DEVELOPMENT OF VALIDATED RP-HPLC METHOD FOR THE
ESTIMATION OF WITHAFERIN – A IN WITHANIA SOMNIFERA, ITS
EXTRACT AND POLYHERBAL FORMULATION.

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ABSTRACT
A simple, rapid and specific reversed phase HPLC method has been developed for the
estimation of Withaferin - A in Polyherbal formulation containing Withaferin - A and its
extract. HPLC analysis was performed on C18 column using mixture of Acetonitrile and
Methanol containing 0.05 % Triethylamine as isocratic mobile phase at a flow rate of 1.0
ml per minute at detection wavelength of 227 nm. The method was validated for
accuracy, precision, linearity, specificity and sensitivity in accordance with International
conference on Harmonization guidelines. Validation revealed the method is specific,
accurate, precise, reliable and reproducible. Good linear correlation coefficients (r2>
0.999) was obtained for calibration plots in the range of 5 – 30 µg/ml. Intraday and
Interday RSD of retention times and peak areas were less than 2.0 %. Average Percent
Recovery was 97.31 %. The method was successfully used for quantitative analysis of
this marker compound in Polyherbal formulation.

Keywords: HPLC; Withaferin A, Withaniasomnifera, Indian ginseng, Polyherbal
formulation.

INTRODUCTION
Traditional medicine from all the ancient civilizations has come upfront during the last
decade, throughout the world as pressing need for the alternatives is mounting [1]. There
is some overlap in the herbal and Ayurvedic medicines. Effort is on to provide the
scientific validity to these medicines by applying the methodology of modern medicine.
Withaniasomnifera (L.) Dunal. (Solanaceae) is a valued herb, up to 1.5 m high shrub with
ovate leaves and greenish-yellow flowers can be found in western India and is locally
known as Ashwagandha [2]. The dried root and preparation thereof are traditionally used
as tonic, hypnotic, sedative and diuretic [3]. The root contains majorly Withanolides –
steroidal lactones with ergostane, this includes Withanone, Withaferin A, Withanolides,
Withasomidienone and Withanolide C [4]. Withaferin - A (4β, 27-dihyrox-5β-6-epoxy-1-
oxowitha-2, 24-dienolide) is main Withanolilide for which notable activities including
anti-inflammatory, anti-convulsive, anti-tumor and anti-oxidant have been reported \[5, 6, 7, 8\]. Withaferin - A has also been reported to have immunosuppressive action on B-lymphocyte proliferation \[9\]. Withanolides are ergostane type of steroids with atoms C-22 and C-26, bridged by a δ-lactone functionally and an oxidized C-1 position. These compounds are specific for the solanaceae family, and in particular, for the genus Withania, and thus they are used as marker compounds \[10\]. The biological activities of withanolides, especially of the dominant Withaferin – A has been studied extensively and reported from the roots and leaves of the species of Withania\[11\]. The number of analytical reports for the determination of Withanolides is comparatively small. Besides a TLC method for the quantification of Withaferin – A \[12\], a few HPLC methods are described in literature. Most of them showed disadvantages either, the acetylation of Withaferin – A is required prior to analysis \[13\], the separation time is long \[14\], or the compounds are not baseline separated and elute, more or less with the injection peak \[15\]. A part of our efforts was to develop a HPLC method suitable for direct determination of Withaferin – A in \textit{withaniasomnifera}, its extract and Polyherbal formulation.

MATERIALS AND METHODS

Powdered drug was purchased from different vendors, LallubhaiVrajlal Gandhi (LVG) Pvt. Ltd., Ahmedabad, India and DishantAyurvedic Pvt. Ltd., Santej, India. Extracts were purchased from Phytoconcentrates Pvt. Ltd., Ahmedabad and Arke Formulations Pvt. Ltd., Rajkot. Polyherbal (Capsule) formulation was procured from Sunrise Remedies Pvt. Ltd., Santej. Pure Withaferin – A was purchased from Natural Remedies Pvt. Ltd., Bangalore.

Preparation of Standard solution

A stock solution of 100 μg/ml was prepared by dissolving 10.0 mg of Withaferin – A in 100.0 ml of HPLC grade methanol.

Preparation of Sample solution

Accurately weighed one gram of powders and extracts of \textit{withaniasomnifera} were refluxed with methanol for 30.0 minutes and filtered. Methanolic extracts were evaporated to dryness. The residue was redissolved in methanol, filtered through 0.45 μm membrane filter and used for HPLC analysis. For capsule average net content was determined and was refluxed with methanol for 30.0 minutes and filtered. Methanolic
extract was evaporated to dryness. The residue was redissolved in methanol, filtered through 0.45 µm membrane filter and used for HPLC analysis. The analytical HPLC experiments were performed with an Agilent Technologies 1120 compact LC equipped with variable wavelength detector operating at 227 nm. Separation was carried out with C\textsubscript{18} (5 µm) column with Methanol: Acetonitrile (98: 2) containing 0.05 % Triethylamine as an eluent at a flow rate of 1.0 ml/minute. Validation of quantitative method was performed with samples for five injections of 20 µl each.

Validation Parameters of Developed Method\textsuperscript{[16]}

Validation of developed method was carried out as per ICH guidelines. Parameters such as Linearity, Accuracy, Precision, LOD and LOQ were taken up as tests for analytical method Validation.

Linearity
The linearity was evaluated by analyzing different concentration of the standard solutions of Withaferin – A. The Beer-Lambert’s concentration range was found to be 5-30 µg/ml.

Accuracy
To ascertain the accuracy of the proposed methods, recovery studies were carried out by standard addition method at three different levels (80%, 100% & 120%). Average percent recovery for was found to be 97.31 % (table 2).

Precision
The repeatability, intraday and interday variations for determination of Withaferin - A was carried out three times in same day and for three consecutive days and % RSD was calculated. Themethod was found to be precise due to low values of %RSD.

LOD & LOQ
The LOD and LOQ of developed method were calculated by using equations:

Limit of Detection (LOD): 3.3 × σ/S
Limit of Quantification (LOQ): 10 × σ /S

Where, σ = The Standard deviation of the response,
S = Slope of calibration curve.

The results of all validation parameters obtained are shown in table no. 1.
RESULTS AND DISCUSSION

The method discussed in the present work providea convenient and accurate way for analysis of Withafern - A. In proposed method, Linearity was observed in the concentration range of 5-30 µg/ml. Accuracy of proposed methods was ascertained by recovery studies and the results are expressed as % recovery. Average percent recovery for Withaferin – A was found to be 97.31%, values of standard deviation and coefficient of variation were satisfactorily low indicating the accuracy of the method. Based on the results obtained, it is found that the proposed method is accurate, precise, reproducible & economical and can be employed for routine quality control of Withaferin - A in capsule dosage form as well extracts.

CALIBRATION CURVE FOR WITHAFERIN - A

Different concentration of the standard solution of Withaferin - A of 5 – 30 µg were prepared from stock solution and used for HPLC analysis. The calibration data for standard Withaferin – A was obtained by plotting the graph of Concentration Vs Peak area.

![Calibration Curve for Withaferin-A](image-url)
Figure 2
HPLC CHROMATOGRAM OF WITHAFERIN – A

Figure 3
HPLC CHROMATOGRAM OF WITHAFERIN – A (Powder LVG)

Figure 4
HPLC CHROMATOGRAM OF WITHAFERIN – A (Powder Dishant)
Figure 5
HPLC CHROMATOGRAM OF WITHAFERIN – A (Extract Arke)

Figure 6
HPLC CHROMATOGRAM OF WITHAFERIN – A (Extract Phytoconcentrates)

Figure 7
HPLC CHROMATOGRAM OF WITHAFERIN – A (Xytone Capsule)
TABLE 1: REGRESSION ANALYSIS DATA AND SUMMARY OF VALIDATION PARAMETERS FOR PROPOSED METHOD

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Validation Parameters</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Linearity Range</td>
<td>5 – 30 µg/ml</td>
</tr>
<tr>
<td>2</td>
<td>Linearity Equation</td>
<td>y = 53027x + 34065</td>
</tr>
<tr>
<td>3</td>
<td>Slope</td>
<td>53027</td>
</tr>
<tr>
<td>4</td>
<td>Intercept</td>
<td>34065</td>
</tr>
<tr>
<td>5</td>
<td>Correlation Coefficient (R²)</td>
<td>0.999</td>
</tr>
<tr>
<td>6</td>
<td>Precision (% RSD)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Repeatability (n=6)</td>
<td>1.24</td>
</tr>
<tr>
<td></td>
<td>Intraday (n=3)</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>Interday (n=3)</td>
<td>0.76</td>
</tr>
<tr>
<td>7</td>
<td>Accuracy (n=3)</td>
<td>97.31 %</td>
</tr>
<tr>
<td>8</td>
<td>LOD (µg/ml)</td>
<td>1.48 µg/ml</td>
</tr>
<tr>
<td>9</td>
<td>LOQ (µg/ml)</td>
<td>4.48 µg/ml</td>
</tr>
<tr>
<td>10</td>
<td>Theoretical Plates</td>
<td>3347</td>
</tr>
<tr>
<td>11</td>
<td>Asymmetry</td>
<td>0.90151</td>
</tr>
</tbody>
</table>

TABLE 2: RECOVERY DATA OF WITHAFERIN A

<table>
<thead>
<tr>
<th>Amount taken (µg)</th>
<th>Amount added (µg)</th>
<th>Total amount of Withaferin - A (µg)</th>
<th>Amount of Withaferin - A recovered (µg)</th>
<th>% Recovery of Withaferin - A</th>
<th>Average % Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>24</td>
<td>54</td>
<td>51.80</td>
<td>95.92 %</td>
<td>97.31 %</td>
</tr>
<tr>
<td>30</td>
<td>30</td>
<td>60</td>
<td>58.27</td>
<td>97.12 %</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>36</td>
<td>66</td>
<td>65.26</td>
<td>98.88 %</td>
<td></td>
</tr>
</tbody>
</table>

TABLE 3: DATA FOR HPLC DETERMINATION OF WITHAFERIN - CONTENT IN DIFFERENT EXTRACTS AS WELL AS IN POWDER OF WITHANIA SOMNIFERA AND IN POLYHERBAL FORMULATION.

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>POWDER</th>
<th>EXTRACT</th>
<th>Xytone Capsule (Polyherbal Formulation)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LVG (Ahmedabad)</td>
<td>Dishant Ayurvedic (Santej)</td>
<td>Arke Formulation (Rajkot)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phyto Concentrates (Ahmedabad)</td>
<td>Sunrise Remedies Pvt. Ltd. (Santej)</td>
</tr>
<tr>
<td>Withaferin A</td>
<td>1.47 %</td>
<td>1.32 %</td>
<td>1.68 %</td>
</tr>
</tbody>
</table>
CONCLUSION
A validated method has been developed for the estimation of Withaferin - A in Capsule dosage form. Proposed method is simple, accurate and precise. The method is suitable for routine analysis of Withaferin - A in Capsule and Extracts. The simplicity of this method allows for application in laboratories that lack sophisticated analytical instruments such as HPTLC, LC–MS. Detection and quantification limit achieved, describe the method is very sensitive. High recoveries and acceptable % RSD values confirm established method is accurate and precise. Hence, the method is recommended for routine quality control analysis of Withaferin - A.

REFERENCES
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