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RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF WITHA FERIN-A IN RANGER CAPSULE

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ABSTRACT: A simple, accurate, precise and robust reverse phase High Performance Liquid Chromatographic method has been developed and validated for the estimation of Withaferin-A in polyherbal formulation namely Ranger Capsule. The chromatographic method was carried out isocratically with Phenomenex Luna C18 (2) (4.6 x 250 mm, 5 μ) and good resolution was achieved with acetonitrile: water (60: 40v/v) mobile phase. Withaferin- A was detected at retention time 4.04 min using flow rate 1.0 ml/min with UV detection at 230 nm. The calibration curve was achieved to be linear over a range of 2-20 μ g/ml with regression coefficient of 0.9993. The proposed method was found accurate with 99.43 % - 100.64 % recovery. Relative standard deviation of repeatability, intra-day and inter-day variations were found to be 0.79 %, 0.12-1.55 %, 0.04-1.73 %, respectively. The Limit of Detection (LOD) and Limit of Quantification (LOQ) were observed to be 0.05 μ g/ml and 0.16 μ g/ml, respectively. This results shows that method was well validated. In the present study, Withaferin- A was found 80 μ g per capsule. This study reveals that proposed method is quicker and cost effective for routine analysis of Withaferin- A in polyherbal dosage form

INTRODUCTION: *Withania somnifera* (L.) Dunal. (Ashwagandha) is one of the most valued plants in Ayurveda and is commonly used in Indian traditional health care systems. The dried roots of the plant are used in the treatment of nervous and sexual disorders. Roots, leaves and fruit are traditionally used as tonic (the plant is sometimes referred to as Indian ginseng), hypnotic, sedative and diuretic^{1, 2, 3}.

From chemistry point of view, the drug contains group of biologically active constituents known as withanolides. Withaferin- A is therapeutically active withanolide reported to be present in leaves.

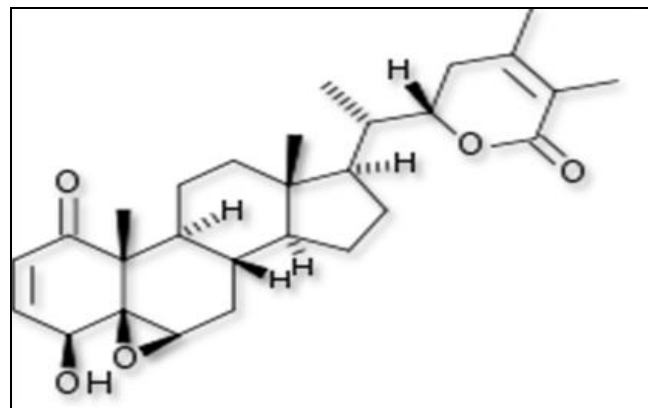


FIG.1: STRUCTURE OF WITHAFERIN A

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Withaferin- A chemically characterized as (4 β , 5 β , 6 β , 22R)-4, 27-Dihydroxy-5, 6:22, 26-diepoxyergosta-2, 24-diene-1, 26-dione, is one of the main withanolide active principles isolated from the plant (**Fig. 1**). Withaferin- A inhibits cyclooxygenase-2 (COX-2) but not cyclooxygenase-1 (COX-1), desired for a non-ulcerating anti-inflammatory/chemotherapeutic drug⁴.

Ranger capsule is polyherbal dosage form that contains multiple Ayurvedic herbs including *Withania somnifera* (L.) Dunal. (Ashwagandha) as one of the major ingredient. This capsule contains other key ingredients such as *Asparagus racemosus* (Shatavari), *Mucuna pruriens* (Kauncha), *Embllica officinalis* (Amalaki), *Centella asiatica* (Mandukparni), *Vitis vinifera* (Draksha), *Nardostachys jatamansi* (Jatamansi), *Tribulus terrestris* (Gokshur), *Zingiber officinale* (Shunthi), *Tinospora cordifolia* (Guduchi), *Terminalia arjuna* (Arjun).

Literature review reveals that there are few methods available for estimation of Withaferin- A individually and simultaneously by HPTLC, HPLC⁵⁻¹². But no such method is available for estimation of Withaferin- A in Polyherbal formulation. Other than this, reported HPLC method is not suitable to resolve the Withaferin- A peak from the other herbal compounds in formulation. Therefore an attempt has been made to develop the method, which can resolve the peak for Withaferin- A from other herbal compounds and can easily quantify for routine quality control analysis. The proposed method is optimized and validated as per the International Conference on Harmonization (ICH) Q2 (R1) guideline¹³.

MATERIALS AND METHODS:

Apparatus:

The chromatography was performed on a Shimadzu LC-20AT, SPD-20A HPLC instrument equipped with UV detector UV-20A with wavelength range 190-700 nm and Spinchrom LC Solution software; Phenomenex Luna C18(2) (4.6 x 250 mm, 5 μ particle size) was used as stationary phase. Rheodyne manual injector with 20 μ l capacity loop was used. Shimadzu Unibloc AUX220 with Capacity of 10 mg -220 g as an analytical balance;

pH meter made of Toshcon and Sonicator Toshcon SW-2 were used in the study.

Reagents and materials:

The reference standard of Withaferin-A was purchased from Natural Remedies Pvt. Ltd., Bangalore, India. Ranger capsule is manufactured and marketed by Vasu Healthcare Pvt. Ltd. Raw materials were procured from Vasu Healthcare Pvt. Ltd., Vadodara, India. HPLC grade Methanol, Acetonitrile, Water, Ortho-phosphoric acid (OPA) was purchased from Merck specialties Pvt. Ltd., Mumbai, India.

Selection of Mobile Phase:

The standard and sample solutions were run in different mobile phase systems. Various proportions of mobile phase were tried using water, acetonitrile and methanol. Also tried the mobile phases with different pH value. It was found that the combination of acetonitrile and water in proportion of 60:40 v/v has given a good resolution, sharp and symmetric peak at retention time 4.04 min.

Optimized chromatographic condition:

TABLE 1: CHROMATOGRAPHIC CONDITION FOR WITHAFERIN-A

Mobile Phase	Acetonitrile : Water (60 :40 % v/v)
Stationary Phase	Phenomenex Luna C18 (4.6 x 250mm, 5 μ particle size)
Wavelength	230 nm
Run time	10 min
Flow Rate	1 mL/min
Injection Volume	20 μ L
Temperature	Ambient
Mode of Operation	Isocratic elution

The optimized chromatographic condition for estimation of Withaferin-A in Ranger capsule is represented in the **Table 1**.

Preparation of Standard Stock Solution:

An accurately weighed quantity of Withaferin-A (10 mg) was transferred to a 10 ml volumetric flask, dissolved and diluted up to the mark with methanol to obtain standard stock solution of 1000 μ g/ml, this solution used as a standard stock solution.

Preparation of Working Standard for calibration curve:

5 ml aliquot from 1000 µg/ml was transferred to 50 ml volumetric flask and made up to the mark with methanol to prepare 100 µg/ml working standard for Withaferin-A. From this working standard mixture, aliquots (0.2, 0.5, 1, 1.5, 2 ml) were pipette out and diluted up to 10 ml to make 2 µg/ml, 5 µg/ml, 10 µg/ml, 15 µg/ml, 20 µg/ml respectively.

Sample Preparation:

Powder was removed from 20 capsules, mixed properly and from that weighed accurately 2.5 g powder in to 25 ml volumetric flask and volume was made up to the mark with methanol, sonicate for 10 min. Then it was filtered with 0.22 µ filter paper to obtain sample stock solution. Aliquot of 4 ml from this sample stock solution was transferred to 10 ml volumetric flask and made up the volume to mark with methanol. This was used as test solution.

Method Validation:

The proposed chromatographic method was validated according to ICH Q2 (R1) guidelines.

System Suitability Test:

System suitability test was performed by injecting six replicates of Withaferin- A working standard solution of 100 µg/ml concentration and observed the parameters viz. tailing factor, theoretical plate and percentage relative standard deviation (% RSD) of peak area.

Specificity:

Specificity of the method was ascertained by the separation of the analyte from other potential components such as impurities, degradants or excipients. A volume of 20 µl of placebo solution, Withaferin- A working standard and sample solution was injected and the chromatogram was recorded.

Linearity:

Three replicate of 2 -20 µg/ml range concentrations of Withaferin- A were injected for linearity study. Calibration curve of linearity was constructed for peak area signal against concentration.

Accuracy:

Accuracy was assessed by standard addition method. Standard was added at three different levels to the sample solution (containing 10.6 µg/ml concentration of Withaferin- A). The results were expressed as a percentage of Withaferin- A recovered in samples.

Precision:

The precision of the method was determined by repeatability and intermediate precision (reproducibility). The repeatability of the instrument was checked by repeatedly injecting and analyzing (n=5) standard solutions of Withaferin-A (10 µg/ml). The results are reported in terms of relative standard deviation (% RSD). Intermediate precision includes intraday and interday precision. The intraday and interday precision of the proposed method were analyzed by three sets of different concentrations (2, 10 and 20 µg/ml) on the same day and on three different days, respectively.

Limit of Detection and Limit of Quantification:

Limit of Detection (LOD) and Limit of Quantification (LOQ) were determined by using the formula based on the standard deviation of y-intercepts of regression lines and the slope. LOD and LOQ were calculated by using equations $LOD = 3.3 \times \sigma/S$ and $LOQ = 10 \times \sigma/S$. Where, σ is standard deviation, S is slope of corresponding calibration curve.

Robustness:

The robustness was assessed by altering the optimized chromatographic condition such as by changing the flow rate, the mobile phase composition and wavelength.

Statistical analysis:

Statistical calculations were carried out with the Microsoft Excel 2007 for Windows software package. Average, Sum, Standard Deviation (STDEV), Regression (RSQ) for Statistical Calculation, and Scattered Chart were used for Linearity; P values > 0.05 were considered to be significant.

RESULTS AND DISCUSSION: Developed and validated RP-HPLC method for the estimation of Withaferin- A in Ranger capsule was found to be

simple, accurate, precise and robust. Several mobile phase combinations were tried and the best resolution was obtained with mobile phase acetonitrile: water (60:40% v/v). Withaferin- A was detected at retention time 4.04 min using column Phenomenex Luna C18 (2) (4.6 x 250 mm, 5 μ), at 230 nm wavelength and 1.0 ml/min flow rate. (Fig.2)

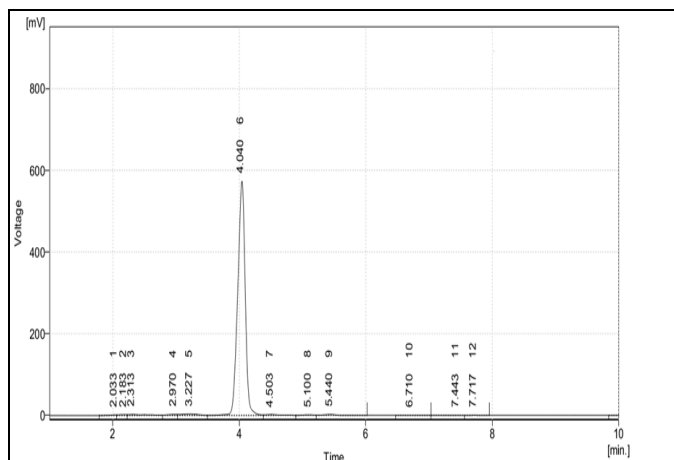


FIG.2: CHROMATOGRAM OF WITHAFERIN-A STANDARD 100μg/ml

TABLE-2: SYSTEM SUITABILITY OF WITHAFERIN-A

Sr. No.	Conc. (μg/mL)	Peak area	Theoretical Plates (N)	Resolution R _s
1	100	4781.63	6248.00	1.91
2		4735.18	6665.00	1.59
3		4794.06	5944.00	2.24
4		4734.08	5636.00	2.03
5		4782.25	6310.00	2.43
6		4697.96	5645.00	1.78
	Mean	4754.19		
	%RSD	0.79		

Conc.- Concentration, μg-microgram, ml- milliliter, RSD- Relative Standard Deviation

The optimized method was validated as per ICH guidelines. % RSD of peak area was found 0.79, theoretical plate was more than 2000 and resolution was achieved within the range of 1.59 – 2.43 %

TABLE 4: ACCURACY FOR WITHAFERIN-A IN RANGER CAPSULE

Amount of withaferin A found (μg)	Amount of Withaferin A added (μg)	Total amount of Withaferin A (μg)	Amount of Withaferin A found (μg) Mean ± SD (n=3)	% Recovery	Mean % Recovery
10.60	8 (80%)	18.60	18.72 ± 0.02	100.64	
10.60	10 (100%)	20.60	20.51 ± 0.01	99.54	99.87
10.60	12 (120%)	22.60	22.47 ± 0.01	99.43	

μg- microgram, SD - Standard Deviation

which showed that the instrument is suitable for further validation of parameters (Table 2).

TABLE 3: LINEARITY FOR WITHAFERIN-A

Conc. (μg/mL)	PA	Slope	Intercept	r ²
2	67.21	32.674	5.4506	0.9993
5	167.71			
10	336.05			
15	504.075			
20	651.24			

Conc. - Concentration, PA- Peak Area, μg-microgram, ml-milliliter

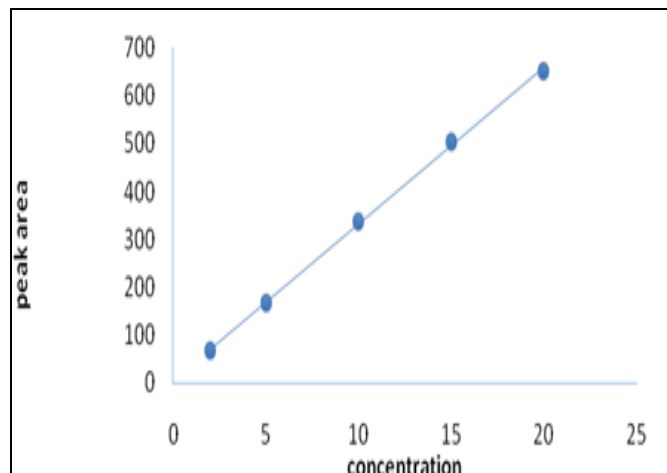


FIG.3: LINEARITY GRAPH OF WITHAFERIN-A

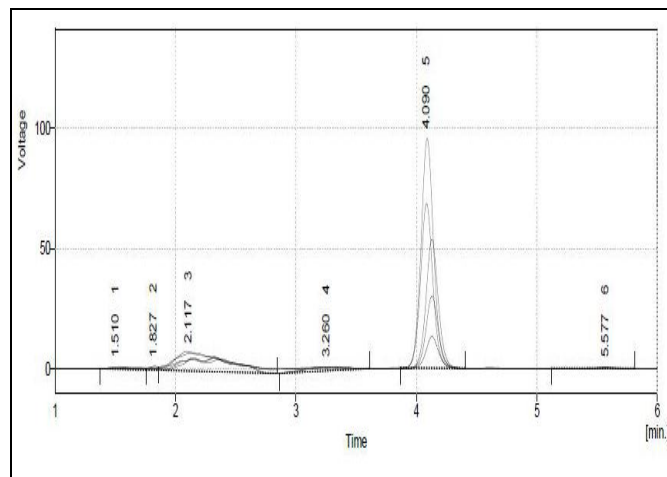


FIG.4: LINEARITY CHROMATOGRAM OF WITHAFERIN A

TABLE 5: REPEATABILITY OF WITHAFERIN-A

Sr. No.	Conc. (µg/mL)	PA	Mean	SD	%RSD
1		336.05			
2		334.26			
3	10	335.16	335.37	0.76	0.23
4		336.12			
5		335.26			

Conc.- Concentration, PA- Peak Area, µg-microgram, mL-milliliter, RSD- Relative Standard Deviation, SD- Standard Deviation.

TABLE 6: A INTRADAY PRECISION OF WITHAFERIN-A

Conc. (µg/mL)	Intraday precision						Mean	SD	%RSD
	2 Hour		4 Hour		6 Hour				
	RT	PA	RT	PA	RT	PA			
2	4.13	67.21	4.13	66.22	4.13	68.31	67.25	1.045482	1.55
10	4.13	336.05	4.13	335.16	4.13	335.26	335.49	0.489268	0.14
20	4.08	651.24	4.09	651.96	4.09	650.36	651.19	0.801332	0.12

Conc.- Concentration, PA- Peak Area, µg-microgram, mL-milliliter, RSD- Relative Standard Deviation, SD- Standard Deviation

TABLE 6: B INTERDAY PRECISION OF WITHAFERIN-A

Conc. (µg/mL)	INTERDAY PRECISION						Mean	SD	%RSD
	Day 1		Day 2		Day 3				
	RT	PA	RT	PA	RT	PA			
2	4.13	68.42	4.13	66.34	4.13	68.31	67.69	1.170427	1.73
10	4.13	334.26	4.13	336.12	4.13	336.05	335.48	1.057199	0.32
20	4.08	650.84	4.09	650.73	4.09	651.24	650.94	0.26839	0.04

Conc.- Concentration, PA- Peak Area, µg-microgram, mL-milliliter, RSD- Relative Standard Deviation, SD- Standard Deviation

TABLE 7: ROBUSTNESS OF METHOD

Parameters	Changes	RT	PA
		(Mean + SD)	(Mean + SD)
Wavelength (nm)	230	4.11±0.00	314.00 ± 1.95
	225	4.10±0.00	393.14± 6.05
	235	4.11±0.00	255.79± 3.85
	Mean	4.10	320.98
	%RSD	0.04%	1.23%
Flow Rate ml/min	0.98	4.19±0.00	330.70± 3.55
	1.00	4.11±0.00	314.00±1.95
	1.02	4.03±0.00	333.67±3.58
	Mean	4.11	326.12
	%RSD	1.94%	0.92%
Mobile Phase	61:39	4.14±0.00	328.03±3.28
	60:40	4.11±0.00	314.00±1.95
	59:41	4.28±0.01	327.62±2.02
	Mean	4.17	323.21±7.98
	%RSD	2.17	2.47%

PA-Peak Area, nm-nanometer, mL-milliliter, RSD-Relative Standard, Deviation, SD- Standard Deviation

A linearity range of 2-20 µg/ml with regression coefficient 0.999 was established (Table 3, Fig 3, 4). The % recovery achieved within 99.43 %-100.64 % for Withaferin- A by standard addition method, suggested that the applied method is accurate. (Table 4). The precision of the proposed method was carried out in terms of the repeatability, inter-day and intra-day time periods.

The % RSD values of repeatability (0.23 %), inter-day (0.04-1.73 %) and intra-day (0.12-1.55 %) variations revealed that the proposed method has good precision level (Table 5, 6a and 6b). The LOD and LOQ values were found to be 0.05 µg/ml and 0.16 µg/ml, respectively. The method was found to be robust with change of ±2 % in wavelength, flow rate and mobile phase ratio

(Table 7). In specificity study, there was an absence of interference from the other compounds, showed that method is specific for the Withaferin-A in the polyherbal formulation. In the present study, quantity of the Withaferin- A was found to be 80 µg per capsule.

CONCLUSION: Proposed study describes new HPLC method for the estimation of Withaferin A in capsule formulation. The method was validated and found to be simple, sensitive, accurate and precise. Percentage of recovery shows that the method is free from interference of the excipients used in the formulation. Therefore the proposed method can be used for routine analysis of estimation of Withaferin A in its capsule formulation.

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CONFLICT: Nil

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