



DEVELOPMENT AND VALIDATION OF A NOVEL HPTLC METHOD FOR SIMULTANEOUS ESTIMATION OF BETA-SITOSTEROL-D-GLUCOSIDE AND WITHAIFERIN A

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ABSTRACT

A new simple, precise, rapid and selective high-performance thin-layer chromatographic (HPTLC) method has been developed for the simultaneous determination of withaferin A and beta-sitosterol-D-glucoside in Ayurvedic formulations containing *Ashwagandha*.

The retention factors of withaferin A and beta-sitosterol-D-glucoside were 0.59 and 0.21, respectively. Linearity was obtained in the range of 5-50 $\mu\text{g mL}^{-1}$ for beta sitosterol- D-glucoside and 0.5-5 $\mu\text{g mL}^{-1}$ for withaferin A.

The developed and validated HPTLC method was employed for standardization of four Ayurvedic formulations for their content of the two markers. Satisfactory recoveries of 103.21-101.66 % and 97.86-99.01 % were obtained for withaferin A and beta-sitosterol-D-glucoside. The results obtained in validation assays indicate the accuracy and reliability of the developed simultaneous HPTLC method for the quantification of withaferin A and beta-sitosterol-D-glucoside in all four *Ashwagandha* formulations.

Keywords: *Withania somnifera*, Ashwagandha, Withaferin A, Beta-sitosterol-D-glucoside, HPTLC

INTRODUCTION

Ayurveda has been a lively system of health care in India with an unbroken practice since 6000 years but growth as an industry has commenced only a few years back. India's share in the global exports of herbal medicines is also low, at around 10 per cent only. Therefore, there is a need to transform Ayurveda into a dynamic, scientifically validated and evidence based industry which takes its roots from rich knowledge base of oral tradition and scriptures¹⁻³.

It is necessary to develop methods for rapid, precise and accurate identification and estimation of active constituents or marker compound/s as the qualitative and quantitative target to assess the authenticity and inherent quality⁴⁻⁵. Through various analytical techniques like TLC, HPLC and HPTLC we can ascertain the presence of these compounds in plants and also quantify them. HPTLC offers many advantages over other chromatographic techniques such as unsurpassed flexibility (esp. stationary and mobile phase), choice of detection, user friendly, rapid and cost effective⁶. Thus, HPTLC is most widely used at industrial level for routine analysis of herbal medicines.

Withania somnifera (L.) Dunal. (Solanaceae) is commonly known as *Ashwagandha*, is a valued herb in Ayurveda and cultivated for centuries in India⁷. The plant is sometimes referred to as Indian ginseng, which is reputed to have adaptogenic, tonic, analgesic, antipyretic, anti-inflammatory, hypnotic, sedative and diuretic and abortifacient properties⁸⁻¹⁴.

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, arishtas) 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.

By considering the demand of this herb, there is a need of simple and rapid analytical method for the manufacturer of plant- based medicines. Thus, the objective of the present work was to develop and validate a High Performance Thin Layer Chromatography method for simultaneous estimation of two biomarkers present in *Ashwagandha* viz., withaferin A and beta-sitosterol-D-glucoside.

MATERIALS AND METHODS

Solvents and chemicals

Standard Withaferin A was provided as a gift sample by Natural

Remedies Bangalore, India. Standard beta sitosterol- D-glucoside was provided as a gift sample by Amsar Pvt. Ltd., India. Traditional formulations containing *Ashwagandha* such as galenicals -Arishta, powders- Churna, tablet form- Vati and even modern dosage form such as Capsule were procured from the local market. All chemicals and reagents used were of analytical grade and purchased from Rankem and S. D. Fine Chemicals, India.

HPTLC instrumentation

The sample solutions were spotted in the form of bands of width 6 mm with a Camag microlitre syringe on precoated silica gel aluminium plate 60F₂₅₄ (20 cm × 10 cm with 250 μm thickness; E. Merck, Darmstadt, Germany, supplied by Anchrom Technologists, Mumbai) using a Camag Linomat V (Switzerland). The plates were pre-washed by methanol and activated at 60°C for 5 min prior to chromatography. A constant application rate of 1.0 $\mu\text{l/s}$ was employed and space between two bands was 5 mm. The slit dimension was kept at 5mm × 0.45 mm and 10 mm/s scanning speed was employed. The slit bandwidth was set at 20 nm, each track was scanned thrice and baseline correction was used. The mobile phase consisted of Chloroform - Methanol 8:2 (v/v) and 15 ml of mobile phase was used per chromatography. Linear ascending development was carried out in 20 cm × 10 cm twin trough glass chamber (Camag, Mut tenz, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 30 min at room temperature (25 °C ± 2) at relative humidity of 60% ± 5. The length of chromatogram run was 8 cm. Subsequent to the scanning, TLC plates were dried in a current of air with the help of an air dryer. Densitometric scanning was performed with Camag TLC scanner III in the reflectance-absorbance mode at 207 nm and operated by Win CATS software (1.3.0 Camag). Concentrations of the compound chromatographed were determined from the intensity of diffusely reflected light. Evaluation was carried out by comparing peak areas with linear regression.

Standard solutions

Standard stock solutions of withaferin A standard were prepared by dissolving 10.0 mg of withaferin A in methanol, yielding 100 mL of a concentration stock = 0.1 mg mL⁻¹. Working standard concentration of 10.0 $\mu\text{g mL}^{-1}$ was prepared by diluting 1ml of stock solution to 10 mL of methanol. Series of dilutions were prepared by aliquoting 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 diluted with the methanol to yield 10 mL of standard solutions containing 0.5, 1.0, 2.0, 3.0 4.0 and 5.0 $\mu\text{g mL}^{-1}$, respectively. Similarly, working standard concentration (100.0 $\mu\text{g mL}^{-1}$) of beta-sitosterol-D-glucoside was prepared by dissolving

10.0mg of beta- sitosterol- D-glucoside in 100 ml of methanol. Series of dilutions were prepared in same manner as of withaferin A to yield final concentration range from 5 to 50 $\mu\text{g mL}^{-1}$.

Sample solutions

Sample solutions were prepared by transferring 2 g of powder equivalent to the average mass of one *Ashwagandha* vati, capsule and churna into a 100 mL volumetric flask. 50 mL of methanol was added and the mixture was macerated on shaker for 24 hrs at room temperature. The flask was then filled to volume with methanol and the extract was filtered through a whatman filter paper. The filtrates were used as sample solution of respective formulations.

Sample solution *Ashwagandharistha* was prepared by drying 10 mL of the content completely and transferring 2 gm of the residue to 50 mL volumetric flask. 25 mL of methanol was added and the mixture was sonicated for one hour. Thereafter, it was filled up to volume with methanol and the solution was filtered through Whatman No. 1 filter paper. The filtrate was then used as sample solutions.

Assay validation

The proposed HPTLC method was validated according to the International Conference on Harmonization guidelines¹⁵⁻²⁴. All measurements were performed in triplicates.

Calibration studies

Linearity was evaluated in the range of 5-50 $\mu\text{g mL}^{-1}$ for beta sitosterol- D-glucoside and 0.5 -5.0 $\mu\text{g mL}^{-1}$ for withaferin A. 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 method was evaluated by repeatability (intra-day) and instrumental precision. Each level of precision was investigated by three sequential replicates of injections of withaferin A and beta sitosterol- D-glucoside at concentrations of 200, 300 and 400 ng mL⁻¹ and 20, 30 and 40 μL^{-1} respectively.

Accuracy studies

In order to evaluate the validity of the proposed method, accuracy was evaluated through the percentage recoveries of known amounts of mixture of withaferin A and beta sitosterol- D-glucoside added to solutions of all commercial products. The analyzed samples were spiked with 80, 100 and 120 % of 0.4 μg withaferin A and 40 μg of beta sitosterol- D-glucoside standard solution. Accuracy was calculated from the following equation:

$$\left[\frac{\text{spiked concentration} - \text{mean concentration}}{\text{spiked concentration}} \right] \times 100.$$

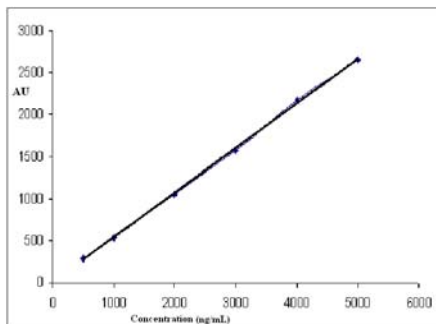


Fig 2: Calibration curve of withaferin A

Robustness

For the determination of the robustness of method, chromatographic parameters, such as mobile phase composition 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

Method optimization

The proposed method gave very good separation and resolution between peaks of the two standards, withaferin A (R_f value = 0.59) and of beta sitosterol- D-glucoside (R_f value = 0.21) as indicated in (Fig. 1).

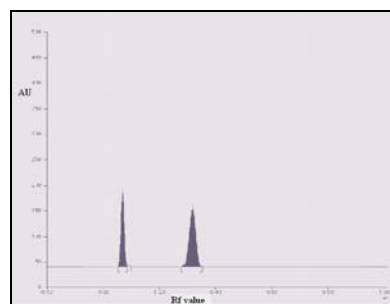


Fig. 1: HPTLC Chromatogram of withaferin A and beta-sitosterol-D-glucoside

Method validation

Linearity, limit of detection and quantitation

Under the above described experimental conditions, linear correlation between the peak area and applied concentration was found to occur in the concentration range of 0.5-5.0 $\mu\text{g mL}^{-1}$ and 5-500 $\mu\text{g mL}^{-1}$ for withaferin A and beta-sitosterol-D-glucoside. The correlation coefficient of withaferin A and beta sitosterol- D-glucoside was found to be 0.9991 and 0.9995 respectively. The peak area (y) is proportional to the concentration of withaferin A (x) following the regression equation $y = 0.5326x + 4.4055$ (Fig 2) and for beta-sitosterol-D-glucoside $y = 0.0403x + 140.45$ (Fig 3). The experimentally derived LOD and LOQ for withaferin A and beta sitosterol- D-glucoside were determined to be 100 and 300 ng mL⁻¹ and 10 and 30 $\mu\text{g mL}^{-1}$, respectively.

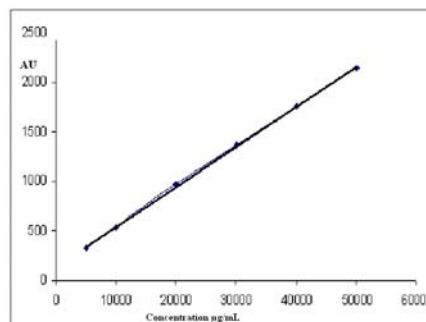


Fig 3: Calibration curve of beta-sitosterol-D-glucoside

Precision

Precision data on repeatability (intra-day) and instrumental variation for three different concentration levels are summarized in Table 1. Both precision studies showed R.S.D. less than 1%, indicating a sufficient 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. Satisfactory recoveries of 101.66-103.21 % and 97.86-99.01 % for withaferin A and beta-sitosterol-D-glucoside respectively indicate that the proposed simultaneous HPTLC method is reliable for the quantification of withaferin A and beta-sitosterol-D-glucoside in all four Ashwagandha formulations (Table 2).

Robustness

The mobile phase composition was altered by ± 2 % changes in the

ratio of chloroform and methanol 8:2 (v/v) and also in detection wavelength (207nm). No changes were observed in retention time and peak shape.

Analysis of Ashwagandha formulations

Validity of the proposed method was applied to standardization for both traditional and modern dosage forms viz. *Ashwagandha* Vati, Arishta, Churna and Capsule. The shape of the peaks was not altered by other substances present in the matrix. The percent content of both viz., withaferin A and beta-sitosterol-D-glucoside for all four formulations are indicated in Table 3.

Stability studies

Stability of withaferin A and beta-sitosterol-D-glucoside in the sample solutions was evaluated to verify whether spontaneous degradation occurred within 3 days. The results were calculated as the percentage of non-degraded content of both the standards at the 21, 48, 72 hours. All formulations showed less than 5% degradation at both investigated temperature (Table 4, 5).

Table 1: Results of precision studies

| | Concentration ($\mu\text{g/ml}$) | | (%RSD) | |
|------------------------|------------------------------------|-----------------------------|--------------|-----------------------------|
| | Withaferin A | Beta-sitosterol-D-glucoside | Withaferin A | Beta-sitosterol-D-glucoside |
| Intra- day precision | 2.0 | 20.0 | 0.006 | 0.007 |
| | 3.0 | 30.0 | 0.057 | 0.069 |
| | 4.0 | 40.0 | 0.059 | 0.067 |
| Instrumental precision | 2.0 | 20.0 | 0.046 | 0.057 |
| | 3.0 | 30.0 | 0.043 | 0.053 |
| | 4.0 | 40.0 | 0.048 | 0.051 |

Table 2: Results of recovery studies

| Formulations | Amount added | | Recovery \pm S.D. (%) | |
|--------------|--------------------------------|---|-------------------------|-----------------------------|
| | Withaferin A (μg) | Beta-sitosterol-D-glucoside (μg) | Withaferin A | Beta-sitosterol-D-glucoside |
| Vati | 3.2 | 32 | 103.21 \pm 0.28 | 98.67 \pm 0.10 |
| | 4.0 | 40 | 104.01 \pm 0.12 | 98.21 \pm 0.13 |
| | 4.8 | 48 | 103.92 \pm 0.31 | 99.01 \pm 0.12 |
| Capsule | 3.2 | 32 | 102.44 \pm 0.12 | 97.13 \pm 0.09 |
| | 4.0 | 40 | 102.38 \pm 0.11 | 97.59 \pm 0.11 |
| | 4.8 | 48 | 103.01 \pm 0.23 | 98.87 \pm 0.27 |
| Churna | 3.2 | 32 | 101.98 \pm 0.11 | 98.06 \pm 0.22 |
| | 4.0 | 40 | 101.36 \pm 0.19 | 98.35 \pm 0.25 |
| | 4.8 | 48 | 102.21 \pm 0.13 | 98.88 \pm 0.19 |
| Arishta | 3.2 | 32 | 103.37 \pm 0.16 | 99.00 \pm 0.09 |
| | 4.0 | 40 | 103.76 \pm 0.11 | 98.79 \pm 0.21 |
| | 4.8 | 48 | 102.99 \pm 0.16 | 98.97 \pm 0.30 |

Table 3: Results of percent content

| Formulations | Percent Content \pm S.D. (%) | |
|--------------|--------------------------------|-----------------------------|
| | Withaferin A | Beta-sitosterol-D-glucoside |
| Vati | 0.698 \pm 0.09 | 0.278 \pm 0.15 |
| Capsule | 0.437 \pm 0.21 | 0.293 \pm 0.07 |
| Churna | 0.458 \pm 0.19 | 0.234 \pm 0.16 |
| Arishta | 0.137 \pm 0.13 | 0.250 \pm 0.11 |

Table 4: Results of stability studies of withaferin A in formulations

| Formulations | Temperature | | | | | |
|--------------|-------------|--------|--------|--------|--------|--------|
| | 4°C | | | 25°C | | |
| | 24 hrs | 48 hrs | 72 hrs | 24 hrs | 48 hrs | 72 hrs |
| Vati | 99.02 | 98.79 | 98.07 | 98.87 | 98.15 | 97.54 |
| Capsule | 98.22 | 97.83 | 97.46 | 98.01 | 97.87 | 97.01 |
| Churna | 98.01 | 97.54 | 96.91 | 97.89 | 97.07 | 96.50 |
| Arishta | 97.89 | 97.03 | 96.75 | 97.51 | 96.94 | 96.17 |

Table 5: Results of stability Studies of Beta-sitosterol-D-glucoside in formulations

| Formulations | Temperature | | | | | |
|--------------|-------------|--------|--------|--------|--------|--------|
| | 4°C | | | 25°C | | |
| | 24 hrs | 48 hrs | 72 hrs | 24 hrs | 48 hrs | 72 hrs |
| Vati | 99.45 | 98.99 | 97.07 | 97.91 | 97.09 | 96.11 |
| Capsule | 98.22 | 97.99 | 96.37 | 97.33 | 96.82 | 96.09 |
| Churna | 97.89 | 97.01 | 95.99 | 97.11 | 96.06 | 95.74 |
| Arishta | 98.61 | 98.13 | 96.55 | 98.32 | 97.97 | 96.17 |

DISCUSSION

A simple, rapid, accurate and convenient method was developed for the first time, for simultaneous estimation of withaferin A and beta-sitosterol-D-glucoside by HPTLC. This method was used to standardize different Ayurvedic medicines containing *Ashwagandha*.

This developed and validated HPTLC method can be used to determine batch to batch variations and routine analysis by herbal manufacturers of *Ashwagandha* formulations.

Thus, these analytical standardization techniques facilitate manufacturers to market their plant based medicines with defined content of respective bioactives and to ensure its quality.

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