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Research Article

MARKER BASED STANDARDISATION OF PLANT BASED FORMULATIONS CONTAINING *BRAHMI* USING BACOSIDE A BY HPTLC

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ABSTRACT

Objective: Marker based standardization using sophisticated methods of analysis such as HPTLC, is one of the globally accepted methods for standardization of herbal products. The aim of the study was to develop and validate simple, precise, rapid and selective high-performance thin-layer chromatographic (HPTLC) method for standardization of commercial formulations containing *Bacopa monnieri* (*Brahmi*) using Bacoside A as Marker compound.

Methods: The mobile phase consisted of toluene: ethyl acetate: methanol: glacial acetic acid and TLC plates precoated with $60GF_{254}$ silica gel was used as the stationary phase. The detection wavelength was 540nm after spraying with 10% sulphuric acid in alcohol reagent. The developed method was well validated by different parameters mentioned in ICH guidelines such as linearity, accuracy, precision etc. The validated method was applied for standardization of Ayurvedic and modern dosage form containing *Brahmi*.

Results: The developed method was validated and the method is accurate, linear, precise and robust. The linear regression data for the calibration plots showed a good linear relationship with r = 0.9998 for bacoside A.

Conclusion: *Bacopa monnieri* (Linn.) Pennel (Family: Scrophulariaceae), commonly known as *Brahmi,* is an important drug for the improvement of intelligence, memory and revitalization of sensory organs. The study revealed that many *Brahmiarishta* contains *Centella asiatica* and not *Bacopa monnieri*, which does not have the nootropic activity. Thus, the method can be used for routine analysis of formulations containing *Bacopa monnieri* for estimation of content of Bacoside A.

Keywords: Bacoside A, *Bacopa monnieri, Brahmi*, Marker, Standardization, HPTLC.

INTRODUCTION

India is sitting on a gold mine of well-recorded and well practiced knowledge of traditional herbal medicine [1]. India is a land to about 17,000 species of higher plants, out of which 7500 are known for their therapeutic uses. Ayurveda, has alone reported approximately 2000 medicinal plant species, followed by Siddha and Unani [2]. But, unlike China, India has not been able to capitalize on this herbal wealth by promoting its use in the developed world despite their renewed interest in herbal medicines. Total global herbal market is of size 62.0 billion dollars, in this India's contribution is only one billion dollars as estimated by Dabur Research Foundation [3]. Nevertheless, among various traditional medicine systems Ayurveda has higher share in market. Thus Ayurvedic system plays a key role to uplift the share of total herbal export of India. Hence, Ayurveda needs immediate and extensive reorientation to gain scientific credibility. But the most important challenges faced by the Ayurvedic medicines arise because of their lack of complete standardization [4-6]. Standardization is defined as adjusting the herbal drug preparation to a defined content of a constituent or a group of substances with known therapeutic activity respectively by adding excipients or by mixing herbal drug extracts [7].With the use of modern methods of analysis such as UV and IR spectroscopy, GC, TLC, HPTLC, HPLC and more, it is possible to set up certain standards and analyze a particular constituent i.e. "Marker compound/s" from Ayurvedic preparations [8]. The European Medicines Agency (EMEA) defines chemical markers as chemically defined constituents or groups of constituents of a herbal medicinal product which are of interest for quality control purposes regardless whether they possess any therapeutic activity. Some medicinal herbs are very limited to certain geographical conditions and thus they are valued more. In such conditions some time the substitutes are used. One of the classic example is *Bacopa monnieri* (*Brahmi)* to which *Centella asiatica* (*Mandukaparni*) is substituted. *Centella asiatica* has some of the common pharmacological effects but the potency is very low [9]. The memory enhancing action is dominated by *Bacopa monnieri* and not by *Centella asiatica* [10]. Thus, by standardizing the medicines containing *Brahmi* one can assure the authenticity and effectiveness of the product.

Brahmi is one of the most explored herb in India since ancient ages. The drug consists of whole plant of *Bacopa monnieri* (Linn.) Pennel from family: Scrophulariaceae [11]. *Brahmi* is an important drug for the improvement of intelligence, memory and revitalization of sensory organs. The nootropic activity has been attributed to the presence of two saponins, namely bacoside A and bacoside B. From these two markers Bacoside A is considered and proved to be more pharmacologically active. Chemically it is 3-(α-L-arabinopyranosyl)-*O-β-D-glucopyranoside-10, 20-dihydroxy-16-keto-dammar-24-ene* [12, 13].

As it is a bioactive compound and found one of the major saponins from *Bacopa monnieri,* it can be considered as a "Marker" compound for Marker Based Standardization.

Nowadays, High-Performance Thin Layer Chromatography (HPTLC) based methods has been an important tool in routine analysis of herbal medicines. Advantage of HPTLC is its ability to analyze several samples simultaneously using a small quantity of mobile phase. Thus, HPTLC facilitates low operating cost, high sample throughput, simplicity and speed, the need for minimum sample clean up, reproducibility, accuracy, reliability and robustness repeated detection of chromatogram with same or different parameters [14-16].

Till date there is no report on standardization of Ayurvedic formulations containing *Brahmi* using bacoside A as a marker. Thus we have made an attempt to develop and validate HPTLC method for Bacoside A as per the ICH guidelines. Thus, to standardize commercial samples of four Ayurvedic preapartions and two modern dosage forms of formulations containing of *Bacopa monnieri* by validated HPTLC method.

MATERIALS AND METHODS

Solvents and chemicals

Standard bacoside A was provided as gift sample by Natural remedies Pvt. Ltd., India as a gift sample. Six formulations of *Brahmi* namely *Gutika, Vati, Churna, Arishta* (two different brands), Tablets and Capsules were procured from the local market.

All chemicals and reagents used were of analytical grade and purchased from Rankem and S. D. Fine Chemicals, India.

HPTLC Instrumentation

The sample solutions were spotted in the form of bands of width 8 mm with a Camag microlitre syringe on precoated silica gel aluminum plate $60F_{254}$ (20 cm × 10 cm with 250 µm thickness; E. Merck, Darmstadt, Germany, supplied by Anchrom Technologists, Mumbai) using a Camag Linomat V (Switzerland). The plates were pre-washed by methanol and activated at 60°C for 5 min prior to chromatography. The slit dimension was kept at 5mm × 0.45 mm and 10 mm/s scanning speed was employed. The slit bandwidth was set at 20 nm, each track was scanned thrice and baseline correction was used. The mobile phase consisted of Toluene: ethyl acetate: methanol: Gla. acetic acid (3:4:2.5:1) v/v and 15 ml of mobile phase was used per chromatography. Linear ascending development was carried out in 20 cm x 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 30 min at room temperature (25 °C ± 2) at relative humidity of 60% ± 5. The length of chromatogram run was 8 cm. Subsequent to the scanning, TLC plates were dried in a current of air with the help of an air dryer. Densitometric scanning was performed with Camag TLC scanner III in the reflectance-absorbance mode at 540 nm after spraying with 10% Sulphuric acid alcohol reagent (Acid alcohol) and operated by Win CATS software (1.3.0 Camag). Concentrations of the compound chromatographed were determined from the intensity of diffusely reflected light. Evaluation was carried out by comparing peak areas with linear regression.

Standard solutions

Reference standard solutions were prepared by dissolving 5.0 mg of bacoside A in 10 ml methanol, yielding stock solution of concentration = 0.5 mg ml^{-1}. From this 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10 µL of this solution were applied using LINOMAT 5 applicator with the band width of 8 mm, which gave different concentration ranging 150- 2500 ng / spot respectively.

Sample solutions

For *Brahmi Vati*

Vati is the traditional solid dosage form similar to tablet which is modern dosage form. The extract or the powdered drug is compressed into vati.

2 g of powdered vati was transferred to 100 ml volumetric flask containing 50 ml of methanol and the mixture was macerated on a shaker for 24 hrs at room temperature. Then 1.0 ml of this extract was diluted to a 10 ml with methanol.

For *Brahmi Chruna*

Churna is a traditional form of Ayurvedic medicine in powder dosage form.

2 g of chruna was transferred to 100 ml volumetric flask containing 50 ml of methanol and the mixture was macerated on a shaker for 24 hrs at room temperature. Then 1.0 ml of this extract was diluted to a 10 ml with methanol.

For *Brahmi Gutika*

Gutika is the traditional solid dosage form similar to vati but bigger in size. The extract or the powdered drug is compressed into gutika.

2 g of powdered *Gutika* was transferred to 100 ml volumetric flask containing 50 ml of methanol and the mixture was macerated on a shaker for 24 hrs at room temperature. Then 1.0 ml of this extract was diluted to a 10 ml with methanol.

For *Brahmi Capsule*

Capsule is a modern solid dosage form in which the powdered crude drug or extract of the herb is filled into the shells of the capsules.

Sample solutions of capsule formulation were prepared same as that of vati by transferring 2 g of capsule contents.

For *Brahmi Tablet*

Tablet is a modern solid dosage form where the powdered crude drug or extracts are compressed in form of tablet.

Sample solutions of tablet formulation were prepared by transferring 2 g of powdered tablet to 100 ml volumetric flask containing 50 ml of methanol and the following the procedure was same as that of vati.

For *Brahmiarishta*

Arishta is a galenical form of medicine. It is also a traditional form which is prepared by natural fermentation process.

Two brands of *Brahmiarishta* namely Brand 1 and Brand 2 were analyzed and the sample preparation was as follows.

10 ml of *Brahmiarishta* (each brand) was evaporated to dryness. 2 g of residue was dissolved in 50 ml methanol in a volumetric flask. 2.5 ml of this extract was diluted to 10 ml with methanol.

A constant application volume of 10.0 µl/s was employed for all the sample solutions.

Assay validation

The proposed HPTLC method was validated according to the International Conference on Harmonization (ICH) guidelines [17-20]. All measurements were performed in triplicates.

Calibration studies

Linearity was evaluated in the range of 500-10000 ng / spot of bacoside A. Peak area *versus* concentration was subjected to least square linear regression analysis and the slope, intercept and correlation coefficient for the calibration were determined. Limit of detection (LOD) and limit of quantitation (LOQ) were determined from the calibration curve using the following expressions: $3\sigma/S$ and $10\sigma/S$, where σ is the standard deviation and S is the slope of the calibration curve.

Precision studies

Precision of the method was evaluated by repeatability (intra-day) and instrumental precision. Each level of precision was investigated by three sequential replicates of injections of bacoside A at concentrations of 1000, 1500 and 2000 ng / spot.

Accuracy studies

In order to evaluate the validity of the proposed method, accuracy was evaluated through the percentage recoveries of known amounts of bacoside A added to solutions of extracts and all commercial products. The analyzed samples were spiked with 80, 100 and 120 % of median concentration (1500 ng) of bacoside A standard solution. For sample preparation of formulations please refer section 8.5. A constant application volume of 10.0 µl/spot was employed for all the sample solutions.

Accuracy was calculated from the following equation:

[(spiked concentration − mean concentration)/spiked concentration] × 100.

Robustness

For the determination of the robustness of method, chromatographic parameters, such as mobile phase composition and chamber saturation time, were intentionally varied to determine their influence on the retention time and quantitative analysis.

Stability studies

Stability of the sample solutions was tested after 24, 48 and 72 hours after preparation and storage at 4.0°C and 25.0°C separately. Stability was assessed by comparing the chromatographic parameters of the solutions after storage with the same characteristics of freshly prepared solutions. There should not be more than 5% of degradable content.

RESULTS AND DISCUSSION

Method optimization

The proposed method gave very good separation and resolution of the standard bacoside A (R_f value = 0.44) as indicated in **(**Figure 1). The shape of the peaks was not altered by other substances present in the matrix (Figure 2).

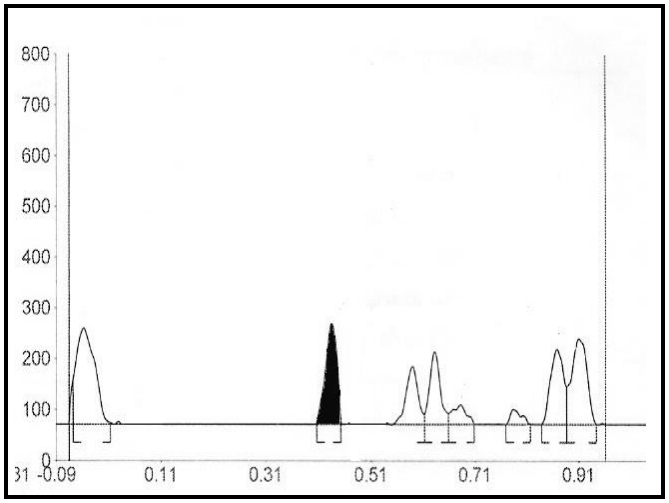

Fig. 1: HPTLC profile of methanol extract of Brahmi vati using optimized parameters

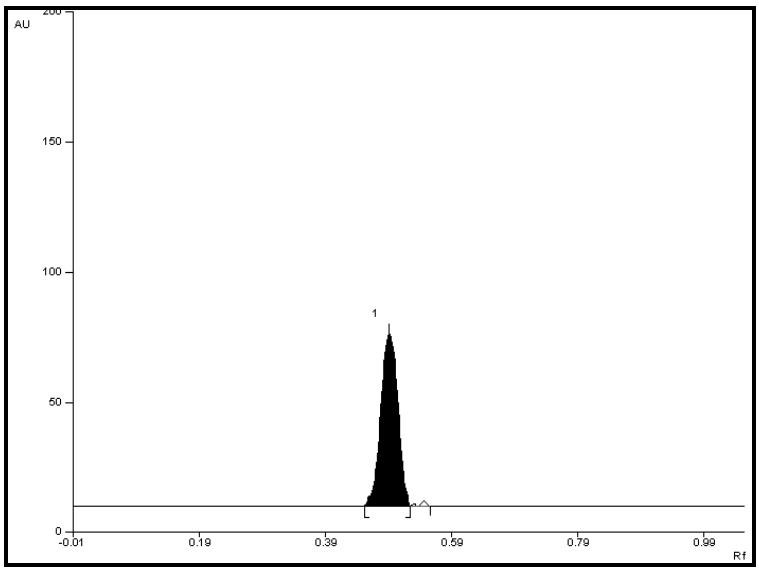

Fig. 2: HPTLC Chromatogram of bacoside A using optimized parameters

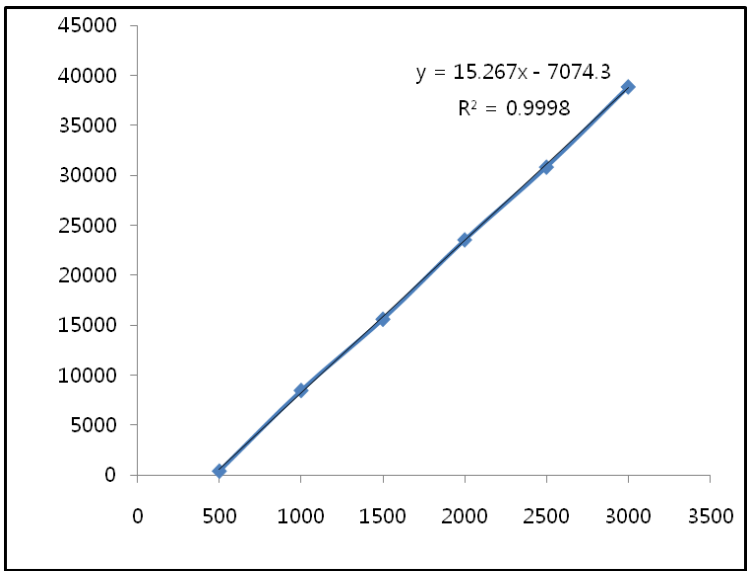

Fig. 3.Calibration curve of Bacoside A

Method validation

Linearity, limit of detection and limit of quantitation

Under the above described experimental conditions, linear correlation between the peak area and applied concentration was obtained in the concentration range of 500-2500 ng / spot of bacoside A. The correlation coefficient of bacoside A was found to be 0.9998. The peak area (*y*) is proportional to the concentration of bacoside A (*x*) following the regression equation y = 15.267x - 7074.3 (Figure 3). The experimentally derived LOD and LOQ for bacoside A were determined to be 150 and 450 ng /spot respectively.

Precision

Precision data on repeatability (intra-day) and instrumental variation for three different concentration levels are summarized in Table 1. Precision studies showed R.S.D. less than 1%, indicating an excellent precision.

Accuracy

Except for two brands of *Aristha* (50.10%, 49.33%), the recoveries from other formulations were in good range of acceptability (97.69-107.97 %) of bacoside A. The proposed HPTLC method is accurate for the quantification of bacoside A in five formulations containing *Bacopa monnieri* (Table 2).

Robustness

The mobile phase composition was altered by ± 5 % changes in the ratio of methanol and glacial acetic acid. The two composition of methanol were tried 2.625 (+5% of 2.5) and 2.375 (-5% of 2.5) and of Glacial acetic acid 1.05 and 0.95 (+5% and -5% of 1) was tried (Table 3.1). The chamber saturation time was altered from 15 min to 30 min (Table 3.2). The altered conditions did not cause any change retention factor and peak shape, thus the method is robust.

Table 1: Results of Precision Studies Bacoside A

Type of Precision	Intra-day			Inter-day		
	AUC for concentration of Bacoside A (ng/spot)			AUC for concentration of Bacoside A (ng/spot)		
S. No.	1000	1500	2000	1000	1500	2000
1	8565.01	15542.35	23544.58	8541.28	15748.49	23453.26
2	8578.19	15640.02	23457.65	8598.46	15684.15	23326.56
3	8588.16	15630.97	23315.16	8578.69	15840.86	23609.29
Mean	8577.12	15604.4	23439.1	8572.81	15757.8	23463
% RSD	0.13538	0.34585	0.49416	0.33874	0.49989	0.60358

Table 2: Recovery studies for Bacoside A in various herbal products

Extracts & Formula-tions	Amount added in µg	AUC			Recovery ± R.S.D
		Formula-tions	Standard	Standard spiked formulations	
Brahmi Vati	1200	775.4	1463.9	2368.5	105.77±0.02
	1500	775.4	1820.8	2726.26	105.01±0.54
	1800	775.4	2157.8	3084.25	105.15±0.17
Brahmi Churna	1200	396.9	1463.9	1857.45	99.82±0.56
	1500	396.9	1820.8	2211.71	99.73±0.94
	1800	396.9	2157.8	2530.43	99.05±0.05
Brahmi Gutika	1200	406.2	1463.9	1820.16	97.33 ±019
	1500	406.2	1820.8	2159.96	96.99 ±0.11
	1800	406.2	2157.8	2488.87	97.07 ±0.23
Brahmi Capsules	1200	865.7	1463.9	2541.12	109.08±0.09
	1500	865.7	1820.8	2936.07	109.29±0.63
	1800	865.7	2157.8	3299.84	109.14±0.58
Brahmi Tablets	1200	807.8	1463.9	2435.71	107.22±0.22
	1500	807.8	1820.8	2815.75	107.12±0.44
	1800	807.8	2157.8	3187.72	107.49±0.56
Brahmiarishta (Brand 1)	1200	-	-	-	-
	1500	-	-	-	-
	1800	-	-	-	-
Brahmiarishta (Brand 2)	1200	-	-	-	-
	1500	-	-	-	-
	1800	-	-	-	-

* There are no accuracy results for both brands of Arishta as there was no peak of Bacoside A was detected.

Stability studies

The results were calculated as the percentage of non-degraded content of bacoside A standard at the 21, 48, 72 hours. All formulations showed less than 3.11% degradation at both investigated temperature. The highest non degradant percent was found in *Brahmi* churna (99.90%) at 4°C in 24 hrs, whereas the least observed in *Brahmi* Ghutika (96.89 %). (Table 4)

Quantitative analysis of plant based formulations for content Bacoside A

The validated method was applied to standardization for both traditional and modern dosage forms viz. *Brahmi* Vati, Churna, Gutika, Capsule, Tablet, Arishta Brand 1 and Brand 2.

The percent content of bacoside A for all seven formulations are indicated in Table 5.

This method could detect bacoside A with a wide range of concentration from 0.010 – 0.079%. The bacoside A content was highest in *Brahmi* tablet (modern dosage form) and least was in *Brahmi* vati. But if per unit dosage is considered then the content of bacoside A is more in *Brahmi* vati (0.55mg) and least in *Brahmi* capsule (0.06 mg).

Table 3.1: Robustness (Mobile phase variation) studies of Bacoside A

S. No.	Mobile phase composition (v/v)				R_f	AUC
	Toluene	Ethyl acetate	Methanol	Formic acid		
1	3	4	2.5	1	0.44	15550.1 ±0.54
2	3	4	2.625	1	0.44	15487.66 ±0.47
3	3	4	2.375	1	0.44	15589.48 ±0.21
4	3	4	2.5	1.05	0.44	15471.06 ±0.61
5	3	4	2.5	0.95	0.44	15508.99 ±0.59
R.S.D.	-	-		-	0	0.31

Table 3.2: Robustness (Chamber saturation time variation) studies of Bacoside A

Sr. No	Chamber saturation time (min)	R_f	AUC
1	20	0.44	15550.1 ±0.54
2	15	0.44	15598.14 ±0.23
3	25	0.44	15560.11 ±0.74
4	30	0.44	15591.15 ±0.48
5	35	0.44	15487.55 ±0.68
R.S.D.	-	0	0.28

Table 4: Stability Studies of Bacoside A in formulations in various herbal products

Formulations	Temperature					
	4°C			25°C		
	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs
Vati	99.86	99.23	98.11	98.99	98.58	97.23
Churna	99.90	99.81	98.69	99.01	98.56	97.09
Ghutika	99.14	98.61	97.30	98.02	97.14	96.89
Capsules	99.60	98.54	98.83	99.43	98.85	97.91
Tablets	99.79	98.69	97.86	99.39	98.78	98.12

Table 5: Percent Content of Bacoside A in various formulations containing *Brahmi*

Extracts & Formulations	Percent Content ± S.D. (%)	Weight of per unit	Content per unit dosage form (mg)
Brahmi Vati	0.022 ± 0.11	250 mg	0.55 mg
Brahmi Churna	0.017 ±0.34	3.0 g (tbsp)	0.51 mg/tbsp
Brahmi Gutika	0.010 ±0.21	350 mg	0.35 mg
Brahmi Capsules	0.03 ± 0.42	200 mg	0.06 mg
Brahmi Tablets	0.079 ± 0.63	250 mg	0.1975 mg
*Brahmi*arishta (Brand 1)	----	2 g	--
*Brahmi*arishta (Brand 2)	---	2g	--

CONCLUSION

A simple, convenient, accurate method was developed and validated for bacoside A using HPTLC. Both the brands of *Brahmiarishta* did not show any peak corresponding to bacoside A. It was observed that both the brands of Arishta labelled as *Brahmi* did not contain *Bacopa monnieri* but the substituent.

This may be due to the addition of substitute ie; *Centella asiatica* instead of *Bacopa monnieri.*

The method was used to standardize formulations containing *Brahmi.* The analysis enabled to quantify the marker compound and even to detect the adulterant.

The method can very well employed for the analysis of *Brahmi* products for routine quality control analysis to detect the percent content of the bioactive compound.

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