. RESEARCH METHODS

2.1. Material

The research sample is tubers grown from the *R. glutinosa varieties 19* harvested at 2 locations: Bach Luu commune - Song Lo - Vinh Phuc (code DH2102) and Dan Quyen commune - Tam Nong - Phu Tho (code DH2104) in March 2021, processed according to the Vietnam Pharmacopoeia V in 2017, treatise *Rehmannia.*[5]

**Corresponding author.*
Email: Loandhhv@gmail.com

https://doi.org/10.52111/qnjs.2023.17107

The sample was created by Dr. Nguyen Van Huy, Center for Medicinal Materials, Institute of Applied Research and Development. The scientific name is *Rehmannia glutinosa*, the family of snout flowers (Scrophulariaceae). The specimen is kept at the Center for Medicinal Materials, Institute of Applied Research and Development, code VNC/DH192101.

2.2. Chemicals, raw materials

Standard substance verbascoside with a purity of 98.14% (lot number RFS-M01101910014) was purchased from Chengdu Herbpurify, China. Other chemicals included acetonitrile (Merck), phosphoric acid (Merck), methanol (Merck), and double-distilled water as standard for high-performance liquid chromatography (HPLC).

2.3. Appliances

Shimadzu HPLC meter, LC-20AD pump, SPD-20A UV Vis detector, SIL-20A automatic sample injection system, CTO-20A thermostat, Electronic analytical balance (Switzerland), and reflux extraction device were deployed.

2.4. Chromatographic conditions

Using a gemini column C18 (250 × 4.6 mm, 5 µm) and chromatographic conditions such as selection of detection wavelength, mobile phase composition, flow rate, and injection volume were referenced based on the previous studies.[2,8-10]

2.5. Standard solution

Standard verbascoside was dissolved in methanol to obtain a solution containing 1000 µg/mL.

2.6. Test solution

Accuratedly weighted 0.8 g of medicinal powder were dissovled in a flask containing 50 ml of methanol (MeOH). The soltion was then placed in a reflux extraction for 1.5 h for cooling. A 20 mL of the obtained filtrate was collected and recovered in the solvent under vacuum condition to nearly dry. The mobile phase then dissolved and transfered entirely so a 5 mL volumetric flask, made up to the mark with the mobile phase before being filtered through a 0.45 µm filter.

2.7. Quantitative process appraisal

Verification of the verbascoside quantification process, including criteria: relevance, specificity, repeatability, linear correlation, precision, the limit of detection (LOD) and limit of quantitation (LOQ) was accorded to the Guidelines No. 32/2018/TT-BYT of the Ministry of Health, Decision No. 07/2013/QD-QLD of the Drug Administration of Vietnam and refered to the regulations of the International Conference on Harmonisation, 2005 (ICH).[11-13]

2.7.1. Suitability

Standard verbascoside solution (concentration 80 µg/ml) and chromatography were prepared six times. The parameters of retention time (t_R), peak area (S_{peak}), mean value, and relative standard deviation (RSD) of S_{peak} were determined. If RSD < 2%, the system is highly relevant.[11-13]

2.7.2. Specificity

Specificity was tested by analyzing the blanks, standard verbascoside solutions, and test solutions. Blank samples shall not give an analytical signal.[11-13]

2.7.3. Repeatability

Chromatography was performed six times for the test solution. If the RSD of verbascoside is ≤ 2%, then the procedure has good repeatability.[11-13]

2.7.4. Linear correlation

From the standard solution of 1000 µg/ml, 5 samples were prepared with concentrations of 20 µg/mL, 40 µg/mL, 80 g/mL, 160 µg/mL and 320 µg/mL for conducting HPLC analysis. The correlation of S_{peak} with verbascoside concentration according to the function $Y = aX + b$ by the method of least squares was investigated. If the correlation coefficient $r \geq 0.9990$, the quantitative process has good linearity.[11-13]

2.7.5. Accuracy

Solution without standard addition: the test solution used in the experiment.

Standard addition solution: Take the test solution and add 25 µg/mL, 50 µg/mL and 100 g/mL verbascoside standard quantities to the test sample. Each level of titration was repeated six times.

The verbascoside content is calculated based on the function Y = aX + b. The accuracy must be in the range of 98 ÷ 102%, and the range has RSD ≤ 2%.[11-13]

2.7.6. Limit of detection (LOD) and limit of quantification (LOQ)

The test solution is gradually diluted into samples LOD1, LOD2, LOD3, LOD4, etc. In turn, 20 µL of each sample is injected into the HPLC system. The S/N ratio (Signal to Noise ratio) was determined. S is the signal height of verbascoside, and N is the background noise. LOD is accepted at a concentration with S/N = 3. LOQ is accepted at a concentration with S/N = 10.[11-13]

2.8. Data processing

The data were processed using Microsoft Excel 2016 and SPSS statistic 20.0 software for correlation function and statistical processing.

3. RESULTS

3.1. Results of selection of chromatographic conditions

The quantification process of verbascoside was conducted to investigate the chromatographic conditions of the HPLC analytical system. The result was that the suitable chromatographic conditions were selected using a gemini column C18 (250 × 4.6 mm, 5 µm), UV detector 334 nm, mobile phase MeCN - phosphoric acid 0.1% (16/84, v/v), flow rate 0.8 mL/min, injection volume 20 µL, analyte retention time 4, 16 minutes.

3.2. Quantitative process appraisal

3.2.1. Suitability

The results of the suitability assessment of the procedure are presented in Table 1, showing that the relative standard deviations of t_R (RSD = 0.29) and S_{peak} (RSD = 0.30) are both < 2%, so the HPLC system has high suitability and ensures the stability of the verbascosid[12,13] quantification procedure.

Table 1. Results of HPLC system suitability verification.

No	Retention time (Minute)	Peak area (mAU.s)
1	4,15	206159
2	4,16	207190
3	4,17	207115
4	4,15	207103
5	4,18	208113
6	4,17	207201
Xtb	*4,16*	*207146,8*
RSD (%)	*0,29*	*0,30*

3.2.2. Specificity

The results of the specificity evaluation of the procedure are shown in the chromatogram (SKD) (Figure 1). The blank sample (1) did not give any peak on the SKD. On the test solution (3), a peak with a corresponding retention time compared to the verbascoside tR on the standard solution (2) shows that the verbascoside tR in the two samples (2 and 3) is similar (approx. 4.16 minutes). High specificity HPLC system and test procedure were confirmed.[12,13]

3.2.3. Linear correlation

The results of the correlation evaluation between S_{peak} and verbascoside concentrations showed that they had a very tight linear correlation (r = 0.9997 > 0.9990) and were simulated by

https://doi.org/10.52111/qnjs.2023.17107

the function Y = 2349X + 7259.6. The results of testing the existence of correlation coefficients and parameters show a linear correlation between concentration and peak area ($p \approx 0 < 0.05$).[12,13]

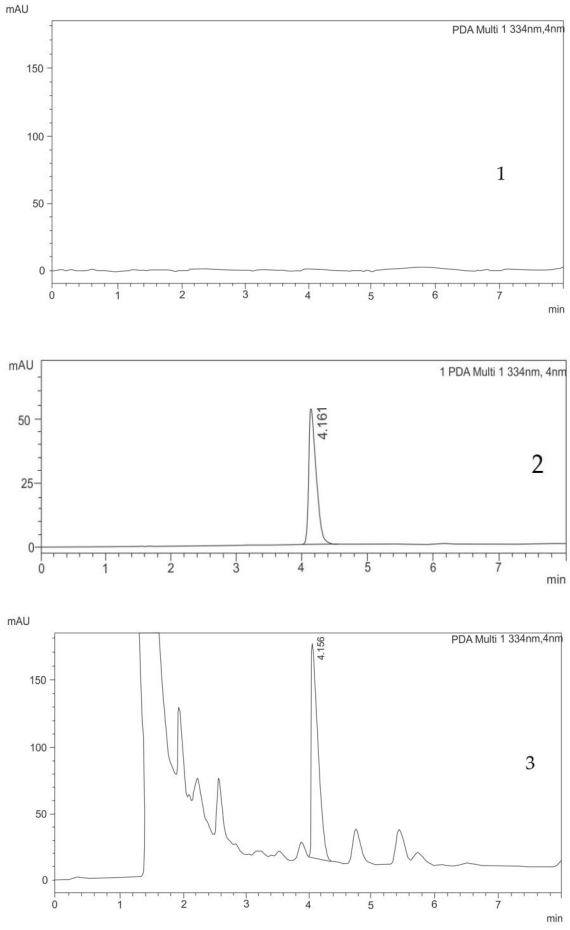

Figure 1. Process specificity assessment chromatogram: (1) White pattern, (2) Standard solution verbascosid, (3) Solution for testing sample.

Table 2. Correlation between S_{peak} and verbascoside concentration.

Concentration (µg/ml)	20	40	80	160	320
S_{pic} (mAU.s)	46870	98620	207103	383595	756511
Y = 2349X + 7259,6					
R^2 = 0,9994; r = 0,9997					

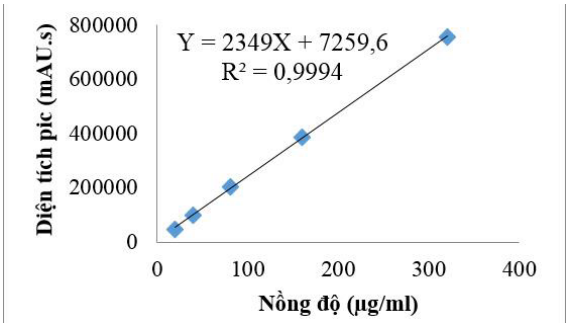

Figure 2. Correlation between S_{peak} and verbascoside concentration.

3.2.4. Repeatability

The repeatability of the procedure was evaluated through 6 replicate tests. The results showed that the average content of verbascoside in the test sample was 0.032%, with RSD = 1.63% < 2%, so the procedure has high repeatability.[12,13]

Table 3. Test solution repeatability evaluation results.

Parameters	Lặp 1	Lặp 2	Lặp 3	Lặp 4	Lặp 5	Lặp 6	Statistics
Weight of sample (g)	0,806	0,815	0,812	0,803	0,819	0,814	Mean (g) = 0,812 RSD (%) = 0,73
Verbascosid content (%)	0,031	0,032	0,032	0,031	0,032	0,032	Mean (%) = 0,032 RSD (%) = 1,63

3.2.5. Accuracy

The results of the procedure correctness evaluation showed that the recovery rate of verbascoside was from 98.24 ÷ 99.64% and within the allowable limit (98 ÷ 102%) with RSD from 1.37 ÷ 1.87% (RSD ≤ 2%), indicating that the procedure has high accuracy.[12,13]

Table 4. Process accuracy.

Time	The concentration of the additional standard (µg/ml)	Recovery concentration (µg/ml)	Accuracy (%)	RSD (%)
Within a day (n = 6)	25	24,91	99,64	1,87
	50	49,12	98,24	1,53
	100	98,67	98,67	1,37
Consecutive days (n = 6)	25	24,82	99,28	1,84
	50	49,62	99,24	1,65
	100	99,40	99,40	1,51

3.2.6. LOD and LOQ verbascosid

The LOD and LOQ values of verbascoside in the test solution were determined, at a concentration of 1.5 µg/mL producing an S/N ratio of 3 (LOD = 1.5 µg/mL), at a concentration of 4.9 µg/ml producing an S/N ratio of 10 (LOQ = 4.9 µg/mL).

3.2.7. Quantitative results of verbascoside in samples grown in Vinh Phuc, Phu Tho

Applying the validated procedure to quantify verbascoside in the root samples obtained from the *R. glutinosa* varieties 19 grown in Bach Luu - Song Lo - Vinh Phuc and Dan Quyen - Tam Nong - Phu Tho. The results are summarized in Table 5.

Table 5. Results of quantification of verbascoside in the samples grown in Vinh Phuc, Phu Tho.

No	Locations	Verbascosid content (%)
1	Bach Luu - Song Lo - Vinh Phuc	0,028
2	Dan Quyen - Tam Nong - Phu Tho	0,027

The verbascoside content in the *R. glutinosa* varieties 19 samples obtained at all locations was comparable to that of the Vietnam Pharmacopoeia V (verbascoside content ≥ 0.02%).

4. DISCUSSION

Verbascoside is a major active ingredient in the *R. glutinosa* and has important biological activities such as anti-inflammatory, re-epithelialization,[3-5] diuretic, antioxidant, wound-healing, and nervous system-protective effects.[3,6,7] This active ingredient is being interested in the development of medicines and supplement foods. Determining verbascoside content in medicinal herbs grown in different locations contributes to assessing the quality of medicinal herbs according to the soil conditions of each region.

The quantification process of verbascoside by the HPLC method has the advantages of high sensitivity, accuracy, reliability, and reasonable cost. Most of the laboratories in Vietnam are equipped with HPLC analysis systems; therefore, this process is easy to apply, it has high popularity in practice testing and evaluating the quality of medicinal herbs. The study was conducted to verify the quantification process of verbascoside in the tubers of the *R. glutinosa* varieties 19. The results showed that the procedure achieved the suitability of the system, good specificity, and a strong linear correlation (r = 0.9997), high recovery rate (99.1%) and high accuracy and repeatability (RSD < 2%), meeting the requirements of the Ministry of Health.[11,12] This result is similar to the research results of some authors, such as Gu et al. (2021), who evaluated the verbascoside content in 33 samples of *R. glutinosa* collected in six provinces of China (Henan, Anhui, Sichuan, Zhejiang, Shandong, Shanxi) by HPLC method that gave the results from 0.020 ÷ 0.26%.[10] The study of Tae et al. quantified verbascoside in 21 samples in Korea and China by HPLC with an average content of 0.07%.[8] Thus, using the HPLC method is suitable for the quantification of verbascoside.

In this study, chromatographic conditions of the HPLC system were used such as the selection of mobile phase acetonitrile (MeCN) - phosphoric acid 0.1% (16/84, v/v), flow rate

https://doi.org/10.52111/qnjs.2023.17107

0.8 mL/min., an injection volume of 20 µL, measured at 334 nm, is suitable, simple and efficient for determining the presence of verbascoside by HPLC. The process has some different chromatographic conditions compared with some procedures for the quantification of verbascoside described in the Vietnam Pharmacopoeia V, which used the mobile phase solvent system MeCN - acetic acid 0.1%.[2] While the study of Gu. et al. used MeCN - aqueous phosphate system of 0.02%, a flow rate of 1 ml/min, injection volume of 10,[1] measured at wavelength 205 nm,[10] Tae et al. used MeCN–water solvent system, flow rate 0.3 mL/min, injection volume 10 µL, measured at 205 nm,[8] and Li et al. (2020) used a flow rate of 0.5 mL/min.[9]

The process applied in this study to analyze *R. glutinosa* varieties 19 samples grown in Vinh Phuc, Phu Tho gave high suitability. The results of the analysis of real samples showed that the verbascoside content was from 0.027% ÷ 0.028%, the result met the requirements of the Vietnam Pharmacopoeia V (the minimum required verbascoside content was 0.02%).[2] Thus, the *R. glutinosa* varieties 19 planted in Vinh Phuc and Phu Tho produces good quality medicinal herbs, serving as a basis for expanding cultivation in this area.

There are no other publications on the quality evaluation of the *R. glutinosa* varieties 19 grown in Vietnam. The results in this paper will contribute to the development of a quantitative process for verbascoside for the medicinal plant – the *R. glutinosa* varieties 19 grown in Vietnam.

5. CONCLUSION

The study has established a procedure for the quantification of verbascoside in root grown from *R. glutinosa* varieties 19 by HPLC. The results of the process validation showed that there was a strong linear correlation (r = 0.9997) between S_{pic} and verbascoside concentrations, the functional form Y = 2349X + 7259.6. Quantitative results of verbascoside in the medicinal samples of *R. glutinosa* varieties 19 grown in Vinh Phuc, Phu Tho reached 0.027% ÷ 0.028%, the result met the requirements as prescribed by the Vietnam Pharmacopoeia V.

Acknowledgment

The research results are funded by an independent state-level project, code ĐTL.CN-03/17.

REFERENCES

1. Department of Crop Production, Ministry of Agriculture and Rural Development. Notice No. 909/TB-TT-CLT dated 31 July 2020 about the announcement of circulation of the variety Dia Hoang 19, 2020.
2. Ministry of Health. *Vietnam Pharmacopoeia V*, Medicine Publishing House, 2017.
3. R. X. Zhang, M. X. Li, Z. P. Jia. *Rehmannia glutinosa:* a review of botany, chemistry and pharmacology, *Journal of Ethnopharmacology,* **2008**, *117*, 199-214.
4. J. H. Lee, J. Y. Lee, H. S. Kang, C. H. Jeong, H. Moon, W. K. Whang. The effect of acetonide on histamine release and arachidonic acid release in RBL-2H3 mast cells, *Archives Pharmacal Research,* **2006**, *29*, 508-513.
5. O. Nigro, A. Tuzi, T. Tartaro, A. Giaquinto, I. Vallini, G. Pinotti. Biological effects of verbascoside and its anti-inflammatory activity on oral mucositis: a review of the literature, *Anti Cancer Drugs*, **2020**, *31*(1), 1-5.
6. W. T. Li, R. X. Deng, X. S. Jing, J. P. Chen, D. Yang, J. G. Shen. Acteoside ameliorates experimental autoimmune encephalomyelitis by inhibiting peroxynitrite-mediated mitophagy activation, *Free Radical Biological Medicines*, **2020**, *146*, 79-91.
7. A. Kalina, K. Liudmila, E. O. Ilkay, I. G. Milen. Verbascoside - A review of its occurrence, (bio) synthesis and pharmacological significance, *Biotechnology Advances*, **2014**, *32*, 1065-1076.

8. H. W. Tae, K. R. Sung, S. K. Sam, S. Jongheon. Simultaneous Analysis of Bioactive Metabolites from *Rehmannia glutinosa* by HPLC-DAD-MS/MS, *Natural Product Sciences,* **2010**, *16*(2), 116-122.

9. H. Y. Li, J. J. Fang, H. D. Shen, X. Q. Zhang, X. P. Ding, J. F. Liu. A quantity-effect research strategy for comparison of antioxidant activity and quality of Rehmanniae Radix and Rehmannia Radix Praeparata by online HPLC-UV-ABTS assay, *BMC Complementary Medicine and Therapies*, **2020**, *20*(16), 1-10.

10. M. Gu, Y. P. Yuan, Z. N. Qin, N. N. Shi, Y. P. Wang, H. Q. Zhai, Z. Z. Qian. A combined quality evaluation method that integrates chemical constituents, appearance traits and origins of raw Rehmanniae Radix pieces, *Chinese Journal of Natural Medicines,* **2021**, *10*(7), 551-560.

11. Ministry of Health. ASEAN guidelines on appraisal of analytical process, Circular 32/2018/TT-BYT Regulating the registration of circulation of drugs and medicinal ingredients, 2018.

12. Drug Management Department. Appendix 8 - Appraisal of analytical procedures by high-performance liquid chromatography (HPLC), decision No. 07/QD-QLD dated 11 January 2013, on the issuance of the Drug Registration Manual.

13. International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use. Validation of analytical procedures: Text and Methodology, 2005.

https://doi.org/10.52111/qnjs.2023.17107