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QUANTITATIVE DETERMINATION OF BERBERINE HYDROCHLORIDE IN THE TAN THONG PHONG VISCOUS EXTRACTS BY HPLC

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Summary

Quantitative Determination of Berberine Hydrochloride in the Tan thong phong Viscous Extracts by HPLC

An HPLC method was proposed for quantification of berberine hydrochloride in the Tan thong phong viscous extracts (Extractum spissum) which is prepared from *Cortex Phellodendri chinensis*, *Rhizoma Atractylodis*, *Radix Achyranthis bidentatae*, *Herba Siegesbeckiae*, *Rhizoma Anemarrhenae*, *Fructus Chaenomelis* using ethanol and water. The chromatography was established as: Using the Shim-pack GIST C_{18} column (250 mm × 4.6 mm, 5 µm); The mobile phase includes acetonitrile - 0.05% orthophosphoric acid solution (gradient program); The detection wavelength was 347 nm; Flow rate was 1 mL/min; The column temperature was set at 20°C; Injection volume was 5 µL. The method was validated for the specificity (RSD of peak area = 0.28%; RSD of t_R = 0.05%); linearity ranging from 11.29 - 361.23 µg/mL (r = 0.9999), precision (RSD < 3.7%) and high accuracy (recovery: 97.77% - 100.36%). The limits of detection (LOD) and quantification (LOQ) were 0.052µg/mL and 0.172 µg/mL, respectively. As for practical application, the tested samples showed the content of berberine hydrochloride was 0.88 ± 0.06%.

Keywords: *Tan thong phong, Viscous extract, Berberine hydrochloride, BBR, HPLC.*

1. Introduction

The remedy for Tan thong phong includes the following herbs: *Cortex Phellodendri chinensis, Rhizoma Atractylodis, Radix Achyranthis bidentatae, Herba Siegesbeckiae, Rhizoma Anemarrhenae, Fructus Chaenomelis* which was modified from the Tam dieu remedy, has the function of clearing heat and drying dampness and the purpose of curing dampness-heat pouring downward, manifested as reddened, swollen, and painful feet and knees, heaviness in the lower limbs, yellow and scanty urine [1],[2]. The viscous extract made from this remedy is a semi-finished product for the preparation of several products such as granules, capsules, etc. It is necessary to study to standardize and determine the content of active ingredients in the viscous extract. Berberine hydrochloride (BBR), a benzylisoquinoline alkaloid, is the main active ingredient of *Cortex Phellodendri chinensis* (the king of the remedy), has the effect of lowering blood uric acid [3],[4], protecting the kidneys [4], anti-inflammatory due to monosodium urate crystals causing inflammation in gout [5], antibacterial, anti-inflammatory, anti-cancer [6]... These effects showed that BBR plays an important role in the effects of *Cortex Phellodendri chinensis* and the whole remedy. Therefore, the determination of the content of BBR has an important meaning in the quality inspection of the Tan thong phong viscous extracts. Some documents have published methods of quantifying BBR in medicinal herbs [1],[7] and in preparations [1],[8] but there has been no quantitative study of this substance in viscous extract. This study was carried out to develop a quantitative method by high performance liquid chromatography and determine the BBR content in the viscous extract of Tan thong phong as a basis for developing the quality standard of this viscous extract.

2. Materials and methods

2.1. Materials and chemicals

Herbs were provided by VCP Pharmaceutical Joint Stock Company: *Cortex Phellodendri chinensis, Rhizoma Atractylodis, Radix Achyranthis bidentatae, Herba Siegesbeckiae, Rhizoma Anemarrhenae, Fructus Chaenomelis* meeting the standards of the 2015 Chinese Pharmacopoeia [1]. The Tan thong phong viscous extracts: Processing herbs, extracting them with 70% ethanol solvent, filtering, pooling the filtrate, distilling, and recovering the solvent under reduced pressure by vacuum rotary evaporator to a viscous liquid, then continuously boiling down in a water-bath at 80°C to viscous extracts (moisture < 20%). The total weight of each batch of medicinal herbs extracted according to the dried herbs is 995.08 g. The percentage of *Cortex Phellodendri chinensis* herb in the formula is 11.05%. The average ratio of extracts compared with average medicinal herbs is 24.76% (n=3) (calculated by dried medicinal herbs, dried extracts).

The preparation of placebo viscous extracts: Prepare as the Tan thong phong viscous extracts, leave *Cortex Phellodendri chinensis* out.

2.2. Chemicals, standard substance

Berberine hydrochloride ($C_{20}H_{17}NO_4.HCl$) (BBR) (87.89%, Lot No. WS.0317168.03) as reference substance was purchased from National Institute of Drug Quality Control. Methanol, acetonitrile, orthophosphoric acid (HPLC-grade) were purchased from Merck; purified water for HPLC was provided by Hanoi University of

Pharmacy; methanol (China) for sample extraction.

2.3. Instruments

Chromatography was conducted with Shimadzu (Japan) HPLC equipment comprising pump (LC-30AD), PDA detector (SPD-M20A), Autosampler (SIL-20A), column oven (CTO-10AS), and Shim-pack GIST C_{18} column (250 mm × 4.6 mm, 5 µm). The instrument was controled by using LabSolutions software (Shimadzu Japan) installed with the equipment.

2.4. Methods

Determination of moisture content of the viscous extracts: Method for the determination of mass loss due to drying [9]: About 1 g of the samples was weighed accurately and dried at 105°C for 5 h (to constant weight), then reweighed. The moisture of the samples was calculated according to the formula:

$$H(\%) = \frac{m_0 - m_1}{m_0} \times 100$$

m_0, m_1: *mass of the samples before and after drying.*

Samples preparation: Samples were extracted by using the ultrasonic method. Extraction solvent and extraction time were investigated to select the extraction method for the highest BBR content.

Standard solutions: BBR standard (87.89% content) was weighed accurately 4.11 mg into a 10 mL volumetric flask. Added about 8 mL of methanol into the flask, then sonicated until the mixture was completely dissolved. Next methanol was added up to the mark. The original standard solution being obtained had a concentration of 361.23 µg/mL. From this original standard solution, a series of standard solutions with concentrations in the range from 11.29 to 361.23 µg/mL was prepared.

Test solutions: The viscous extracts were accurately weighed about 0.2 g of into a 50 mL conical flask with a ground stopper. Added exactly 25 mL of solvent, then the conical flask with mixture was weighed, and sonicated for 30 minutes. The mixture was left to cool down, reweighed and then replenished the lost mass of the mixture with solvent. It was shaken and filtered through a 0.45 µm syringe membrane filter to obtain a chromatographic injection solution.

Test sample plus standard solutions: Absorbed 1 mL of test solution into the sample vial, added 0.1 mL of original standard solution, and mixed well.

Placebo solution: Placebo extract was weighed accurately about 0.2 g into a 50 mL ground-stoppered conical flask and processed the sample as for the test sample.

Test sample 50% plus standard solutions: Weighed accurately about 0.1 g of the extract into a 50 mL conical flask with ground stoppered. Preparing BBR standard solution: BBR was weighed accurately 24.04 mg then dissolved with methanol in a 20 mL volumetric flask to obtain a BBR standard solution with a concentration of 1056.44 µg/mL. Standard solution was added at three levels approximately 25%, 50%, and 75% of the BBR content in the 50% test sample to obtain a sample with approximately 75%, 100% and 125% content of the test sample by accurately adding 0.41 mL, 0.82 mL and 1.23 mL, respectively to the sample. Processed the sample as for the test sample, each level of standard addition was repeated 3 times.

Chromatographic conditions: Referring to some documents [1],[7],[8] and investigating mobile phase, column temperature, and detection wavelength to select chromatographic conditions as follows:

- Stationary phase: Shim-pack GIST C_{18} column (250 mm × 4.6 mm, 5 µm).
- Mobile phase: The mobile phase including acetonitrile (mobile phase A) and orthophosphoric acid 0.05% (mobile phase B) was surveyed according to the isocraticand gradient elution program.
- Flow rate: 1 mL/min.
- Column temperature: The column temperature was surveyed at 20°C, 30°C and 40°C to choose the column temperature for sharp, well-resolved BBR peaks.
- Sample injection volume: 5 µL.
- Detection wavelength: The BBR standard solution was analyzed with a PDA detector and recorded the spectrum in the range of 200-400 nm to select the wavelength for maximum absorption.

5 µL of the standard solutions were separately injected into the HPLC system, recorded the chromatogram and the area of the BBR peak. A linear regression line showing the dependence of peak area on standard solution concentration (µg/mL) according to the equation $y = ax + b$.

5 µL of the test solution was injected, recorded the chromatogram and the area of the BBR peak. The concentration of BBR in the test solution (µg/mL) was calculated by the formula:

$$C_t = \frac{S_t - b}{a}$$

The absolute BBR content in the dried extract was calculated by the formula:

$$X(\%) = C_t \times \frac{0.25}{m_{cd} \times (100 - H)}$$

S_t: Peak area of BBR in the chromatogram obtained of the test solution

C_t: Concentration of BBR in the test solution (µg/mL).

a: The slope of the linear regression line.

b: The intercept value of the linear regression line.

m_{cd}: Mass of the viscous extracts (g).

H: Moisture of the viscous extract (%).

HPLC method validation: The HPLC method was validated according to AOAC [10] and ICH [11] guidelines including specification, *System suitability*, linearity and range, precision (repeatability, intermediate precision), accuracy, LOD and LOQ.

Specification: Performing chromatography of solutions: solvent, placebo sample, standard sample, test sample, and test sample plus standard under the selected conditions. Requirements: In the chromatogram of the test solution, the analyte peak must have the same retention time as the BBR's peak in the standard solution chromatogram. If there is an auxiliary peak, the peak of the analyte must be completely separated from this peak. In the chromatograms of the placebo sample, there was no peak with a retention time corresponding to the BBR peak.

System suitability: The compatibility of a chromatographic system was determined by analyzing a BBR's standard solution of the appropriate concentration for 6 times in duplicate under the selected chromatographic conditions. Recording the parameters of the retention time and the area of the BBR's peak to calculate the RSD (%) of the retention time and peak area.

Linearity and range between concentration and peak area: A series of BBR standard solutions at appropriate concentrations was prepared for the chromatography with the selected condition above. Constructed a linear regression equation between peak area and concentration of BBR in standard solutions.

Survey on the precision (repeatability and intermediate precision) of the method: 6 independent test samples were repeatedly qualified by 2 different technicians on 2 different days with same methods as operating and chromatographic conditions above. Calculated the RSD of the quantitative result. Requirement: RSD $\leq$ 3.7% (AOAC's recommended level is from 0.1% to less than 1%) [10].

Survey of method accuracy: The method's accuracy was determined by adding standard to the test sample at a concentration of 50%. At each level, standardization process was performed on 3 independent samples. These samples were processed and analyzed according to the procedure to determine the percentage (%) of extra BBR recovered relative to the added amount (recovery rate). Requirement: The recovery rates at all 3 levels of standard addition are in the range of 95-105% (AOAC's recommendation at concentrations from 0.1% to less than 1%) [10].

Limit of detection (LOD) and limit of quantitation (LOQ) determination: The test solution of known concentration was gradually diluted and then was injected into the chromatographic system. Determined the LOD detection limit at the concentration for the S/N ratio ~ 3, calculated the LOQ (LOQ = 3.3×LOD).

Quantitative application of BBR in Tan thong phong viscous extracts: The developed method above was used to quantify BBR in some samples of Tan thong phong viscous extracts.

3. Results and discussion

3.1. Investigation of chromatographic conditions and extraction methods.

Selection of wavelength:

The concentration of BBR standard solution at 45.16 µg/mL was analyzed with a PDA detector and recorded its spectra in the range of 200-400 nm. The BBR results showed maximum absorption at three wavelengths 231, 266 and 347 nm. At 347 nm for a stable baseline chromatogram, the most compact, well-balanced BBR peak was selected as the quantification wavelength. This wavelength is close to the regulation of choosing wavelength 345 nm in some subjects: *Coptis chinensis*, Nhi dieu hoan remedy, Tam dieu hoan remedy [1] and the study of Ho Canh Hau et al. [8] but it is different from that regulation specified in two subjects: *Cortex Phellodendri chinensis* and Tu dieu hoan remedy whose wavelength of 265 nm [1] and the study of Kamal Y.T. et al. whose wavelength of 266 nm [7].

Mobile phase investigation:

Test sample and BBR standard sample (at a concentration of 45.16 µg/mL) were analyzed with a solvent system including acetonitrile (mobile phase A) and 0.05% orthophosphoric acid (mobile phase B) with the A:B ratio of 45:55 (v/v). The chromatograms showed that the BBR's peak has a short retention time (about 3.8 minutes) but cannot separate the substance and the BBR's peak tail was still attached to other peaks (Fig. 1a). Therefore, we investigated gradient elution programs with mobile phase A ratio varying from 10% to 80% for a period of 25 min and chose the program for BBR peak chromatography at a retention time of about 9.7 minutes separate from the other peaks; the peak tail was compact (Fig. 1b). The elution program in gradient as the following: 0-5 min (10-40% A); 5-10 minutes (40-80% A); 10-15 minutes (80-40% A); 15-25 min (40-10% A). This is also the mobile phase program chosen by Kamal Y.T. et al. in the quantitative study of BBR in *Berberis aristata* and preparations [7].

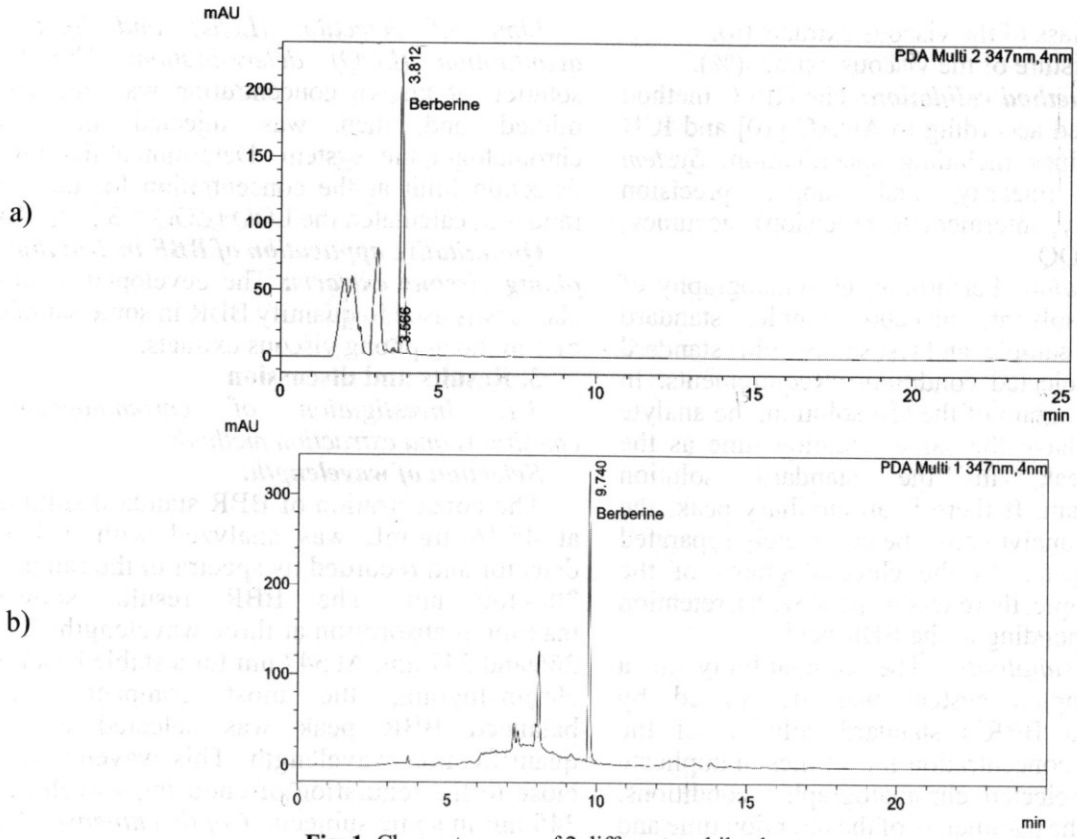

Fig. 1. Chromatograms of 2 different mobile phase program.
a) mobile phase A:B ratio of 45:55 (v/v), b) gradient elution program.

Column temperature investigation:

Analyzed the sample at column temperature of 20, 30 and 40°C to obtain the BBR retention time of 9.740 minutes, 9.716 minutes, and 9.677 minutes, respectively, which are not much different. The column temperature of 20°C giving sharp, well-resolved BBR peak was selected for quantification.

Investigation of extraction solvent:

Weighed accurately about 0.2 g of the viscous extracts. According to the method of sample preparation, the samples were ultrasonic extracted for 30 minutes with methanol of three concentrations 50%, 70% and 100%. The samples then were quantified by HPLC. The results of the peak area of BBR calculated on 0.2 g of extract with 50%, 70%, 100% methanol respectively are: 1368875; 1547867 and 1418536 (mAU.s). The 70% methanol with the highest BBR peak area was selected for the extraction.

Investigation of extraction time:

Weighed accurately about 0.2 g of the viscous extracts. According to the method of sample preparation, the samples were ultrasonic extracted for 15 minutes, 30 minutes and 45 minutes with 70% methanol. The samples then were quantified by HPLC. The results of BBR peak area calculated on 0.2 g high with extraction time of 15, 30 and 45 minutes respectively are: 1287284; 1418536 and 1381494 (mAU.s). Extraction time of 15 minutes and 45 minutes gave lower extraction efficiency, 90.74% and 97.39%, respectively, compared with 30-minute extraction; therefore, 30-minute time was chosen to extract the viscous extracts.

3.2. Validation of developed HPLC method

Specification:

The results presented in Fig. 2 showed that in the chromatograms of the test sample and the test sample plus standard, the peak had a retention time corresponding to the peak on the standard solution's chromatogram. The peaks in the chromatograms of the test sample and the test sample plus standard were completely separated. BBR was detected at a retention time of about 9.7 minutes, wich was completely separated from other peaks. BBR's peak was well-balanced, and its tail width was small. Meanwhile, on the chromatogram of the placebo sample, there was no peak with a retention time corresponding to the retention time of the peak in the BBR standard solution's chromatogram. On the other hand, comparing the spectrum of the peaks between the test sample and the standard sample (Fig. 3) resulted in a match ratio of 0.9995 with the maximum absorption at three wavelengths 231, 266 and 347 nm. Therefore, the analytical method was specific for simultaneous determination of selectivity and specificity.

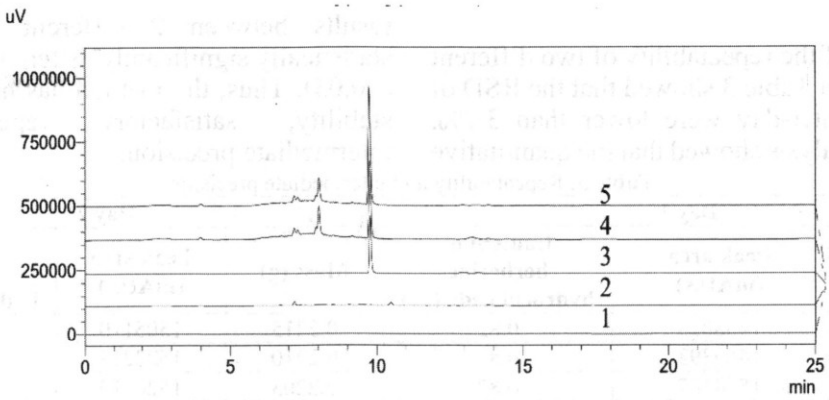

Fig. 2. Chromatography of the studied samples recorded at 347 nm
1: Solvent; 2: Placebo; **3**: BBR standard, **4**: Test sample, **5**: Test sample plus standard.

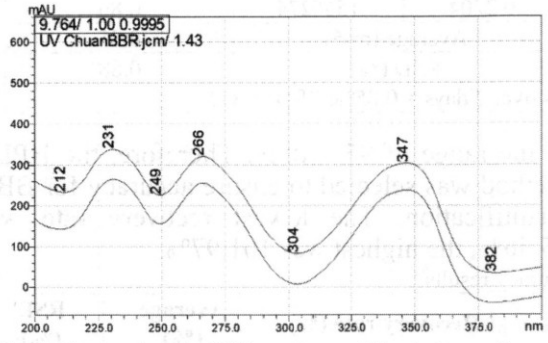

Fig. 3. Comparing the UV spectrum of the test sample and the BBR standard

System suitability:

Analyzed a BBR's standard solution concentration of 45.16 µg/mL for 6 times, recorded the values of retention time and peak area. The suitability of the system presented in Table 1 showed that the relative standard deviation (RSD%) of retention time (0.03%) and peak area (0.28%) of BBR were both less than 2%, the number of theoretical places is 67713. Thus, the selected chromatographic conditions had good repeatability in terms of retention time and peak area of BBR, and the HPLC system used in this study was suitable and ensured the stability of the quantitative determination of BBRs.

Table 1. System suitability results (n=6)

	Retention time (min)	Peak Area (mAU*s)	Number of theoretical places
Average	9.722	1014113	67713
RSD (%)	0.03	0.28	2.03

Linearity and Range:

The linearity results presented in Table 2 and Fig. 4 showed that in the investigated concentration range from 11.29 µg/mL to 361.23 µg/mL, there was a satisfactory linear correlation between peak area and BBR concentration with correlation coefficients r = 0.9999. The calibration curve had a high linearity to ensure quantitative analysis of BBR.

Table 2. Results of BBR quantitative linear range BBR

Concentration (µg/mL)	11.29	22.58	45.16	90.31	180.62	361.23
Peak area (mAU.s)	255290	509152	1013908	2037081	4076996	8340241
Regression equation:	y = 23082x -30505					
Correlation coefficient (r):	0.9999					

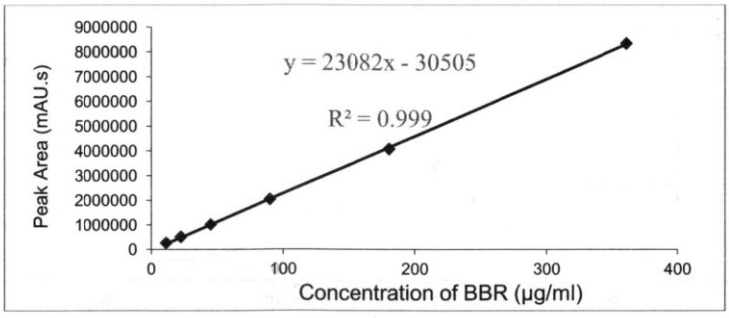

Fig. 4. The correlation curves between concentration and peak area of BBR

Precision:

The results of the repeatability of two different days presented in Table 3 showed that the RSD of intra-day and inter-day were lower than 3.7%. Using Anova analysis showed that the quantitative results between 2 different days were not statistically significantly different (*P-value* = 0.49 > 0.05). Thus, the method has high repeatability, stability, satisfactory repeatability, and intermediate precision.

Table 3. Repeatability and intermediate precision

Day 1			Day 2		
Mass (g)	**Peak area (mAU.s)**	**Content of berberine hydrochloride (%)**	**Mass (g)**	**Peak area (mAU.s)**	**Content of berberine hydrochloride (%)**
0.2205	1458838	0.82	0.2215	1505110	0.84
0.2205	1469393	0.83	0.2210	1522213	0.85
0.2212	1554167	0.87	0.2203	1520919	0.85
0.2214	1488793	0.83	0.2202	1528473	0.86
0.2204	1511128	0.85	0.2204	1517623	0.85
0.2206	1550586	0.87	0.2204	1540274	0.86
Average (n=6)		0.85	Average (n=6)		0.85
RSD (%)		2.57	RSD (%)		0.88
Average BBR content from 12 qualifications over 2 days = 0.85%; RSD=1.87%					

Accuracy:

The accuracy results presented in Table 4 showed that the average recovery rates at each concentration level from 97.77% to 100.36% were in the range of 95 -105%. Therefore, the HPLC method was selected to ensure accuracy for BBR quantification. The lowest recovery rate was 95.46%, the highest was 101.97%.

Table 4. Method accuracy results

Levels	Spiked (µg/mL)	Recovery (µg/mL)	Recovery rate (%)	Average (%)	RSD (%)
75%	17.33	17.18	99.13	98.98	2.17
	17.33	16.76	96.75		
	17.33	17.51	101.04		
100%	34.65	35.32	101.92	100.36	2.74
	34.65	33.67	97.18		
	34.65	35.33	101.97		
125%	51.98	49.62	95.46	97.77	2.31
	51.98	50.87	97.87		
	51.98	51.97	99.98		

Limit of detection (LOD) and limit of quantification (LOQ):

At concentration of 0.052 µg/mL, on a chromatogram, BBR's peak was still balanced and separated (Fig. 5) with an S/N ratio of 4.40. Continuously diluting to a concentration of 0.039 µg/mL could not detect the BBR's peak. Thus, the limit of detection (LOD) was a solution with a concentration of 0.052 µg/mL. This LOD value is higher than the study of Ho Canh Hau et al. (LOD = 0.02 µg/mL) [8] but lower than the study of Kamal Y.T. et al. (LOD = 1.5 µg/mL). [7]. Limit of quantitation (LOQ) = 3.3×LOD = 0.172 µg/mL.

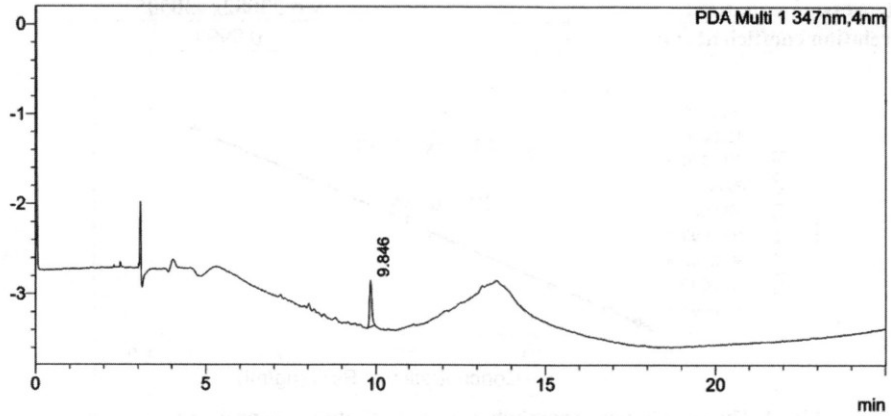

Fig. 5. Chromatogram of BBR at LOD

3.3. Quantification of BBR in viscous extract samples

From the developed method, quantification of BBR in viscous extract samples was carried out. Preparation of the test sample solution and conduct chromatography under the conditions described on the 3 samples. The result of BBR content in Tan thong phong viscous extracts was 0.88 ± 0.06%.

4. Conclusion

The method for quantification of berberine hydrochloride in Tan thong phong viscous extracts by HPLC had been developed and validated analytical: Shim-pack GIST C_{18} column (250 mm x 4.6 mm, 5 µm), flow rate: 1 mL/min, column temperature: 20°C, and mobile phase acetonitrile : orthophosphoric acid 0.05% (gradient elution). Analytical method with high sensitivity, specificity, linear ranging from 11.29 to 361.23 µg/mL (r = 0.9999), good repeatability and intermediate precision (RSD < 3.7%), high accuracy (97.77% to 100.36%), LOD and LOQ were 0.052 µg/mL and 0.172 µg/mL, respectively. The berberine hydrochloride content in Tan thong phong viscous extracts was 0.88 ± 0.06%. It was necessary to continue investigating the berberine hydrochloride content in Tan thong phong viscous extracts to develop quantitative criteria and quality standards for this viscous extract.

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METHOD DEVELOPMENT FOR QUANTIFICATION OF PAEDEROSIDIC ACID FROM *PAEDERIA SCANDENS* BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Summary

Method Development for Quantification of Paederosidic Acid from *Paederia scandens* by High Performance Liquid Chromatography

A simple and sensitive high performance liquid chromatography (HPLC-DAD) method for quantification of paederosidic acid from *Paederia scandens* aerial part was developed and validated. The chromatographic separations were obtained by a Shimadzu C18 column (250×4.6mm, 5µm) using isocratic mode with 0.1% phosphoric and acetonitrile (85:15, v/v). Detection was set at a wavelength of 234 nm. The validation results displayed good accuracy, repeatability, linearity, and selectivity. The contents of paederosidic acid in *Paederia sc* acid *andens* samples were 0.0075 -0.2339%. The obtained results provide useful information for the quality testing of the *Paederia scandens* in Vietnam.

Keywords: *Paederia scandens* (Lour.) Merr., *Paederosidic acid, HPLC.*

1. Introduction

Paederia scandens (Lour.) is a perennial herb belonging to the *Paederia* L. genus of Rubiaceae. The vine Paederia scandens is widely distributed in Asia, particularly in Vietnam [1]. Many parts of the plant, involving the roots, leaves, barks, and fruits