RESEARCH ARTICLE

An Innovative HPTLC-based Approach for the Quantification of Withaferin A in Combinations of Herbal Compounds

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ABSTRACT

Withaferin A in herbal mixed dose forms was quantified utilizing a new destructive reagent that is quick, accurate, and economical. Toluene, ethyl acetate, and formic acid were mixed in a mobile phase with a ratio of 5:4:2 v/v/v, and separation was carried out at a detection wavelength of 254 nm. In accordance with ICH guidelines, the procedure was verified. A range of 90 to 630 ng/band was determined for the calibration. The method was validated in three separate experiments with varying concentrations of the standard additive. The limit of detection (LoD) of 2.43 ng and the limit of quantitation (LoQ) of 7.54 ng were noted. During system adaptability testing, %RSD was under 2. Using the same approach, unchanged, on nine different formulations proved the method's separation and quantification efficiency.

Keywords: Withaferin A, HPTLC, Validation, Herbal formulation.

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INTRODUCTION

The Ayurvedic medical system embraces *Withania somnifera* herb in high regard due to its numerous advantageous uses, including as a tonic, sedative, diuretic, and immunomodulatory agent.[1] The plant also has excellent antioxidant, antistress, hypoglycemic, diuretic, and hypochlesteromic properties.[2] There are several distinct types of chemical compounds found in *W. somnifera*. Withanolides are the most medicinally significant[3]. To identify and quantify the active components of *Withania,* numerous analytical approaches have been developed, including as thin layer chromatography (TLC), high-performance thin-layer chromatography (HPTLC), nuclear magnetic resonance (NMR), capillary electrochromatography, microfluidic electrochromatography, and many more.[4,5] *W.* in both its crude and refined pharmaceutical forms.[6] Applying the established analytical approach to different formulations did not yield the same results in terms of component separation and quantification. The overarching goal of this study was to establish and verify a reliable method for withaferin- A quantification that could be used across many formulations independently of one another.[7,8]

MATERIALS AND METHODS

Instruments

Many other tools were included in HPTLC's equipment, in addition to a 100 µL Hamilton syringe, a UV with dual wavelength UV light, a CamagLinomate five applicator, and a TLC scanner 3. The method development process made use of precoated TLC plates (20 X 10) made of silica gel G60F254.

Materials and Chemicals

All of the analytical-grade solvents were bought from Modern Science Labs Private Limited in Nashik, India. Natural Remedies Pvt. Ltd. made the acquisition of standard withaferin A. India, Ltd. The local market was scouted for polyherbal compositions.

Methods Used in Experiments

Making a withaferin a standard stock solution

Using a volumetric flask, a 300 µg/mL withaferin-A standard solution was made. This was accomplished by combining 3 mg of accurately measured withaferin-A with 10 mL of methanol to get the required volume.[9,10]

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Withaferin a calibration curve standard solution preparation

A final concentration of 90 µg/mL was achieved by properly diluting the standard stock solution with methanol. A percolated TLC plate was able to reach a concentration of 90 to 630 ng/band when the proper amounts were used.[11]

Sample solution preparation

The solution became colorless after refluxing a precisely measured amount of 2 g of *W. somnifera* in a hydro-alcoholic solvent (2:3 V/V) for 15 minutes each time. About 20 mL of hexane were used three times to remove the filtrate. About 10 mL of chloroform was used for each of the three extractions of the hydro-alcoholic extract. After drying off the chloroform extract, dissolve it in 10 mL of methanol.[12]

Validation Method

In accordance with International Council of Harmonization (ICH) guidelines, the procedure was verified.

Range and linearity

For this calibration curve, we utilized a withaferin-A concentration range of 90 to 630 ng/band.[13]

Precision

The method's accuracy was evaluated by determining the recovery of withaferin-A at three distinct levels (80, 100, and 120%) using the usual addition method. The examined 280 ng/band samples were spiked with 336, 280, and 224 ng/band of additional concentration, and the resulting mixes were scrutinized using the suggested approach. At each level, all samples underwent recovery analysis three times.[14,15]

Accuracy method reliability

Accuracy in measuring peak area following the suggested procedure, we ran seven separate cans of the same location with a solution of Withaferin A (270 ng/band) to ensure technique accuracy.[16,17]

Repeatability in the context of a sample

Applying the withaferin A test solution (270 ng/band) seven times and analyzing each time according to the suggested manner ensured procedure precision.[18]

Reproducibility, where precision is intermediatePrecision within and between days for the intra-day precision, we prepared three distinct concentrations (270, 360, and 450 ng/band) of withaferin A test solution and tested it three times using the proposed approach at different times throughout the same day, each time with fresh solutions. The intra-day precision was achieved by making three separate test solutions of withaferin A at varying concentrations (270, 360, and 450 ng/band) and then analyzing them at different times on different days using the proposed approach. Each time, new solutions were prepared.[19,20]

Detection and quantification limits

These equations were used to determine LoD and LoQ in accordance with the ICH guidelines.

LoQ = 10 N/S,

LoD = 3.3 N/S

In this case, S is the slope of the matching calibration curve, and N is RSD.[21]

Accuracy

To determine the peak purity, we compared the spectra at three separate levels: start (S), apex (A), and end (E). This allowed us to guarantee that the dosage form of Withaferin-A was free of powdered material and other contaminants.

Testing for System Appropriateness

Modifying the system slightly but intentionally allowed for testing of its appropriateness. The following percentages represent the relative standard deviation (RSD) after adjustments were made to the mobile phase composition in accordance with the specified limits: 30% for the minor solvent component, 2% for the absolute change, and 10% for the main solvent composition[22].

Evaluation of Medicinal Dose Form

Enlisted commercial dosage formulations were successfully subjected to the proposed validated approach. Applying Whatman filter paper no. 41 allowed the sample solution to pass through. Triple applications of 10 µL of each solution were made to the HPTLC plate, followed by development and drying. Scanning at 254 nm followed derivatization with anesaldehyde sulfuric acid and a 15-minute heating to 110°C. Based on the peak areas reported for the standard solutions, withaferin A's quantitative analysis was performed.[23,24]

RESULTS

Developing the Method

Selection of mobile phase

Considerations such as molecular weight, solubility, and sample type (ionic, ionizable, or neutral molecule) should be taken into account while choosing an HPTLC method.

The impact of chromatographic factors, including solvent ratio and mobile phase composition, was investigated in order to optimize the chromatographic settings. After that, we took notes on the chromatograms and determined the resolution and chromatographic retention factor. For the purpose of estimation, we chose the settings that produced the highest resolution and retention factor. A variety of solvent compositions were tested with varying amounts on both the standard and individual dose form solutions of withaferin A. The goal was to find a mobile

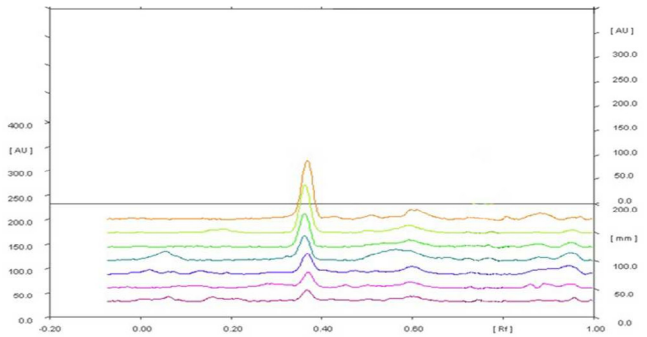

Figure 1: Chromatogram of withaferin-A

Table 1: Results of accuracy

Standard added (ng/band)	*Drug taken (ng/band)*	*Total Amount (ng/band)*	*Recovered Amount (ng/band) ± SD*	*%Recovery*
224	280	504.0	498.3 ± 01.4	98.80
280	280	560.0	559.7 ± 02.0	100.311
336	280	616.0	620.7 ± 02.1	101.091

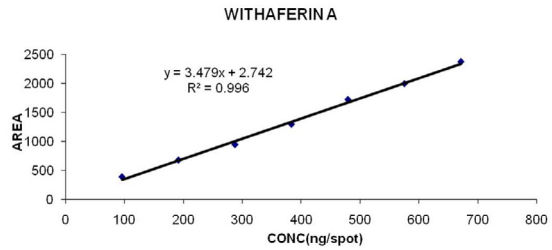

Figure 2: Calibration curve of withaferin-A standard solutions

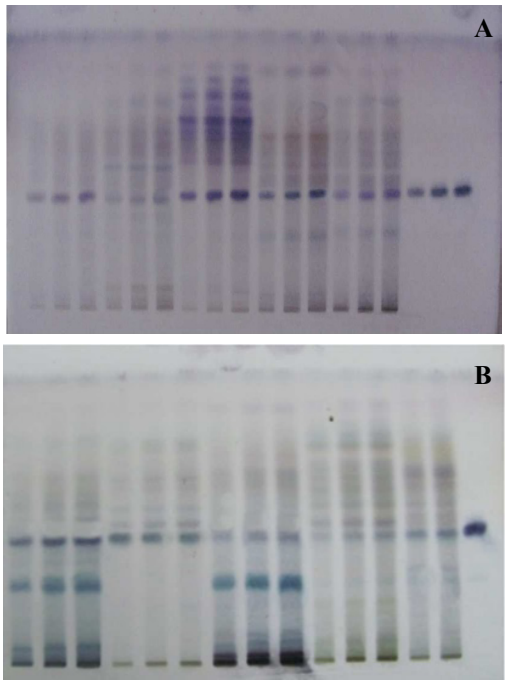

Figure 3 (A and B): Generated and modified plate solutions of several herbal blends applied in triplicate

phase that met the resolution requirements and could separate withaferin-A from dosage forms that were mixed. Finally, with ethyl acetate, toluene, and formic acid as solvent composition, all of the selected formulations showed encouraging outcomes. The ideal combination of toluene:ethyl acetate:formic acid was determined to be 5:4:2 V/V/V after minor tweaks to the selected solvents (Figure 1).

Validation of Methods

The approach underwent validation in accordance with the ICH recommendation. Inside the calibration range, the response was determined to be linear according to the regression equation y = 3.479x + 2.742 (1, 2) (Figure 2). The three independent recovery results ranged from 99.47 to 101.09. There was no interference from other components, hence the approach was

Table 2: Method validation data

Parameters	*Values*
Repeatability on peak area	0.46
Accuracy	99.47–101.09
Linearity and range	90–630 ng/band
Intraday precision	1.5–2.08
Repeatability on sample use	1.89
LoD	2.59 ng
Precision inter-day	1.49–2.27
Specificity	Specific
LoQ	7.87 ng
System suitability	Suitable (%RSD less than 2)

Table 3: Assay of marketed formulations

Code	*Product*	*Withaferin A (% W/W)*
M1	Tablets abana	2.85
M2	Capsule spark	2.65
M3	Capsule streswin	2.22
M4	Tablets Artho-N-	2.49
M5	Tablets sumenta	2.60
M6	Tablets amycordial	2.53
M7	Tablets ostolief	2.99
M8	Capsule bravobol	2.74
M9	Capsule motif	2.45

determined to be specific. Based on the results, the LoQ and LoD were determined to be 1.43 and 7.54 ng, respectively. Peak area measurement reproducibility was determined to be 0.46 and sample application repeatability to be 1.89. Deliberate adjustments to the mobile phase, pH, etc., had %RSD < 2 according to the system suitability data. This table summarizes the findings from the technique validation (Tables 1 and 2).

Determination of Pharmacological Dose Form

Withaferin A had an average Rf value of 0.35–0.39 ± 0.01. Nine distinct herbal combination dosage forms had their withaferin-A concentrations estimated using the established and validated approach[25] (Figure 3 a and b). Here is a table that summarizes the assay results (Table 3).

CONCLUSION

For the previously mentioned herbal combination dosage forms of withaferin-A, the procedure was determined to be exact, rapid, accurate, thorough, and economical. With no

interference from other ingredients or drugs in the product, the assay results demonstrate that this method is appropriate for regular evaluation of withaferin-A in its combination medicine forms.

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