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Research Article

Marker Based Standardization of Extracts and Formulations of *Achyranthes aspera*

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ABSTRACT
There is great demand of herbal medicines in the developed as well as developing countries as they are considered to be effective and safe. Although they are as efficacious as allopathic medicines and safer as compared to allopathic medicines, they are not globally accepted. The major reason behind this fact is that they lack proper standardization technique. The present paper discusses a new, simple, accurate and reproducible analytical method using betaine as marker. This developed and validated method was applied for marker based standardization of extracts and marketed formulations containing *Achyranthes aspera.* The method employs C18 column as stationary phase and acetonitrile: water (10:90, v/v) as mobile phase. Flow rate of the mobile phase was kept at 1.0 ml/min and detection was carried out at 205 nm. The developed method was validated as per ICH guidelines. Linearity was obtained over the range of 15-70 µg/ml. LOD and LOQ were found to be 5 µg/ml and 15 µg/ml, respectively. The recovery of betaine was between 107.28 % -108.35 %. The method is reproducible and robust. The developed and validated method can be used as quality control tool for the analysis of extract and herbal formulations containing *Achyranthes aspera* using betaine as marker.
KEY WORDS: *Achyranthes aspera,* Betaine, Herbal formulations, Marker based standardization

INTRODUCTION

Herbal drugs also referred to as botanicals, biomedicines or herbal supplements are used for the prevention and treatment of various health ailments since ancient times.[1] Herbal drugs are essential part of traditional medicines in several countries including China and India.[2,3] India has a well-established and ancient system of medicine known as Ayurveda. WHO has also identified the importance of traditional medicine and has created strategies, guidelines and standards for botanical medicines.[4]

The major hurdle in the wider acceptability of these plant-based medicines across the globe is that they lack proper standardization technique. Therefore, proper identification and standardization techniques using modern analytical methods have to be adopted for manufacturing of herbal formulations. There is an urgent need to provide the evidence of quality of herbal medicines in terms of standardization using sophisticated methods. It is imperative to develop fast, sensitive, accurate and reproducible method of analysis for standardization of herbal formulations. One of the methods for ensuring quality of herbal drugs is marker- based standardization which involves identification of major and unique component/s in herbs known as markers and development of appropriate method for their analysis using sophisticated techniques.

Achyranthes aspera Linn., commonly known as Apamarga, is a variable, erect, annual to perennial herb. It can grow up to 1.5 m high. The genus name is derived from Greek word *achyr* means chaff or bran and the species name refers to the rough texture of spikes.[5-8] In India it is distributed along roadsides and waste places.[9]Wwhole plant, leaves, seeds, roots,
flowers and fruits are used for medicinal purpose.[10] In the Vedas, Apamarga has been described as a divine medicine. It is famous as a herbal lithotriptic agent (that breaks the urinary stones) and is a diuretic. It is also used as bitter tonic, expectorant, carminative, digestive, anti-inflammatory and blood-purifier. Juice of fresh leaves is given in diarrohoea.[11-13] Two main alkaloids, betaine and achyranthine have been identified from this plant. Other chemical constituents include hentriacontane, ecdysterone and two glycosides of oleanolic acid and amino acids.[6] Achyranthine is yellow coloured semisolid which is highly hygroscopic and betaine is present in substantial amount and can be used as marker for standardization of extracts and formulations containing *Achyranthes aspera.*

MATERIALS AND METHODS

Reagents and standards: All the solvents and purified water were of HPLC grade from S. D. Fine chemicals, Mumbai, India. All the

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Table 1: Effect of mobile phase composition on retention time and peak symmetry

Mobile phase composition (v/v)	Retention Time(R_T) (min)	Peak Symmetry
Methanol/water (65/35)	2.855	0.521
Methanol/water (25/75)	2.812	0.568
Methanol/water (80/20)	3.019	0.494
Acetonitrile/water (50/50)	2.400	0.580
Acetonitrile/water (25/75)	2.603	0.589
Acetonitrile/water (75/25)	3.051	0.419
Acetonitrile/water (10/90)	2.793	0.606

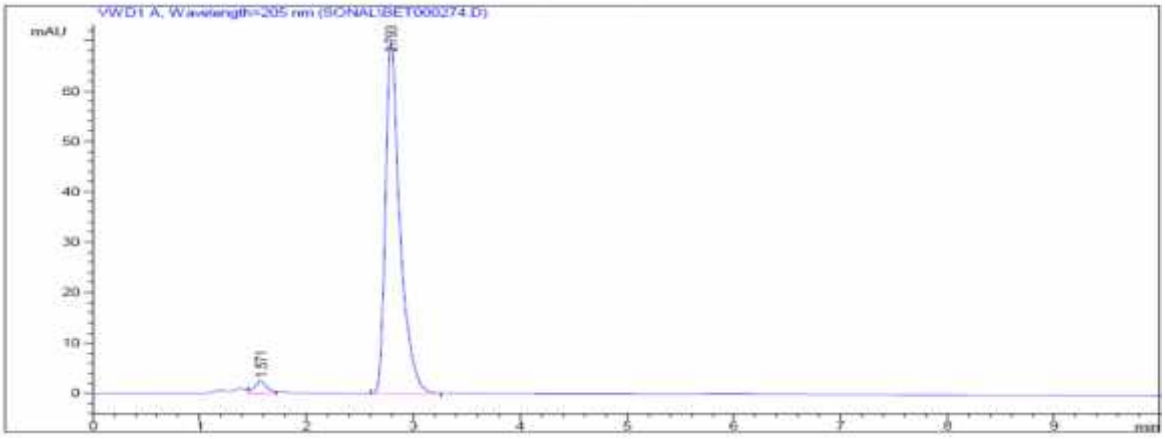

Fig. 1: HPLC chromatogram of standard betaine

Table 2: Results of system suitability parameters for betaine

Compound	Betaine	Standard limits
Number of plates (N)	2405	> 2000
Tailing factor (T)	0.69	< 2.0
R.S.D. of Retention time (t_R)	0.4712	< 2.0 %
R.S.D. of area	0.909	< 2.0 %

Table 3: Results of quantitative determination of betaine

Parameters	Results
Concentration range (µg/ml)	15-70
Regression equation	y = 0.9124x−0.9274
Correlation coefficient (r^2)	0.9998
Limit of Detection (LOD)	5.0 µg/ml
Limit of Quantitation (LOQ)	15.0 µg/ml

Table 4: Percentage of recovery, to evaluate the accuracy of the method for betaine

Component	Amount Present[a] (µg/ml)±S.D.	Amount Found[a] (µg/ml)± S.D.	% Recovery[a]± S.D.
Betaine	30.8653±0.26	33.4434±0.15	108.3591±1.22
	38.6962±0.24	41.7096±0.12	107.7913± 0.97
	46.2987±0.87	49.6580±0.08	107.2807 ± 1.99

[a] *n=3, triplicate injections*

solutions were filtered through 0.2 µ PTFE filter (Goettingen, Germany). Standard of betaine (Purity 98.0 % w/w) was purchased from Sigma-Aldrich, St. Louis, Missouri, USA.

Instrument: HPLC analysis was carried out using model of Agilent Technologies Ltd 1200 series comprising of degasser (G1322A), quaternary pump (G1311A), variable wavelength detector (G1314B) and manual injector (G1328B). Data was analysed by using ChemStation software version B.04.01.

Preparation of solutions

Preparation of standard solution: A stock solution of 1000 ppm was prepared by dissolving 100 mg of standard betaine in 100 ml of mobile phase (acetonitrile: water, 10:90, v/v). From this stock solution, a working standard of 100 ppm was prepared. Appropriate aliquots of working standard of betaine was taken in 10 ml volumetric flasks and diluted up to mark with mobile phase to obtain final concentrations of 15, 20, 30, 40, 50, 60 and 70 µg/ml.

Preparation of sample solution: Betaine was isolated from dried seeds of *Achyranthes aspera* using solvent extraction technique.[14] Isolated betaine (10 mg) was dissolved in 100 ml of mobile phase by sonication for 10 min to prepare a stock solution of 100 µg/ ml. From this, various aliquots were taken and diluted with appropriate volume of mobile phase to produce different concentrations which were used to validate the method.

Preparation of sample solutions for marketed formulations

Tablets (Brand I): Twenty tablets were individually weighed; their mean weight was determined and the tablets were triturated. Accurately weighed 20 g of tablet triturate was transferred to 250 ml volumetric flask containing 200 ml of acidified ethanol and extracted. The resulting acidic ethanol extract was made alkaline with ammonia to precipitate the alkaloids. The precipitates were filtered, dried and dissolved (1.245 g) in 5 ml of mobile phase, sonicated for 10 min. Accurately measured 0.02 ml of

Table 5: Validation results for intraday and interday precision studies for betaine

Component	Amount level (µg/ml)	Intra day (% R.S.D.)[a]	Inter day (% R.S.D.)[a]	
		Day 1	Day 1	Day 2
Betaine	20	1.5025	0.3534	0.3361
	40	1.0352	0.7178	1.1830
	60	0.4787	0.4327	0.3288

[a] *n=3, triplicate injections*

Table 6: Results of robustness study for betaine

Variable	Mean Retention time±S.D.	Retention time % R.S.D.	Mean peak area ±S.D.	Peak area % R.S.D.
Mobile phase Composition (v/v)				
Acetonitrile: Water (10.5:89.5)	2.806±0.00	0.2316	32.9589±0.44	1.3635
Acetonitrile: Water (9.5:90.5)	2.826±0.00	0.1415	33.4416±0.24	0.7197
Flow rate (ml/min)				
0.9	3.127±0.00	0.1751	35.2452±0.35	1.0095
1.1	2.552±0.00	0.3016	27.7383±0.29	1.0743

[a] *n=6, six injections*

Table 7: Percent content of betaine in extracts and formulations containing *Achyranthes*

Extracts & Formulations	% w/w of betaine[a] ± S.D.	% mg of betaine[a] ± S.D.
Extract I (Gujarat)	0.0801±0.01	-
Extract II (Tamilnadu)	0.0688±0.00	-
Extract III (Maharashtra)	0.0566±0.00	-
Tablet (Brand –I)	6.3805±0.82	1.9200±0.24/Tablet
Tablet (Brand –II)	5.8078±0.49	2.1743±0.18/Tablet
Tablet (Brand –III)	18.3382±0.99	5.4627±0.29/Tablet

[a] *n=3, triplicate injections*

above solution was further diluted to 10 ml with mobile phase, sonicated, filtered and injected in HPLC.

Tablets (Brand II): Twenty tablets were individually weighed; their mean weight was determined and the tablets were triturated. Accurately weighed 20 g of tablet triturate was transferred to 250 ml volumetric flask containing 200 ml of acidified ethanol and extracted. The resulting acidic ethanol extract was made alkaline with ammonia to precipitate the alkaloids. The precipitates were filtered, dried and dissolved (1.156 g) in 5 ml of mobile phase, sonicated for 10 min. Accurately measured 0.02 ml of above solution was further diluted to 10 ml with mobile phase, sonicated, filtered and injected in HPLC.

Tablets (Brand III): Twenty tablets were individually weighed; their mean weight was determined and the tablets were triturated. Accurately weighed 20 g of tablet triturate was transferred to 250 ml volumetric flask containing 200 ml of acidified ethanol and extracted. The resulting acidic ethanol extract was made alkaline with ammonia to precipitate the alkaloids. The precipitates were filtered, dried and dissolved (1.032 g) in 5 ml of mobile phase, sonicated for 10 min. Accurately measured 0.02 ml of above solution was further diluted to 10 ml with mobile phase, sonicated, filtered and injected in HPLC.

Method development: To determine λ_{max} of betaine, UV spectrum of 10 µg/ml of standard betaine was carried out. Various combination of methanol with water and acetonitrile with water were tried and their effects on peak symmetry and peak shape were observed.

Method validation [15]**:** The developed method was validated according to the ICH guidelines.

System suitability studies were carried out to confirm that the proposed method was able to produce results with high reproducibility. It was evaluated by injecting six replicates of standard solutions of betaine and analyzing various parameters such as peak area, number of theoretical plates (N) and tailing factor (T). To determine the linear relationship, appropriate aliquots of betaine stock solutions were taken in 10 ml volumetric flasks and diluted up to mark with mobile phase to obtain final concentrations of 15-70 µg/ml. Duplicate injections using 20 µl loop were made and chromatograms were recorded at 205 nm. Quantitation was carried out by keeping peak area and concentrations of compound to straight line equation and correlation coefficient (r^2) was determined. The LOD and LOQ were calculated based on signal to noise ratio method. Betaine solutions in increasing concentrations were injected until signal to noise ratio of 3:1 and 10:1 were obtained for determination of LOD and LOQ, respectively. The accuracy of the method was evaluated through the analyte recovery test at three concentration levels. Known amount of sample was spiked with 32, 40 and 48 µg/ml of the standard solutions of betaine and % recovery was found out by following formula: % Recovery= Measured value /True value X 100. The intraday and interday precision studies were carried out by estimating responses on same day and on different day for three different concentrations (20, 40 and 60 µg/ml) and

results obtained interms of relative standard deviation (% R.S.D.). The robustness of the developed method related to the variation in retention time and area was studied by changing the mobile phase composition and flow rate. The developed and validated method was applied to determine betaine content in three extracts and different brands of marketed tablets containing *Achyranthes aspera.* For extracts, seeds of *Achyranthes aspera* were procured from three different geographical regions of India (Gujarat, Tamilnadu and Maharashtra) and extracts were prepared.

RESULTS AND DISCUSSION

λ_{max} of betaine was found to be 205 nm which was used as detection wavelength. Acetonitrile: water in ratio of 10: 90 gave peak symmetry of 0.6 and better peak shape than other mobile phase combinations at flow rate of 1.0 ml/min (Table 1).

Thus the following chromatographic conditions based on retention time, peak symmetry and number of theoretical plates were selected:

Column: C_{18} (Waters, 300 mm X 3.9 mm, 5µm)
Mobile Phase: Acetonitrile: Water (10:90)
Flow rate: 1.0 ml/min
Detection Wavelength: 205 nm

Betaine eluted at mean retention time of 2.793 min (Figure 1). The above developed method was validated as per ICH guidelines for various parameters such as system suitability, specificity, linearity, LOD, LOQ, accuracy, precision and robustness. The results of system suitability studies in comparison with the required limits are shown in Table 2. A linear relationship was found between concentration and area in the range of 15-70 µg/ml. The results are tabulated Table 3. Accuracy was found to be in the range of 107.28 to 108.35 % (Table 4). The low intra and interday R.S.D. obtained during repeatability studies indicates that the proposed method is precise (Table 5). Robustness was evaluated by deliberately changing method parameters and their effect on peak area and retention time was observed. Only one parameter was altered at a time keeping the other parameters constant. RSD was lower than 2.0 % for the method parameters such as mobile phase composition and flow rate indicating that the developed method was robust (Table 6). The validated method was applied for quantitation of betaine in different extracts and marketed formulations. The % w/w of betaine was found to be higher in Extract I. In case of analysis of formulations, brand-III of tablets contained higher amount of betaine (Table 7).

CONCLUSIONS

A simple, rapid, accurate and precise method has been successfully developed and validated for betaine. The developed method was applied for quantitative determination of betaine in three extracts and different brands of tablets containing *Achyranthes aspera.* This method can be used as an analytical tool for standardization of extracts and herbal medicines containing *Achyranthes aspera* using betaine as marker compound.

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