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Original article

HPLC analysis and standardization of Brahmi vati – An Ayurvedic poly-herbal formulation

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ABSTRACT

Objectives: The aim of the present study was to standardize Brahmi vati (BV) by simultaneous quantitative estimation of Bacoside A_3 and Piperine adopting HPLC–UV method. BV very important Ayurvedic polyherbo formulation used to treat epilepsy and mental disorders containing thirty eight ingredients including *Bacopa monnieri* L. and *Piper longum* L.

Materials and methods: An HPLC–UV method was developed for the standardization of BV in light of simultaneous quantitative estimation of Bacoside A_3 and Piperine, the major constituents of *B. monnieri* L. and *P. longum* L. respectively. The developed method was validated on parameters including linearity, precision, accuracy and robustness.

Results: The HPLC analysis showed significant increase in amount of Bacoside A_3 and Piperine in the in-house sample of BV when compared with all three different marketed samples of the same. Results showed variations in the amount of Bacoside A_3 and Piperine in different samples which indicate non-uniformity in their quality which will lead to difference in their therapeutic effects.

Conclusion: The outcome of the present investigation underlines the importance of standardization of Ayurvedic formulations. The developed method may be further used to standardize other samples of BV or other formulations containing Bacoside A_3 and Piperine.

1. Introduction

Ayurveda, the ancient system of plant based medicines is gaining recognition throughout the world and many herbal drugs are now clinically tested and accepted for manufacturing in present scenario.[1,2] Ayurvedic formulations have been used to cure the diseases in both humans and animals for centuries in the Indian subcontinent. These formulations are prepared by particular traditional processing methods which involve the use of several herbs and minerals. The Ayurvedic formulations are available in different forms such as powder, decoction, fresh juice, vati, oil, clarified butter preparations and alcoholic preparation.[3] As per Ayurvedic Formulary of India (AFI), Brahmi vati (BV) is comprised of Brahmi (*Bacopa monnieri*) and another thirty-seven herbal and animal ingredients along with nine bhasmas. BV is one of the most important formulations used to cure mental disorders, epilepsy as well as to improve the memory. This polyherbo-mineral formulation also has cardio-protective and antipyretic actions but still scientific evidences are lacking. The ingredients involved in the preparation of BV are presented in Table 1.[4] The whole herb of Brahmi (*B. monnieri* L.) is used for alleviating the mental and cardiovascular conditions since ancient time. In a recent study, the alcoholic extract of *B. monnieri* enhanced the learning ability in rats.[5,6] Alcoholic extract of *B. monnieri* has shown cognition facilitating effect in normal rats and inhibited the amnesic effects of scopolamine and immobilization stress.[7,8] In behavioral response studies, alcoholic extract of *B. monnieri* facilitated the cognitive function and augmented the mental retention capacity.[9]

The chief plant ingredient of BV, Brahmi (*B. monnieri* L., family Scrophulariaceae) is a native plant of India. The plant is reported to contain steroidal saponin, alkaloid and glycosides etc.[10] Number of formulations containing Bacoside as active constituent has been already in market as memory plus, Brahmi Ghrita, Sarasvati ristha, Ratnagiri rasa and Smritisagar rasa. *Piper longum* fruits, another chief constituent of BV, have alkaloids as the main constituent.[11] Bacoside A_3 the main triterpenoid saponins now regarded as responsible for the characteristic neuropharmacological effects of the B. *monnieri*.[12] Piperine, the main alkaloid of *P. longum*, protects against neurodegeneration and cognitive impairment in animal model of cognitive deficit like condition of Alzheimer's disease.[13]

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Table 1
Ingredients of Brahmi vati formulation.

Name	Botanical name/Source	Part used	Quantity
Abhraka bhasma	Calcined Biotite mica	–	6 g
Sangeyasaba pisti	Processed Jadite	–	6 g
Akik bhasma	Calcined agate	–	6 g
Manikya pisti	Processed ruby	–	6 g
Chandrodaya	Calcined sulfur and mercury	–	6 g
Pravala bhasma	Calcined red coral	–	6 g
Kaharuba pisti	Processed amber	–	6 g
Svarna bhasma	Calcined gold foil	–	6 g
Mukta bhasma	Calcined pearl	–	6 g
Jayaphala	*Myristica fragrans* Houtt.	Seed	4 g
Lavanga	*Eugenia caryophyllata* Thunb.	Flower bud	4 g
Kustha	*Saussurea lappa* C.B. Clarke.	Root	4 g
Jatipatri	*Myristica fragrans* Houtt.	Aril/Mace	4 g
Krsnajiraka	*Carum carvi* Linn.	Fruit	4 g
Pippali	*Piper longum* Linn.	Fruit	4 g
Tvak	*Cinnamomum zeylanicum* Blume.	Stem bark	4 g
Anisuna	*Pimpinella anisum* Linn.	Fruit	4 g
Ashwagandha	*Withania somnifera* Dunal.	Root	4 g
Akarkara	*Anacyclus pyrethrum* DC.	Root	4 g
Dhanyaka	*Coriandrum sativum* Linn.	Fruit	4 g
Vansalochana	*Bambusa arundinacea* Retz. Willd.	Silicious concretion	4 g
Ela	*Elettaria cardamomum* Linn.	Seed	4 g
Sankhapuspi	*Convolvulus pluricaulis* Choisy.	Plant	4 g
Sveta Chandana	*Santalum album* Linn.	Heart wood	4 g
Surpha	*Foeniculum vulgare* Mill.	Fruit	4 g
Patra	*Cinnamomum tamala* Buch. Ham.	Leaf	4 g
Nagakesara	*Mesua ferrea* Linn.	Androcium	4 g
Rumimastagi	*Pistacia lentiscus* Linn.	Exudates	4 g
Pippalimula	*Piper longum* Linn.	Root	4 g
Chitraka	*Plumbago zeylanica* Linn.	Root	4 g
Kulinjana	*Alpinia galanga* Willd.	Rhizome	4 g
Kasturi/Lata kasturi	*Hibiscus abelmoschus* Linn.	Seed	18 g
Amber	Ambergris	Substance produced from digestive system of sperm whales	18 g
Brahmi	*Bacopa monnieri* Linn.	Plant	18 g
Nisotha	*Operculina turpethum* Linn.	Root	18 g
Aguru	*Aquilaria agallocha* Roxb.	Heart wood	18 g
Kumkuma	*Crocus sativus* Linn.	Stigma	18 g
Brahmi svarasa	*Bacopa monnieri* Linn.	Plant	Q.S.

The aim of the present study was to develop high performance liquid chromatography–ultra violet (HPLC–UV) method to separate and quantify the Bacoside A_3 (the main constituent of Brahmi) and Piperine (main constituent of *P. longum*) from BV samples as a tool for standardization. The proposed method was validated as per guidelines of the International Conference on Harmonization (ICH).[14–16] The structures of Bacoside A_3 and Piperine are presented in Fig. 1. The content of Bacoside A_3 in *B. monnieri* and Piperine in *P. longum* were also evaluated to get the individual percent yield so that expected yield in the formulation can also be calculated.

The fingerprint analysis by HPLC/HPTLC is considered as the most important approach in standardization of the Ayurvedic product involving marker compound.[17] In a recent study, HPLC technique has been used to estimate eugenol from different marketed Ayurvedic formulation as commercial formulations like *Caturjata Churna*, *Lavangadi Vati*, *Jatiphaladi Churna*, *Sitopaladi Churna* and clove oil.[18] Recently, the researchers have developed HPTLC analytical profile of Brahmi Ghrita: A polyherbal Ayurvedic formulation.[19] Therefore, fast, sensitive and accurate quality control tests for Ayurvedic formulations are desired which will be in alignment with these modern technologies.[20] Keeping in view these facts, the Ayurvedic polyherbo-mineral formulation – BV was prepared using the guidelines as per AFI. The separation of the Bacoside A_3 and Piperine was performed on isocratic HPLC system equipped with UV detector. Quantitative estimation of Bacoside A_3 and Piperine were performed at 345 nm. The HPLC analysis of in-house Brahmi vati (IBV) and three marketed samples (BV1, BV2 and BV3) suggested difference in chromatographic patterns.

2. Materials and methods

2.1. Reagents

The solvents used were of HPLC grade and were used without further purification. Water HPLC grade (Batch No. LO9A/0609/2912/53), Acetonitrile HPLC grade (Batch No. E10A/0210/2005/53) and Acetic acid for HPLC (Batch No. B10A/0610/0302/53) were purchased from S D Fine Chemical Limited, Mumbai. Methanol HPLC grade (Batch No.888168043) was purchased from Qualigens Fine Chemicals, Mumbai. The reference compounds, Piperine was purchased from Sigma Aldrich. Bacoside A (consisting of Bacoside A_3 and A_2 in 18% and 81% ratio respectively) was procured as a gift sample from Indian Institute of Integrative Medicine, Jammu, India.

2.2. Preparation of Brahmi vati

For the preparation of Brahmi vati, all minerals and metals were processed to get bhasma and pistis. Bhasmas were prepared by traditional process, involving two steps – shodhana (purification) and maran (calcinations) of gem/mineral/metals with specified plant materials.[21] Akik bhasma, Abhrak bhasma, Praval bhasma and Mukta bhasma were prepared by this process. Kaharuba pisti, Manikya pisti and Sangeyasaba pisti were prepared by triturating the minerals with specified plant materials. Chandrodaya and Swarna bhasma were purchased from Ayurvedic Pharmacy, Institute of Medical Sciences, Banaras Hindu University, Varanasi. All bhasma and pistis were packed in glass bottles, labeled and stored in cool and hygienic place. Afterward, all the pulverized plant materials were sieved to find respected fine powders. For preparation of BV, powdered Chandrodaya, Saffron, *Abelmoschus* and Ambara were mixed together. In this, one by one bhasma and pistis were added and mixed well. In last, the powder mixture was mixed with fresh juice of *B. monnieri* and ted. Pills about 250 mg were prepared by hand rolling, dried in shade and packed in sterilized polyethylene pouches, labeled as IBV and stored in a cool and hygienic place.[22]

2.3. Marketed samples

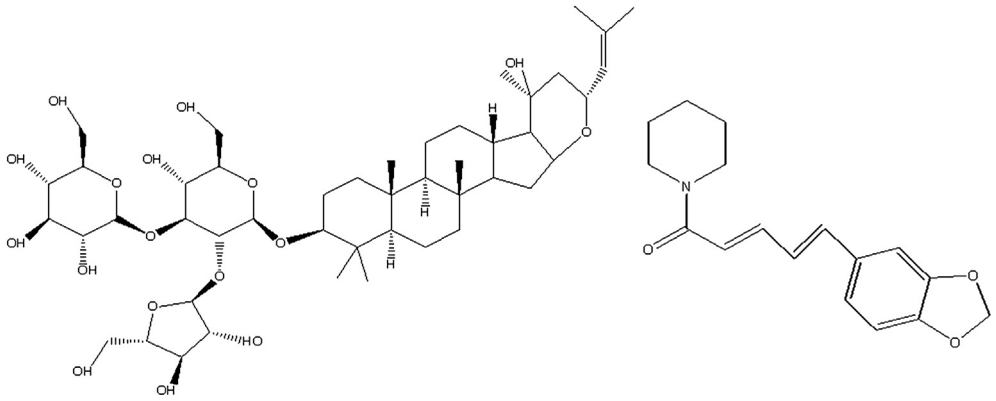
Three marketed samples of BV, of three different manufactures were purchased from local market and labeled as BV1, BV2 and BV3.

2.4. HPLC system and conditions

The HPLC system (Shimadzu Co., Japan), consisting LC-20AT pump, UV detector (Shimadzu SPD-20 A), Rheodyne 7725 I (CA, USA) manual injector with 20 μl loop and phenomenex C-18(2) column (250 × 4.6 μm ID, 5 μm) with a compatible guard column was used. The mobile phase consisted of Sodium acetate buffer and Acetonitrile (65:35 v/v), pH 3.2 adjusted with acetic acid The mobile phase was filtered through a 0.45 μm cellulose nitrate filter membrane and was degassed prior to use.

2.5. Sample preparation for HPLC analysis

Dried *P. longum* fruits were powdered and 1 g of this was extracted in 250 ml of HPLC grade methanol. The extract was



(i) Bacoside A3 (ii) Piperine

Fig. 1. Structure (i) Bacoside A_3, (ii) Piperine.

filtered and 0.1 ml of this was transferred to a 100 ml volumetric flask and volume was made up to the mark. The resulting solution was used for quantification. Dried *B. monnieri* whole plant was powdered and 1 g of this was extracted in 100 ml of HPLC grade methanol. The extract was filtered and 0.2 ml from this was transferred to a 100 ml volumetric flask and volume was made up to the mark. The resulting solution was used for quantification.

20 tablets (250 × 20 = 5000 mg = 5 gm) of each formulation including IBV, BV1, BV2 and BV3 were extracted in HPLC grade 500 ml methanol separately. The extracts were filtered through 0.45 μm filter (Millipore). 0.1 ml from above were transferred in a 10 ml volumetric flask and volume was made up to 10 ml. 20 μl of the prepared samples were injected for quantification.

2.6. Preparation of calibration curves

To prepare standard solution of Bacoside A, 1 mg was accurately weighed and dissolved in 100 ml of HPLC grade methanol. Volumes of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1 ml of this solution were transferred into 10 ml volumetric flask and volume was made up to 10 ml with methanol. This yielded the solution of Bacoside A in the concentration range 100–1000 ng/ml. The Bacoside A_3 is only 18% in each dilution, thus concentration of the marker in terms of Bacoside A_3 is 18–180 ng/ml, 20 μl of each solution were injected to injection loop and run for calibration curve of Bacoside A_3. For Piperine, 1 mg of Piperine was accurately weighed and dissolved in 1 ml of HPLC grade methanol. From this solution 100 μl was transferred to a 100 ml volumetric flask and volume was made up to 100 ml. Volumes of 0.2, 0.4, 0.6 and 0.8 ml of this solution were transferred into 10 ml volumetric flask and volume was made up to 10 ml. 20 μl of each solution were injected and run for calibration curve of Piperine.

2.7. Method validation

The validity of the proposed HPLC method was assessed by linearity, precision, accuracy and robustness, limit of detection (LOD) and limit of quantification (LOQ). The linearity for Bacoside A_3 was 18–126 ng/ml whereas for Piperine, it was 20–80 ng/ml. Precision was studied to find out intra and interday variations in the method for Bacoside A_3 and Piperine two times onsame day and different day, respectively. The % RSD was calculated which should be <2%. Intraday precision was done on the same day and interday precision was done on the different day. % RSD was calculated for each case. Accuracy was performed by recovery study of both the marker compounds as known amount of standard component was added into preanalyzed sample and subjects them to the proposed HPLC method. The study was carried out at two different concentration levels for both the markers. Findings are presented in Table 3.

For robustness study, changes in mobile phase (from 65:35 to 63:37 and 67:33) were made and any change in retention time was measured. Limit of detection (LOD) and limit of quantification (LOQ) were verified as per the International Conference on Harmonization (ICH) guidelines.[14]

3. Results and discussion

3.1. HPLC conditions optimization

The aim was to develop a method for the quantitative determination of two important constituents of BV which are Bacoside and Piperine. These ingredients are common in various Ayurvedic formulations as Brahmi vati, Brahmi Rasayana and Brahmi Ghrita etc. There is no HPLC–UV method reported till date for simultaneous estimation of Bacoside A_3 and Piperine. In the present work, HPLC method was developed, validated and top priority was given for complete separation of Bacoside A_3 and Piperine from Brahmi vati (BV).

The mobile phase was chosen after several trials, which consisted of Sodium acetate buffer and Acetonitrile (65:35 v/v), pH 3.2 adjusted with acetic acid was finally selected in order to achieve optimum separation, high sensitivity and good peak shape. The detection wavelength was chosen at 345 nm since Bacoside A_3 and Piperine have better absorption and sensitivity at this wavelength.

In the present study, a shorter run time (30 min) and complete separation of Bacoside A_3 and Piperine was achieved. The retention time for Bacoside A_3 and Piperine were found to be 6.83 min and 9.52 min respectively. The chromatogram also showed several other unidentified peaks. The peaks in the chromatogram of formulations were identified by comparison of the retention time with those corresponding to the marker compounds. Fig. 2 represents characteristic chromatogram of standard Bacoside A_3 and Piperine and Fig. 3 represents peaks of Bacoside A_3 and Piperine in Brahmi vati formulation (IBV).

3.2. Calibration and method validation

The HPLC method was validated by defining the linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy

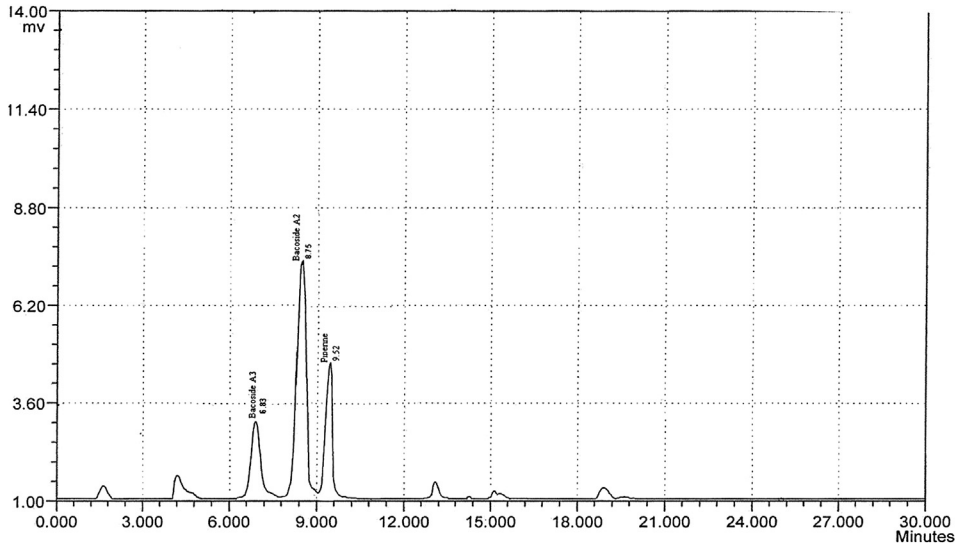


Fig. 2. Chromatogram representing peaks of Bacoside A_3 and Piperine as marker compound.

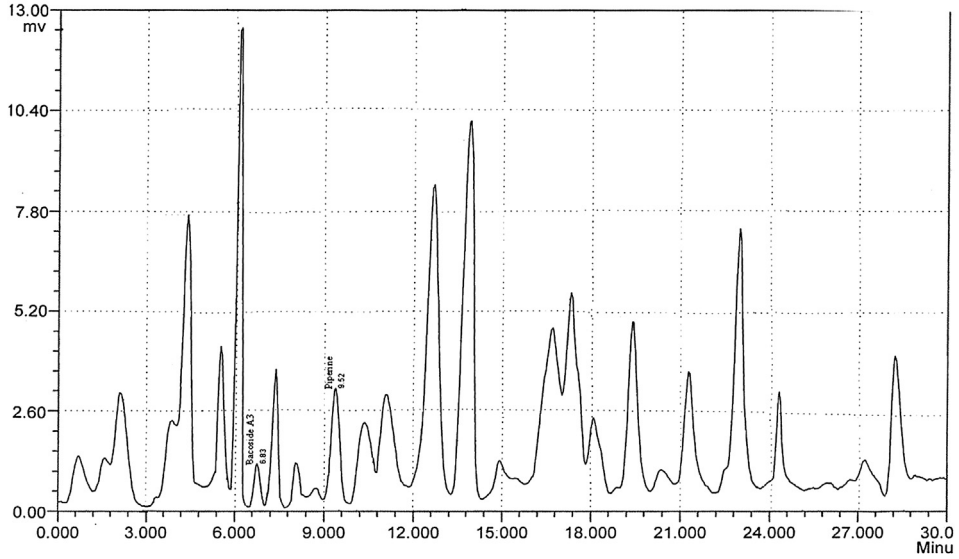


Fig. 3. Chromatogram representing peaks of Bacoside A_3 and Piperine in Brahmi vati formulation (IBV).

and robustness. The method was evaluated by determining the precision in the retention time of both markers in a standard sample as well as in the marketed formulations and a low RSD value (0.55% and 0.29%) indicated high precision of the method. It was observed that the other constituents present in the formulation did not interfere with any of the markers indicating specificity of the method.

A linear relationship between peak areas and concentrations was observed over given range for both compounds (Table 2). Standard solutions of Bacoside A (A_3 and A_2 in 18% and 81% ratio respectively) in the range of 100–1000 ng/ml were prepared which consists of 18–180 ng/ml Bacoside A_3 and of Piperine in the range of 10–100 ng/ml were prepared and analyzed. The regression equations of these curves are shown in Table 2 and their coefficients of regression (R^2) were 0.998 confirming the linearity of the method. A signal three times higher than noise was regarded as the detection limit. The LOD value for Bacoside A_3 and Piperine were found to be 4 ng/ml and 5 ng/ml and LOQ value were found to be 13.2 ng/ml and 16.5 ng/ml respectively.

The intra and interday precisions (expressed as RSD) and accuracy (expressed as recovery) for the two analytes were determined by spiked samples with the standard solutions ($n = 6$),

Table 2
Linearity, LOD, LOQ, regression curves of the HPLC method.

Parameter/Marker	Linearity R range (ng/ml)	LOD (ng/ml)	LOQ (ng/ml)	Calibration curve	R^2	tR (min)
Bacoside A_3	18–180	4	13.2	$Y = 572.5X + 326.5$	0.998	6.83 ± 0.015
Piperine	20–80	5	16.5	$Y = 110.1X - 61.96$	0.998	9.52 ± 0.011

Table 3
Intraday and interday precision and accuracy.

Marker	Intraday		Interday	
	Mean ± SD (ng/ml)	RSD (%)	Mean ± SD (ng/ml)	RSD (%)
Bacoside A_3	71.5 ± 1.049	1.46	71 ± 0.894	1.25
Piperine	37.83 ± 0.752	1.98	38.33 ± 0.516	1.34

consecutively, using the analytical method described above. The coefficient variations of intra and interday studies were both less than 2.0%. The results of the recovery of Bacoside A_3 and Piperine ranged between 98.0 and 101.66%. The precision as well as the reproducibility of this method was satisfactory (Tables 3 and 4). The robustness of the method was investigated under changed conditions which include changes of mobile phase composition. Changes in mobile phase (from 65:35 to 63:37 and 67:33) showed robustness of the method as insignificant change in retention time was observed and % RSD was less than 2 in each case (Table 5). The degree of reproducibility of the results obtained as a result of small deliberate variations in the mobile phase composition has proven that the method is robust.

The in-house and marketed samples of BV were analyzed by the present method.

3.3. RP-HPLC analysis and quantitation of markers in in-house and marketed samples of Brahmi vati

RP-HPLC analysis of the prepared formulation showed the presence of many peaks due to the presence of several ingredients in BV. Two markers, Bacoside A_3 and Piperine were identified and separated in this formulation. The amounts of these marker constituents present per g of the formulation are given in Table 6.

Chromatogram of in-house preparation showed a large number of peaks and differs markedly from marketed samples in number as well as peak area. The larger number of peaks in in-house preparation is may be due to the presence of several ingredients in this formulation. Use of exhausted material, wrong material, storage conditions, different altitude and area of drug collection and adoption of different manufacturing processes are the expected regions behind the difference in chromatograms of in-house and marketed samples of the same formulation.

The Bacoside A (Bacoside A_3 and A_2) is a saponin from *B. monnieri* and reported to have antiepileptic, anticancer, hepatoprotective, memory enhancing and antidepressant activities.[23–27] Piperine, an alkaloid found in *P. longum* L. reported to have antidepressant, antioxidant, anti-inflammatory, antihypertensive, antitumor, hepatoprotective and anti-asthmatic properties.[28] Presence of adequate amount of these two marker compounds in BV establishes its identity and ensures the quality and efficacy of the formulation. In the result of quantitative analysis, *B. monnieri* showed the presence of 0.273% of Bacoside A_3 and *P. longum* (PL) showed the presence of 1.487% of Piperine (Table 6).

The BV formulation of 250 mg contains 18 mg of BM and 6 mg of PL. So by this data the expected amount of Bacoside A_3 and Piperine must be 196.56 μg and 356.88 μg per g of BV. The result of quantitative analysis revealed that IBV and BV1 formulations have Bacoside A_3 and Piperine in 5% range of the expected value while, BV2 and BV3 have less than −5% values of the expected amount. The present study revealed that the marketed samples have lack of uniformity in Bacoside A_3 and Piperine content. There are different variety of Brahmi and Piper plants available having a different percent yield of Bacoside A and Piperine respectively. Use of plant materials having a low percent yield of secondary metabolites may result in a low quality formulation. Another reason responsible for lack of uniformity, may be the unsuitable storage condition, of raw materials and the finished products which can reduce the active constituents of the formulation.

Table 4
Recovery studies of Bacoside A_3 and Piperine.

Marker	Basal amount (ng)	Amount added (ng)	Amount recovered (ng)	% Mean recovery
Bacoside A_3	72	72	142.99	98.61
	72	90	160.99	98.88
Piperine	50	50	99.00	98.00
	50	60	110.97	101.66

Table 5
Robustness of the method by changing the composition of the mobile phase of Bacoside A_3 (54 ng/ml) and Piperine (40 ng/ml).

Mobile phase composition			Bacoside A_3		Piperine	
Sodium acetate buffer:Acetonitrile			Mean tR ± SD	% RSD	Mean tR ± SD	% RSD
Original	Used	Levels				
65:35	63:37	−2	6.84 ± 0.08	1.28	9.52 ± 0.13	1.41
	65:35 Original	0	6.83 ± 0.03	0.43	9.52 ± 0.02	0.27
	67:33	+2	6.81 ± 0.09	1.34	9.53 ± 0.14	1.53

Table 6
Quantification of Bacoside A_3 and Piperine in the in-house and marketed formulations of Brahmi vati

	Bacoside A_3 Mean(μg/gm) ± S.D, $n = 6$	Piperine Mean(μg/gm) ± S.D, $n = 6$
BM	2733 ± 32.890	–
PL	–	14,870 ± 64.443
IBV	192 ± 3.742	343 ± 17.527
BV1	188 ± 4.336	340.5 ± 13.065
BV2	95.33 ± 3.559	152.16 ± 13.288
BV3	71.66 ± 4.320	126 ± 11.662

BM: *B. monnieri*, PL: *P. longum*, IBV: In-house standard preparation, BV1–BV3: Marketed samples.

4. Conclusion

The results obtained indicate that there is a lack of uniformity in the amount of marker compounds present in the same formulation marketed by different manufactures. So there should be a set of standards for every traditional formulation. The developed method is fast, precise and accurate and may be useful for evaluation of quality of BV in future.

Conflicts of interest

All authors have none to declare.

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