Original Article

Determination of Saponin Glycosides in *Bacopa monnieri* by Reversed Phase High Performance Liquid Chromatography

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ABSTRACT

A reversed phase HPLC system was developed for the quantitative analysis of bioactive saponin glycosides in the medicinal plant, *Bacopa monnieri* (L.) Wettst (Brahmi). Five dammarane saponin glycosides namely Bacoside A_3 (**1**), Bacopaside II (**2**), 3-*O*-[α-L-arabinofuranosyl(1→2)-{β-D-glucopyranosyl-(1→3)}-α-L-arabinopyranosyl] jujubogenin (**3**), Bacopasaponin C (**4**) and Bacopaside I (**5**) were analyzed on a C-18 column. With an isocratic elution of 0.2% phosphoric acid and acetonitrile (65:35 v/v), the separation was obtained in a total run time of 20 min. The method was validated and the applicability of this HPLC method for analyzing saponin glycosides from Brahmi is demonstrated.

Key words: *Bacopa monnieri*, RP HPLC analysis, saponin glycosides

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Introduction

Bacopa monnieri (L.) Wettst., commonly known as Brahmi, has been used for a long time in Ayurvedic medicine as nervine tonic for promoting mental health and improving memory[1]. Recently, several studies have been published on the biological effects of this plant, especially for a therapeutic potential in treatment or prevention of neurological diseases and improvement of higher order cognitive processes.[1-5] Triterpenoid saponins, the major components in Brahmi, were reported to be responsible for the cognitive enhancing activity of Brahmi.[1,6]

Although several commercial products of Brahmi claimed for memory improvement have been launched in the market, only a few reports regarding quality control methods were published. Estimation procedures by using high performance thin layer chromatography (HPTLC)[7] and ultraviolet (UV) detector[8] for the determination of "Bacoside A," the mixture of active saponins in Brahmi were reported. More precise high performance liquid chromatography (HPLC) methods for quantification of each saponin are available.[9-11] However, in all methods, gradient elution is used and more than 30 min run time is needed. In the present study, we described a simple and accurate isocratic reversed phase (RP) HPLC procedure for a determination of five major saponins in Brahmi. The method was validated and its applicability for quality control purpose was discussed.

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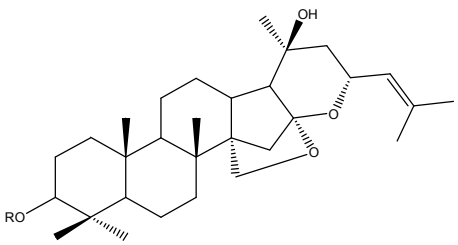
Materials and Methods

Plant materials

The aerial parts of Brahmi were collected from Phetburi, Bangkok and Phitsanulok provinces, Thailand and named as Brahmi A, B, and C, respectively. The plants were identified by Associate Professor Dr. Wongsatit Chuakul, Faculty of Pharmacy, Mahidol University, Thailand. The voucher specimens (Phrompittayarat 001, 002 and 003) were kept at the herbarium of Mahidol University, Bangkok, Thailand. In addition, different parts of Brahmi C, i.e. shoots (10 cm cut from the apex), lower parts (the rest of the aerial part below "shoot") and roots, were collected. All plant materials were cut into small pieces and dried for 3 days. Then, they were coarsely air-powdered.

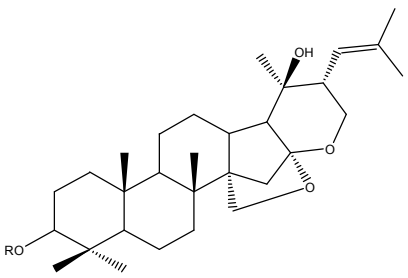
Chemicals

Acetonitrile and methanol (HPLC grade) were purchased from Labscan Asia, Thailand. Orthophosphoric acid (AR grade) was purchased from BDH, England. The saponin reference standards, Bacoside A_3 (**1**), Bacopaside II (**2**), 3-*O*-[α-L-arabinofuranosyl (1→2)-{β-D-glucopyranosyl-(1→3)}-α-L-arabinopyranosyl] jujubogenin (**3**), Bacopasaponin C (**4**) were obtained as a gift from Professor I. Khan, the National Center for Natural Products Research, MS, USA. Bacopaside I **(5)** was purchased from ChromaDex, CA, USA. Structures of compounds **1** – **5** are shown in Figure 1. Bacoside A was purchased from Natural Remedies, Bangalore, India.



Jujubogenin

Saponin glycosides	**R**
Bacoside A_3 (**1**)	α-L-arabinofuranosyl(1→2)-[β-D-glucopyranosyl-(1→3)]-β-D-glucopyranosyl
Bacopasaponin C isomer (**3**)	α-L-arabinofuranosyl(1→2)-[β-D-glucopyranosyl-(1→3)]-α-L-arabinopyranosyl



Pseudojujubogenin

Saponin glycosides	**R**
Bacopaside II (**2**)	α-L-arabinofuranosyl(1→2)-[β-D-glucopyranosyl-(1→3)]-β-D-glucopyranosyl
Bacopasaponin C (**4**)	α-L-arabinofuranosyl(1→2)-[β-D-glucopyranosyl-(1→3)]-α-L-arabinopyranosyl
Bacopaside I (**5**)	α-L-arabinofuranosyl(1→2)-[6-*O*-sulfonyl-β-D-glucopyranosyl-(1→3)]-α-L-arabinopyranosyl

Figure 1 Structures of five saponin glycosides (**1**-**5)**

Sample preparation

The dried material of Brahmi was soaked in water for 24 hrs and then squeezed to discard water before 4-hr percolation with 7 mL of 95% ethanol per 1 g of dried plant for 3 times. The ethanolic extract was filtrated and dried under reduced pressure. Each extract (10 mg) was redissolved in 10 mL methanol and filtrated through a 0.45 µm nylon filter prior to injection into the chromatographic system.

HPLC Analysis

The separation was performed using a Shimadzu HPLC system equipped with a SPD-M10AVP photodiode array detector (PDA), an LC-10ATVP pump (Shimadzu, Japan) and a Rheodyne injector with 20 µL loop. A Luna RP-18 column (150x4.6 mm, 5 µm particle size) was used together with a Phenomenex RP-18 guard column (Torrance, CA, USA). The mobile phase consisted of 0.2% phosphoric acid and acetonitrile (65:35 v/v). The pH of the mobile phase was adjusted to 3.0 with 5 M NaOH. The flow rate and total run time were 1.0 mL/min and 20 min, respectively. All peaks were integrated at the wavelength of 205 nm. They were initially assigned by comparing retention times with standards, and confirmed with characteristic spectra obtained from the PDA.

Method validation

The HPLC method was validated for linearity, limit of detection (LOD), precision and accuracy. Brahmi A extract was used as a sample for the method validation. The linearity of the method was evaluated in the 7.8 to 500.0 µg/mL range. Seven concentrations of five standard saponins were chosen for generating the calibration curves. Three determinations (n = 3) were carried out for each solution. The correlation graphs were constructed by plotting the obtained peak areas versus the injected concentrations. The LOD was determined by a serial dilution of the mixture of five standards. The concentration that gave a signal to noise ratio of 3 was regarded as minimal detectable amount recorded. For specificity test, peak purity was confirmed by studying the PDA data of all relevant peaks. Intra- and inter-day precisions were determined by the analysis of two concentrations (2 and 5 mg/mL) for each analyte and expressed as percentage of relative standard deviation (% RSD). In the intra-day precision experiment, five determinations (n = 5) were carried out for each concentration for any given day. The inter-day precision was measured in triplicate (n = 3) for three consecutive days. The accuracy of the method was evaluated by analyzing the mixture that prepared by adding 20 µg/mL of standard saponins to the Brahmi extract, containing a known amount of the analyzed saponins. Three determinations (n = 3) were carried out.

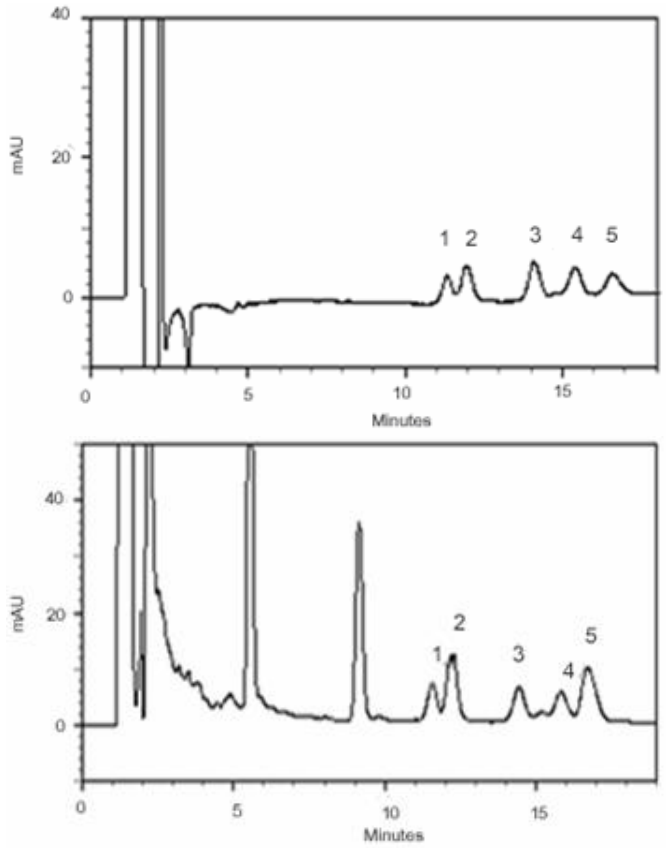
Results and Discussions

In order to optimize the HPLC system for determination of five saponin glycosides (**1** - **5**) in Brahmi, we various column systems were tested. These included column types (normal phase and reversed phase columns), lengths (150 - 250 mm), particle sizes (3 – 5 µm) and sources (Phenomenex, Altech, Water). Various mobile phases were also used. Finally, the experiments showed that a Luna RP 18 (Phenomenex, CA, USA) with an isocratic elution of 0.2% phosphoric acid and acetonitrile (65:35 v/v; pH 3.0) gave the best separation in the shortest time.

The chromatograms of the mixture of five saponin reference standards and Brahmi extract are presented in Figure 2. Due to their poor chromophores, most of the Brahmi triterpenoid saponins can only be detected at wavelength of 205 nm. The retention time of compound 4 slightly shifted. The identification of the peak was confirmed by adding the standard compound to the extract. All saponins gave LOD within a range of 0.3 - 0.6 µg/mL (Table 1).

Analytical data of each reference standard at seven concentrations were obtained from at least three experiments. Calibration curves were generated by linear regression based on the peak areas. The linearity was obtained within the range of injected concentrations (7.8

- 500 µg/mL). The equations are shown in Table 1. Linearity with high squares of correlation coefficient (r^2 = 0.9998 – 0.9999) were obtained for each reference standard. Peak purity was investigated by studying the PDA data of all peaks of interest; no indications for impurities were found.



2A

2B

Figure 2 RP HPLC-chromatograms of the mixture of five reference standards (20 µg/mL) (2A) and Brahmi extract (2 mg/mL) (2B) under optimized RP HPLC conditions (column = Luna C-18, 5 µm, 100 mm × 4.6 mm; mobile phase = 0.2% phosphoric acid and acetonitrile (65:35 v/v, pH 3.0); flow rate = 1 mL/min; detection wavelength = 205 nm; injected sample volume = 20 µL). Peak assignments were matched with compounds shown in Figure 1.

Table 1 Calibration data for the RP HPLC analyses of five saponins (n = 3)

Analyte	Regression equation	r^2	LOD (µg/mL)
1	$y = 1.6886(10^{-4})x + 0.1058$	0.9998	0.62
2	$y = 1.3499(10^{-4})x - 0.3357$	0.9998	0.62
3	$y = 1.7869(10^{-4})x - 1.6754$	0.9999	0.31
4	$y = 1.3479(10^{-4})x - 1.8996$	0.9999	0.31
5	$y = 1.7449(10^{-4})x - 1.5552$	0.9999	0.31

Note: Y reflects the peak area (mAU); x = concentration of the compound (µg/ml); r^2 = square of correlation coefficient; LOD = limit of detection

Retention times shifted slightly since we recycled the mobile phase. However, it did not hamper the analysis. The precision of the assay was shown as % RSD. % RSD not higher than 4 and 10 were found for intra-day and inter-day assays, respectively (Table 2). Recovery was in the range of 85 to 105% (Table 3)

which was in the acceptable range (85-115%) according to general analytical guideline[12-14]. This indicated that this RP HPLC method had adequate accuracy. Moreover, compared with the HPLC methods developed by Ganzera et al.[10] and Deepak et al.[11] which all were gradient elution systems, our method is more practical and simple. Isocratic elution was used allowing the recirculation of the mobile phase which can save cost of the analysis. Also a shorter run time (20 min) was obtained.

Table 2 Intra- and inter-day precision of the investigated saponins under optimized RP HPLC conditions

Analyte	Conc. (mg/mL)	% Intra-day RSD (n = 5)	% Inter-day RSD (n = 3)
1	2.0	3.22	5.12
	5.0	1.17	7.11
2	2.0	0.28	2.20
	5.0	0.74	1.69
3	2.0	2.12	9.89
	5.0	1.58	9.39
4	2.0	1.04	5.70
	5.0	1.83	4.29
5	2.0	2.82	3.45
	5.0	0.59	3.20

Table 3 Recoveries of the investigated saponins from Brahmi shoot extract by using RP HPLC

Analyte	% Recovery (%RSD)
1	92.24 (5.94)
2	85.75 (8.56)
3	93.84 (9.12)
4	90.92 (4.87)
5	103.05 (5.11)

Note: Values are shown as mean percentage of recovery from three experiments.

Aerial parts of Brahmi collected from three places in Thailand were analyzed. A typical HPLC chromatogram of the extract is shown in Figure 2B. All samples gave the similar HPLC fingerprints. Five major saponins were detected and quantified (Tables 4). The dominant compound was either Bacopaside II (**2**; 0.27-0.59%) or Bacopaside I (**5**, 0.35-0.71%). The total amounts of saponins in the samples were varied from 1.11 to 2.16%. It is noted that the presence of Bacopaside I (**5**) in the HPLC analyses of Brahmi have not been mentioned in the reports of Ganzera et al.[10] and Deepak et al.[11]. However, Ganzera et al. also found an unidentified peak of saponin in their chromatogram with the same pattern of the peak which was identified as Bacopaside I in our analysis.

Bacoside A is known as marker for standardization of the commercial product of Brahmi. However, it has just been recently reported as a mixture of four saponins by Deepak et al.[14]. The composition of Bacoside A was confirmed to consist of compounds 1-4 (Table 4). Compound 3, the jujubogenin isomer of bacopasaponin C, was present in the highest concentration which was in agreement with the previous study.

In the general harvesting procedure of Brahmi, only the shoots are collected. The other parts are spared to promote the further growth of this plant. In order to confirm that the quantity of saponins in the shoots was higher than other parts, the amounts of saponins in the different parts were also analyzed (Table 5). The highest saponin content (2.03%) was obtained from the shoots whereas the roots contained around four times less saponins (0.51%).

In conclusion, the present study describes an RP HPLC method as a practical method for the quantification of triterpenoid saponins in the medicinal plant, Brahmi. At optimized conditions, a separation of five saponins was feasible within 20 min. Furthermore, the method was validated for linearity, limit of detection, accuracy, precision, and intra- and inter-day variations.

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Table 4 The amount of the investigated saponins in Bacoside A and in Brahmi from different sources analyzed by using RP HPLC (n = 3)

Analyte*	Amount of saponins as mean % w/w (% relative standard deviation)			
	Brahmi A	Brahmi B	Brahmi C	Bacoside A
1	0.34 (3.17)	0.07 (7.80)	0.27 (5.13)	17.20 (1.74)
2	0.59 (3.14)	0.27 (4.74)	0.53 (3.32)	12.45 (1.93)
3	0.32 (2.75)	0.22 (4.14)	0.24 (4.84)	24.62 (2.19)
4	0.21 (1.77)	0.15 (5.46)	0.22 (9.29)	18.84 (2.44)
5	0.71 (3.02)	0.41 (2.31)	0.35 (2.22)	-
Total	2.17	1.11	1.62	73.11

* Brahmi A, B and C were collected from the provinces of Phetchaburi, Bangkok and Phitsanulok, respectively.

Table 5 The amount of saponins in different parts of Brahmi (n = 3)

Analyte	Amount of saponins as mean % w/w (% relative standard deviation)		
	Shoot[a]	Lower part[b]	Root
1	0.31 (4.01)	0.13 (3.42)	0.10 (9.68)
2	0.64 (1.16)	0.32 (7.16)	0.08 (4.70)
3	0.34 (1.86)	0.10 (7.57)	0.15 (4.00)
4	0.23 (2.84)	0.07 (3.18)	0.10 (7.08)
5	0.51 (1.56)	0.19 (6.84)	0.08 (5.21)
Total	2.03	0.81	0.51

[a] Shoot = the part containing stems and leaves that was cut at 10 cm from the apex of Brahmi
[b] Lower part = the rest of the aerial part below the shoot

References

1. Singh HK, Dhawan BN. Neuropsychopharmacoloical effects of the Ayurvedic nootropic *Bacopa monniera* Linn. (Brahmi). *Indian J Pharmacol* 1997;29:S359-S365.
2. Russo A, Borrelli F, Campisi A, Acquaviva R, Raciti G, Vanella A. Nitric oxide-related toxicity in cultured astrocytes: effect of *Bacopa monniera*. *Life Sci* 2003;73: 1517-1526.
3. Das A, Shanker G, Nath C, Pal R, Singh S, Singh HK. A comparative study in rodents of standardized extracts of *Bacopa monniera* and *Ginkgo biloba* anticholinesterase and cognitive enhancing activities. *Pharmacol Biochem Behav* 2002;73:893-900.
4. Vohora D, Pal SN, Pillai KK. Protection from phenytoin-induced cognitive deficit by *Bacopa monniera*, a reputed Indian nootropic plant. *J Ethnopharmacol* 2000;71:383-390.
5. Stough C, Lloyd J, Clarke J, et al. The chronic effects of an extract of *Bacopa monniera* (Brahmi) on cognitive function in healthy human subjects. *Psychopharmacol* 2001;156: 481-488.
6. Russo A, Borrelli F. *Bacopa monniera*, a reputed nootropic plant: an overview. *Phytomedicine* 2005;12:305-317.
7. Gupta AP, Mathur S, Gupta MM, Kumar S. Effect of the method of drying on the bacopaside A content of the

harvested Bacopa monniera shoots revealed using a high performance thin layer chromatography method. *J Med Arom Plant Sci* 1998;20:1052-1055.

8. Pal R, Sarin JPS. Quantitative determination of bacosides by UV-spectrophotometry. *Ind J Pharmaceut Sci* 1992; 53:711-714.
9. Renukappa T, Roos G, Klaiber I, Volger B, Kraus W. Application of high-performance liquid chromatography coupled to nuclear magnetic resonance spectrometry, mass spectrometry and bioassay for the determination of active saponins from *Bacopa monniera* Wettst. *J Chromatogr A* 1999;847:109-116.
10. Ganzera M, Gampenrieder J, Pawar RS, Khan IA, Stuppner H. Separation of the major triterpenoid saponins in *Bacopa monniera* by high-performance liquid chromatography. *Anal Chim Acta* 2004;516:149-154.
11. Deepak M, Sanggli GK, Arun PC, Amit A. Quantitative determination of the major saponin mixture Bacoside A in *Bacopa monnieri* by HPLC. *Phytochem Anal* 2005;16: 24-29.
12. Ermer J, Miller JHMcB. Method validation in pharmaceutical analysis: a guide to best practice. New York. John Wiley & Sons Inc, 2005.
13. Chan CC, Lee YC, Lam H, Zhang XM. Analytical method validation and instrument performance verification. New Jersey. John Wiley & Sons Inc, 2004.
14. Miller JM, Crowther JB, Miller J. Analytical chemistry in a GMP environment: a practical guide. New York. John Wiley & Sons Inc, 2000.