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MARKER BASED STANDARDIZATION OF FORMULATIONS CONTAINING *ASHWAGANDHA* USING WITHAFERIN A BY HPLC

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ABSTRACT

A high performance liquid chromatography (HPLC) method for the determination of Withaferin A in *Withania somnifera* formulations was developed. The developed and validated method was successfully applied to analyze the content of Withaferin A in different marketed formulations containing *Ashwagandha*. The method comprised of a simple isocratic system with reserve phase (RP)-18 column, acetonitrile and water as mobile phase and Withaferin A was detailed at 215 nm. The optimized mobile phase enabled the efficient separation of various components present in formulations within a 10 min analysis, with a retention time of 6.3 minutes for Withaferin A. The method was validated as per International conference of harmonization (ICH) guidelines with a view to demonstrate its selectivity, linearity, precision, accuracy and robustness. The linearity range was 100-1000 ng mL^{-1} and the limit of detection (LOD) and limit of quantification (LOQ) were found to be 30 and 90 ng respectively. The accuracy assay performed revealed a recovery of 96.43-106.57 % from the various marketed formulations analyzed. The inter-day and intra-day reproducibilities were found to have an R.S.D. less than 0.1 %, indicating very good precision.

Key words: *Ashwagandha*, *Withania somnifera*, Withaferin A, standardization, high performance liquid chromatography.

INTRODUCTION

In recent years, there has been great demand for plant derived products in developed countries. These products are increasingly being sought out as medicinal products,

nutraceuticals and cosmetics. [1] There are around 6000 herbal manufacturers in India. More than 4000 units are producing Ayurveda medicines. [2]

Commercialization of the manufacture of these medicines to meet this increased demand has caused a decline in their quality, primarily due to a lack of adequate regulations pertaining to herbal medicines. Hence, the need of the hour is to evolve a systematic approach and to develop well-designed and validated methodologies for the standardization of herbal formulations. [3, 4]

This can be achieved only if the herbal products are evaluated and analyzed using sophisticated modern techniques of standardization such as UV- visible, TLC, HPLC, HPTLC, GC-MS, spectrofluorimetric and other methods. [5]

One of the quality control methods using these techniques is by selecting a known active constituent or a marker compound as the qualitative and quantitative target to assess the authenticity and inherent quality. [6, 7]

Withania somnifera (L.) Dunal. (Solanaceae) is a valued herb in Ayurveda, an ancient system of medicine, and as such was used and cultivated for centuries in India. It is locally known as *Ashwagandha* which means ‘smells like a horse’. The plant is sometimes referred to as Indian ginseng. Roots, leaves and preparations thereof are traditionally used as tonic, hypnotic, sedative and diuretic. [8-11]

In Indian market there are many products containing *Ashwagandha* with different therapeutic benefits. The products are available in form of traditional Ayurvedic preparations as well as modern formulations. Ayurvedic formulations such as solid dosage forms (vati, pills, powders), liquid dosage forms (asavas, aristhas) and semisolid dosage forms (ghritas, avlehas) are available. Modern dosage form such as capsules, tablets containing *Ashwagandha* powder and /or extract also exist in market.

Withanolides are specific for the Solanaceae family, in particular for the genus *Withania*, and are used as marker. Withanolides are ergostane type steroids, with atoms C-22 and C-26 bridged by d-lactone functionality and an oxidized C-1 position. [12] Withaferin A is one of the most important Withanolides to which the curative properties of the plants are attributed. [13]

High Performance liquid chromatography (HPLC) is considered as one of the most sensitive reliable methods of analysis. There are number of analytical methods reported for analysis of Withaferin A by HPLC and HPTLC. [14-18] But, the reported methods are very time consuming with very much advanced instrumentation like DAD and ELSD detector and with gradient solvent system. Due to these reasons, herbal manufacturers are reluctant to adopt these methods for the routine analysis of their products.

Thus, we aim at a simple, rapid, accurate and precise HPLC method for standardization of commercial formulations using Withaferin A as a potential marker which can be easily employed in quality control testing of *Ashwagandha* formulations.

EXPERIMENTAL

Solvents and chemicals

All the solvents of analytical reagent grade and were purchased from Rankem and S.D. Fine Chemicals, Mumbai India. Solvents used for HPLC analysis were of HPLC grade and they were filtered and degassed before use. Withaferin A standard compound was obtained from Natural Remedies Bangalore, India. Commercially available brands of *Ashwagandha* Aristha, *Ashwagandha* Vati, *Ashwagandha* Churna, *Ashwagandha* Tablets and *Ashwagandha* Capsules were procured from local market.

Equipment

Isocratic RP-HPLC was performed using Shimadzu LC-2010HT chromatograph equipped with quaternary low-pressure gradient unit pump, high throughput autosampler and UV-VIS detector. A Phenomenex-C18 reverse-phase analytical column (150 mm × 4.6 mm, i.d., 5 µm particle size) was used. The data were analyzed using the EZChrom software.

Chromatographic conditions

The mobile phase was a binary mixture of Acetonitrile–water, 4:6 (v/v). The flow rate was $1mLmin^{-1}$. Absorbance was seen at 215 nm, λmax of Withaferin A.

Preparation of Standard solutions

10.0 mg of Withaferin A was dissolved in 100 mL methanol, yielding a stock solution concentration of 0.1 mg mL^{-1}. Working standard solution of concentration of 1.0 μgmL^{-1} was prepared by diluting 1ml of stock solution to 100 mL with mobile phase. Series of dilutions were prepared by transferring aliquots of 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0

mL of the working standard solution and diluting with the mobile phase to yield 10 mL of standard solutions containing 50, 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 ng mL^{-1}, respectively.

Preparation of Sample solutions

For Ashwagandha 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 Ashwagandha churna

Two brands of churna namely Brand I and Brand II were analyzed.

2 g of *churna* (each brand) 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 Ashwagandha Capsule

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

For Ashwagandha 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 Ashwagandharishta

Two brands of *ashwagandharishta* namely Brand A and Brand B were analyzed and the sample preparation was as follows.

10 mL of *Ashwagandharishta* (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.

Assay validation

The developed RP-HPLC method was validated according to the ICH guidelines. [19-28] All measurements were performed in triplicates.

Calibration studies

Linearity was evaluated in the range 100–1000 ng mL^{-1}. 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 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 developed method was evaluated by repeatability (intra-day) and intermediate precision (inter-day). Each level of precision was investigated by three sequential replicates of injections of Withaferin A at concentrations of 200, 400 and 600 ng mL^{-1}. Repeatability was evaluated on the same day, while intermediate precision was determined by comparing the assays for 2 days.

Accuracy studies

Accuracy studies were carried out through the percentage recoveries of known amounts of Withaferin A added to solutions of extracts of commercial products. The analyzed samples were spiked with 80, 100 and 120 % of 400 ng of standard solution. 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, flow rate and detection wavelength, were intentionally varied to determine their influence on the retention time and quantitative analysis. Intraday variability was studied for the sample, by injecting the same concentration of the sample in triplicate and the standard error mean was calculated.

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.

RESULTS AND DISCUSSION

Method optimization

Conditions giving the shortest and optimum retention time (*tR* = 6.3 min) with no apparent drug decomposition were selected as the optimized conditions for analysis. Analysis was carried out with C_{18} reverse-phase analytical column. Mobile phase was Acetonitrile–water 4:6 (v/v) with flow rate 1mL min^{-1} and detection wavelength 215nm. A representative chromatogram indicating a well isolated peak is shown in (Fig 1).

Method validation

Linearity, limit of detection and quantitation

Under the above described experimental conditions, linear correlation between the peak area and applied concentration was obtained in the concentration range of 100–1000 ng mL^{-1}, as confirmed by the correlation coefficient of 0.9988. The peak area (y) is proportional to the concentration of Withaferin A (*x*) following the regression equation y = 4019x as shown in (Fig 2). The experimentally derived LOD and LOQ for Withaferin A were determined to be 30 and 90 ng mL^{-1}, respectively.

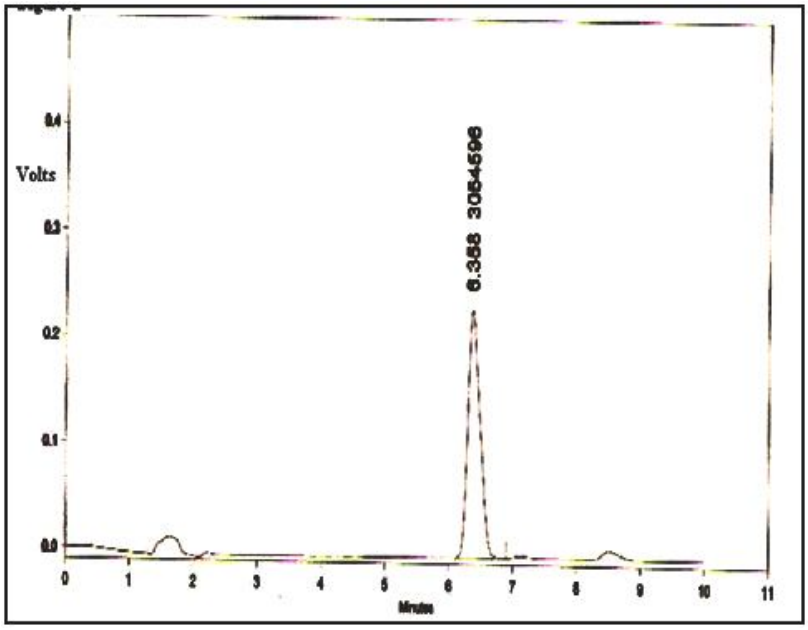

Figure 1: Typical HPLC chromatogram of Withaferin A marker

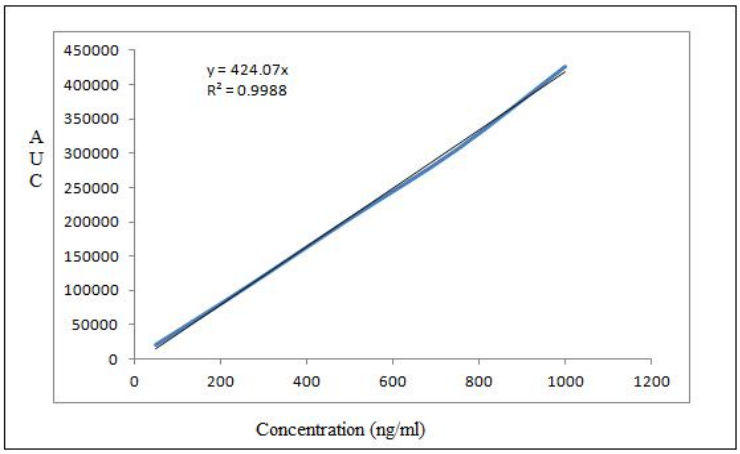

Figure 2: Calibration Curve for Withaferin A

Table 1: Results of precision studies of Withaferin A

	Concentration (ng/ml)	Withaferin A (RSD%)
Intra- day precision	200	0.041
	400	0.047
	600	0.058
Inter - day precision	200	0.052
	400	0.049
	600	0.050

Precision

Precision data on the intra-and inter-day variation for three different concentration levels are summarized in (Table 1). Both inter-and intra-day R.S.D. were less than 2% (0.041-0.058), indicating very good precision.

Accuracy

All the samples of various formulations were spiked with the known amount of standard, and the percent ratios between the recovered and expected concentrations were calculated. The satisfied recoveries of 96.43–106.57 % indicate that the proposed HPLC method is reliable for the quantification of Withaferin A in all seven commercial formulations, (Table 2).

Table 2: Recovery studies of Withaferin A in various formulations

Formulations	**Amount added (% of 400 ng)**	**Recovery ± S.D. (%)**
Vati	80	104.18 ±0.11
	100	104.79 ±0.32
	120	104.56 ±0.09
Capsules	80	101.07 ±0.20
	100	101.15 ±0.41
	120	101.11 ±0.05

Tablets	80	106.29 ±0.31
	100	106.01 ±0.17
	120	106.57 ±0.07
Churna (Brand A)	80	104.88 ±0.13
	100	104.17 ±0.73
	120	104.44 ±0.34
Churna (Brand B)	80	103.99 ±0.22
	100	104.01 ±0.54
	120	104.24 ±0.55
Arishta (Brand I)	80	99.77 ±0.10
	100	99.39 ±0.23
	120	99.12 ±0.12
Arishta (Brand II)	80	96.43 ±0.09
	100	97.39 ±0.13
	120	96.87 ±0.17

Robustness

The mobile phase composition was altered by ± 5 % changes in the ratio of acetonitrile – water, 4:6 (v/v). No changes was observed in retention time and peak shape.

The method was found to be robust at different detection wavelength viz., 211nm, 213 nm, 215nm and 217nm

Among the studied factors, the flow rate in the range of 0.8 – 1.2 $mLmin^{-1}$ has influenced the retention time but there was no significant impact on peak area.

Analysis of commercial formulations

The developed and validated method was used to ascertain the content of Withaferin A in seven marketed formulations containing *Ashwagandha*. The shapes of the peaks were not altered by substances present in the matrix indicating that the method is suitable for analysis. The percent content of Withaferin A was found to be 0.511 ± 0.5 %, 0.759 ± 0.4% and 0.789 ± 0.25% in *Ashwagandha* vati, capsule and tablet respectively. Thus the modern solid dosage forms of *Ashwagandha* contained higher amount of the bio marker Withaferin A as compared to traditional vati formulation.

The two different brands of churna showed 0.770 ± 0.37% and 0.811 ± 0.4% percent content of Withaferin A. The amount of Withaferin A was found to be 0.150% ± 0.3 and 0.144 % ± 0.4 in two different brands of *Ashwagandharishta*.

In traditional Ayurvedic formulations of *Ashwagandha*, highest amount of Withaferin A was found in churna formulation as compared to vati and arishta. *Ashwagandharishta* showed the

least percentage of Withaferin A.

Stability of Withaferin A in the sample solutions was evaluated at 4°C and 25°C for 3 days to verify whether spontaneous degradation occurred. The results were calculated as the percentage of non-degraded Withaferin A at the specified time intervals. All formulations showed less than 4% degradation indicating that the samples were stable at 4° C and 25°C for 3 days.

CONCLUSION

A simple, rapid, accurate and convenient HPLC method was developed and validated for estimation of content of Withaferin A in various *Ashwagandha* formulations. This method can be applied for standardization of products containing *Withania somnifera*. This validated method can be used to determine batch to batch variations and routine analysis by herbal manufacturers of *Ashwagandha*. Thus, these analytical standardization techniques facilitate herbal manufacturers to market their medicines globally with defined content of respective bioactive/s and to ensure its quality.

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