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Research Article

DEVELOPMENT AND VALIDATION OF HPTLC METHOD FOR ANALYSIS OF AQUEOUS *BACOPA MONNIERIA* EXTRACT

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ABSTRACT

Objective: The present study was designed with an objective of developing a validated, simple, accurate and precise HPTLC method for the determination of aqueous extract of *Bacopa monnieria*.

Methods: High performance thin layer chromatography (HPTLC) method was developed and validated for rapid analysis of aqueous extract of dried *Bacopa monnieria* powder. Chromatographic separation was achieved on precoated silica gel HPTLC aluminum plate 60 F_{254} using dichloromethane: methanol (2:4.5 v/v) as mobile phase. Detection was performed at 294 nm wavelength densitometrically.

Results: The limit of detection and quantitation were 300 ng/spot and 1000 ng/spot, respectively. The linear regression analysis data for calibration plots showed good linear relationship (R^2 = 0.999) in the concentration range of 2000-12000 ng/spot. Accuracy (99.34 to 100.59%) and precision (% RSD < 2%) of the developed HPTLC method was in accordance with the ICH guidelines.

Conclusion: The developed procedure can be selectively used in the assay and quality control of *Bacopa monnieria* from various ayurvedic formulations without interference from excipients and auxiliary substances.

Keywords: *Bacopa monnieri*, HPTLC, Validation.

INTRODUCTION

Bacopa monnieri (L.) Wettst., commonly known as Brahmi has long been established in Ayurvedic medicine as nervine tonic for promoting mental health, improving memory and rejuvenating sense organs [1,2]. Additionally, it is reported useful in various neurological disorders like anxiety, epilepsy, depression, insomnia, psychosis and stress [3,4]. *Bacopa monnieri* is considered to possess anti-lipid peroxidative, anti-inflammatory, free radical scavenging, astringent, laxative as well as surfactant activities [5,6]. Bacopa's extensive range of activities is mainly attributed to its major active constituents such as bacosides [7-10], bacosaponins [11-13] along with other constituents' viz. alkaloids and phytosterols [14].

Several analytical methods like spectrophotometry, spectroflurometry, HPTLC and HPLC have been reported for estimation of *Bacopa monnieri* [15-21]. Ingkaninan et al. developed reversed phase-HPLC technique for quality control of several cognitive enhancing commercial products of Bacopa [22]. Shinde et al. reported simultaneous estimation of *Bacopa monnieri* and *Withania somnifera* in marketed formulations by HPTLC [23]. However, in all the reported methods, alcoholic extracts of *Bacopa monnieri* were used for method development. Literature reveals no method for analysis of aqueous extracts of *Bacopa monnieri*. Ayurvedic products are marketed in the form of spansules, capsules, syrups and other similar conventional formulations where their aqueous solubility influence their biological performance.

So, the present study was designed for the development and validation of simple HPTLC method for the determination of aqueous extract of *Bacopa monnieria* from marketed Brahmi syrup. The proposed method is validated as per ICH guidelines [24, 25] and its applicability for quality control purpose of commercial products is discussed.

MATERIALS AND METHOD

Materials

Bacopa monnieria extract dry powder was a gift sample from Tulip Laboratories Pvt. Ltd., Ranjangaon, Maharashtra, India. Marketed Brahmi Syrup containing 75 mg/ml *Bacopa monnieria* was procured from local market, Pune, India. All chemicals and reagents were of analytical grade and purchased from Merck Chemicals, Mumbai, India.

Sample Preparation

Aqueous Solution of Plant Extract

Stock solution of 1 mg/ml was prepared by dissolving 100 mg *Bacopa monnieria* extract dry powder in 100 ml double distilled water. This solution was subjected to magnetic stirring and sonication for 15 min followed by filtration through a 0.45 µm membrane filter (Pall, India) under reduced pressure.

Solution of Marketed Preparation

75 mg/ml of Brahmi Syrup was sonicated as such for 15 min and filtered through 0.45 µm membrane filter under reduced pressure prior to application.

Chromatographic conditions

HPTLC plates were prewashed and activated at 110 °C for 5 min prior to spotting. Working standard solutions in the concentration range of 2000 to 12000 ng/spot were applied (band width: 6 mm) in triplicate with a Camag 100 µL sample syringe (Hamilton, Switzerland) on precoated silica gel HPTLC aluminum plate 60 F_{254}, (20 × 10 cm with 250 µm thickness; E. Merck, Darmstadt, Germany) using a Camag Linomat V automatic sample applicator (Switzerland). Each concentration was applied on the HPTLC plate for six times, repeatedly at 0.1 µl/s constant application rate. The plate was then developed in a twin trough glass chamber (Camag, Switzerland) saturated with the mobile phase. Mobile phase consisted of dichloromethane: methanol (2:4.5 v/v). The optimized chamber saturation time for mobile phase was 20 min. Length of chromatogram run was 8 cm. After linear ascending development, air dried plate was scanned by densitometry using Camag TLC scanner 3 in the reflectance-absorbance mode and operated by CATS software (V 3.15, Camag). Slit dimension was maintained at 5 mm × 0.45 mm. Deuterium lamp was the source of radiation for emitting a continuous UV spectrum between 190 and 400 nm. All determinations were performed at ambient temperature with detection wavelength at 294 nm. Peak areas were plotted against the corresponding concentrations to obtain the linear calibration regression.

Method Validation

As per the ICH guidelines [24, 25], the optimized HPTLC method was validated for following parameters:

Linearity

Linearity was performed by applying standard solution at different concentrations ranging from 2000 to 12000 ng/spot on HPTLC plates. Peak areas were recorded for each concentration. Linearity curve for aqueous *Bacopa monnieri* extract was obtained by plotting a graph of peak area *vs.* applied concentration.

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

Limit of detection is the minimum detectable amount of analyte in a solution; difficult to quantify. The quantification limit of an individual analytical procedure is the minimum amount of analyte in a sample that can be quantitatively determined. The limit of detection (LOD) and limit of quantification (LOQ) were estimated at signal to noise (S/N) ratios of 3:1 and 10:1, respectively, in six replicates.

Specificity

Specificity is the ability of the proposed analytical method to explicitly estimate the analyte in the presence of other components by overlaying *in situ* spectra. The spots for plant extract and marketed preparation are confirmed by comparing the R_f.

Accuracy

Accuracy of the proposed method was established by measurement using the standard addition method whereby a constant amount (similar to label claim) of marketed preparation was blended with three different amounts (corresponding to 80, 100 and 120% of label claim) of dried *Bacopa monnieria* powder. At each level of concentration, six determinations were performed and the results obtained were compared with expected results.

Precision

The method was validated for intraday and interday precision. Intraday precision (method repeatability) was measured by repeating the same procedure three times on the same day for three different concentrations of *Bacopa monnieria* extract (4000, 8000 and 12000 ng/spot). The interday (intermediate) precision of the method was checked by performing similar procedure on different days under the same experimental conditions. Repeatability of sample application and measurement of peak area for analyte were expressed in terms of percent relative standard deviation (% RSD).

Analysis of the marketed preparation

Solution of 75 mg/ml marketed Brahmi preparation was spotted on HPTLC plate in triplicates to calculate definite amount of aqueous *Bacopa monnieria* extract by comparing peak areas of the analyte with the standard calibration curve.

Robustness

Robustness was assessed by deliberately altering the chromatographic conditions and studying the effects on the obtained results.

RESULTS AND DISCUSSION

For optimization, different combinations of mobile phases were investigated. Mobile phase containing dichloromethane: methanol (2:4.5 v/v) showed highest selectivity for better estimation of aqueous Brahmi extract from dried powder of the *Bacopa monnieria*.

Method Validation

Linearity

Aqueous extract of dried *Bacopa monnieria* powder showed linear response in the concentration range of 2000-12000 ng/spot (Fig. 1) and the resultant regression equation was:

$$y = 0.049x + 258.3$$

with correlation coefficient (R^2) of 0.999 (Table 1). There was no significant difference observed in the slope and intercepts of standard curves. Residual analysis was performed to establish linearity (Fig. 2).

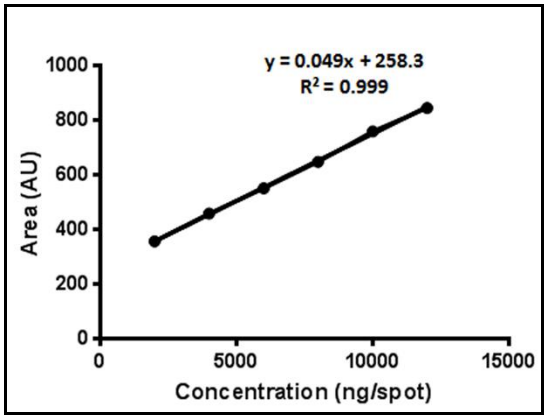

Fig. 1: Linear regression analysis for aqueous extract of *Bacopa monnieria*.

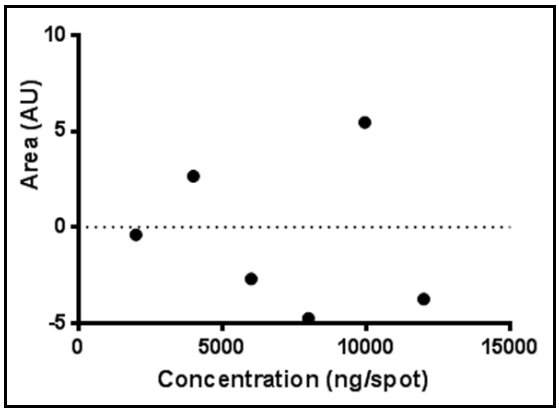

Fig. 2: Residuals plot obtained from linear regression analysis for aqueous extract of *Bacopa monnieria*.

LOD and LOQ

The LOD and LOQ for determination of aqueous *Bacopa monnieria* extract were 300 ng/spot and 1000 ng/spot, respectively (Table 1).

Specificity

The specificity of developed method was confirmed by comparing the respective spectra of *Bacopa monnieria* extract and marketed Brahmi syrup (Fig. 3a and 3b).

Accuracy

As depicted in Table 2, recovery values obtained in the range of 99.34 to 100.59 % with small % RSD (< 1) demonstrated accuracy of the developed HPTLC method for estimation of aqueous *Bacopa monnieria* extract from the marketed preparation.

Precision

The results of the intra-day and inter-day precision experiments are shown in Table 3. The developed method for estimation of aqueous *Bacopa monnieria* extract was found to be precise as % RSD values were < 2, as recommended by ICH guidelines [24].

Analysis of the marketed preparation

Densitogram of the aqueous extract of *Bacopa monnieria* from its marketed preparation illustrated an appropriate shaped peak at R_f 0.75 (Fig. 4).

Table 1: Summary of the validation parameters

Parameters	Results*
Linearity Range	2000-12000 ng/spot
Correlation coefficient (R^2)	0.999
Slope ± RSD	0.049 ± 0.0017
Intercept ± RSD	258.3 ± 8.41
LOD	300 ng/spot
LOQ	1000 ng/spot
Precision	< 2 % RSD
Accuracy	99.34 to 100.59 %
Assay	99.19% w/w

*Statistically significant parameters at p < 0.001

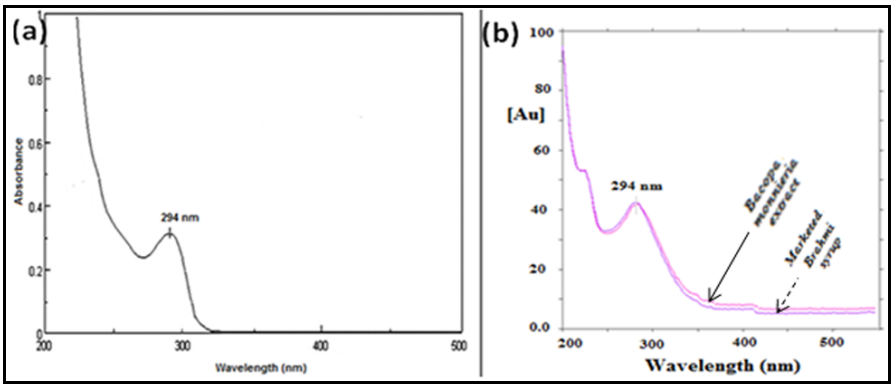

Fig. 3: (a) UV spectrum of *Bacopa monnieria* and (b) HPTLC spectra overlay of *Bacopa monnieria* extract and marketed Brahmi syrup.

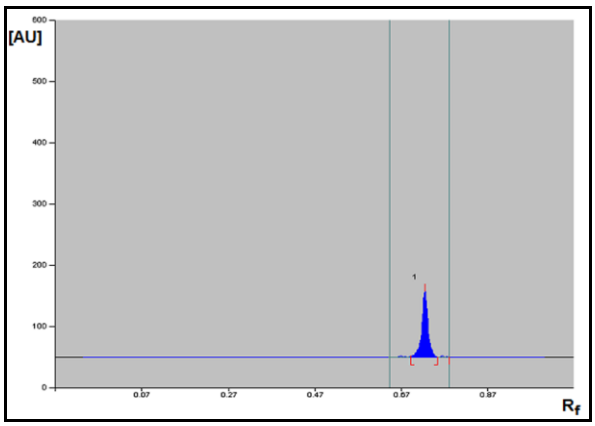

Fig. 4: Densitogram of aqueous *Bacopa monnieria* extract.

Experimental results for the amount of aqueous fraction of *Bacopa monnieria* in marketed Brahmi syrup, expressed as percentage of label claim were in good agreement with the label claims. This indicated that there was no interference from any of the excipients and supplements which are normally present in ayurvedic medicinal products. The proposed HPTLC method estimated 99.19% w/w *Bacopa monnieria* of the label claimed from the marketed Brahmi syrup.

Table 2: Accuracy studies for the determination of aqueous brahmi extract (n=6)

Analyte	Marketed Syrup Label Claim (ng/spot)	Plant Extract Amount added (ng/spot)	Total amount (ng/spot)	Found amount ± SD (ng/spot)	% Recovery	% RSD
Aqueous Brahmi Extract	7500	6000 (80%)	13500	13741.56±0.47	100.59	0.62
	7500	7500 (100%)	15000	14960.33±0.93	99.73	0.17
	7500	9000 (120%)	16500	16392.47±0.26	99.34	0.34

Table 3: Intraday and interday precision of aqueous brahmi extract (n=3)

Analyte	Intraday			Interday		
	Conc. (ng/spot)	Found conc. ± SD (ng/spot)	% RSD	Conc. (ng/spot)	Found conc. ± SD (ng/spot)	% RSD
Aqueous Brahmi Extract	4000	3965.69 ± 14.47	1.7605	4000	4015.43 ± 27.67	0.6519
	8000	7939.44 ± 24.91	1.5988	8000	7923.44 ± 31.05	0.8714
	12000	11909.16 ± 32.51	0.80504	12000	11909.16 ± 26.71	1.2004

Robustness

The standard deviation of peak areas was calculated for each parameter and % RSD was found to be less than 2%. Lower values of % RSD (Table 4) indicated robustness of the method.

Table 4: Robustness testing of the HPTLC method (n=6)

Parameters	SD of peak area	% RSD
Mobile phase composition (±0.2 ml)	5.92	0.93
Amount of mobile phase (±5%)	2.47	1.27
Time from spotting to chromatography (+10 min)	5.71	1.54
Time from chromatography to scanning (+10 min)	4.86	0.59
Development distance (±2 cm)	3.22	1.12
Duration of saturation (±10 min)	4.18	1.33

CONCLUSIONS

The developed HPTLC technique is accurate, precise, specific and significant for routine analysis and quality control of aqueous fractions of dried *Bacopa monnieria* powder. This validated procedure can be selectively used in the assay of Brahmi from various ayurvedic formulations without interference from the auxiliary substances.

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