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VALIDATED RP – HPLC METHOD FOR THE QUANTIFICATION OF ANDROGRAPHOLIDE IN TOXIROAK PREMIX, A POLYHERBAL MYCOTOXIN INHIBITOR

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ABSTRACT: A new simple, rapid, precise, accurate and specific RP-HPLC method was developed to carry out the quantification of Andrographolide in the formulation *vis a vis* its standardization to ensure the batch to batch consistency in mycotoxin inhibition activity of the polyherbal formulation Toxiroak premix. An optimal condition of separation and detection were achieved on a Phenomenex luna C18 column (250 mm x 4.6 mm, 5 µm) using isocratic mixture of Acetonitrile and 0.1 % ortho-phosphoric acid in a ratio of 40:60,v/v as a mobile phase, 1.0 ml/min as flow rate 226 nm as the wavelength for detection and quantification. To verify the the efficiency of chromatographic run, the system suitability was studied by taking into account number of theoretical plates and symmetry / tailing factor which were found to be consistent during repeated injections . The method was validated for linearity, selectivity, accuracy, precision, repeatability and recovery in accordance with the statistical method of validation and is useful for establishing the batch to batch consistency.

INTRODUCTION: Though herbal products have become increasingly popular throughout the world, one of the impediments in its acceptance is the lack of standard quality control profile. The quality of herbal medicine that is, the profile of the constituents in the final product has implications in efficacy and safety.

However, due to the complex nature and inherent variability of the constituents of plant-based drugs, it is difficult to establish quality control parameter though modern analytical technique are expected to help in circumventing this problem [1].

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The development of authentic analytical methods which can reliably profile the phytochemical composition, including quantitative analyses of marker/bioactive compounds and other major constituents, is a major challenge to scientists.

Standardization is an important step for the establishment of a consistent biological activity, a consistent chemical profile or simply a quality assurance program for production and manufacturing of herbal drugs.

Aflatoxins are frequent contaminants of food and feed as a result of mould growth. Losses to farmers or livestock or poultry producers from aflatoxin-contaminated commodities include lower yields, reduced growth rates and decreased feed efficiencies. The ease and frequency with which aflatoxins contaminate agricultural commodities, concomitant with the chronic exposure of animals to these chemicals via contaminated animal feeds, can mean

the difference between profit and loss for the poultry industry. Toxiroak premix, a proprietary polyherbal formulation of AYURVET for poultry, is a mycotoxin inhibitor and acts as a mould inhibitor; it inhibits the biosynthesis of mycotoxins, effectively binds the toxins in the GI tract and helps in bio-neutralization of the toxins in liver.

The mould inhibition and bio-neutralization activity of Toxiroak is due to the presence of certain herbs like *Azadirachta indica*, *Curcuma longa*, *Allium sativum* that are known to inhibit the growth of fungus and block the biosynthesis of mycotoxins.

Presence of herbs like *Andrographis paniculata* and *Solanum nigrum* help in increasing the activity of liver drug metabolizing enzymes leading to detoxification of mycotoxins.

Andrographis paniculata or Kalmegh also known as "King of bitter" is one among the prioritized medicinal plants in India and this herb is reported to possess antihepatotoxicity [2] antifungal activities [3] besides its general use as an immunostimulant agent [4].

The key chemical constituents of the plant are Andrographolide (**Fig. 1**), bisandrographolide, andropanolide, 14-deoxy-11-oxo-andrographolide, 14-deoxyandrographolide, 14-deoxy-11, 12 - didehydroandrographolide, etc.[5].

Various studies carried out have proved that the plant and it's one of the bioactive compounds Andrographolide exhibits significant biological activities against pathogens, hence the inclusion of the plant in formulation will add value to its bio efficacy.

To ensure the batch to batch consistency in term of biological efficacy and chemical profiling, a new HPLC method was developed for the quantification of bioactive compound andrographolide in the formulation *vis a vis* to authenticate the optimum quantity of herb added in turn.

The method was validated on the basis of its selectivity, linearity, precision, accuracy, limit of detection and limit of quantification according to International Conference on Harmonization (ICH) guidelines [6].

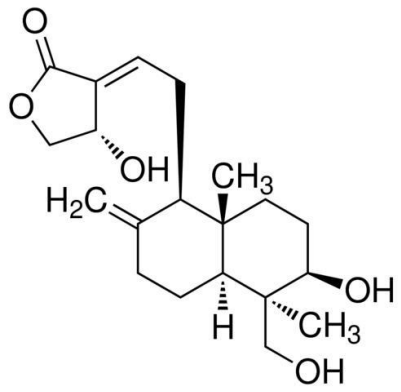

FIG. 1: STRUCTURE OF THE ANDROGRAPHOLIDE

MATERIAL AND METHODS: All the reagents and solvents were of AR or HPLC grade as per requirement. The active compound Andrograholide (MW 350.45) was isolated in our lab and structure was established by interpreting the 1H, 13C & 2D NMR spectra, matching the Rf and spectra with authentic Sigma Aldrich reference standard. Latest controlled samples of Toxiroak premix were obtained from the QA/QC department of AYURVET LTD, Baddi.

Preparation of standard solution of Andrographolide : Accurately weighed around 5 mg of standard Andrograholide was dissolved in 5 ml of methanol obtaining stock concentrations of 1000 µg/ml. Stock solution was further diluted to obtain the dilution range of 40 – 80 µg/ml.

Preparation of test solution : For the quantification of Andrograholide, Toxiroak premix (1g) was extracted with 25 ml of petroleum ether (60ºC – 80ºC) at room temperature (25 ± 5°C), for 10 h and filtered. The defatted sample was extracted with 50 ml of methanol under reflux conditions for 3 hours and filtered. The process was repeated twice. The final volume was made to 100 ml with methanol, filtered the solution through 0.45 µm membrane filter before injecting into HPLC.

High Performance Liquid Chromatography:

- **Conditions and Procedure:** Andrographolide content was analyzed by High Performance Liquid Chromatography (WATERS, binary pump p515 with PDA 2996 detector, USA). The data was acquired on the Empower 2.0 controlling software. Separation was obtained on Phenomenex luna C18 column (250 mm x 4.6 mm, 5 µm) using isocratic mixture of Acetonitrile and 0.1 % ortho-phosphoric acid in a ratio of 40:60, v/v as a mobile phase.

The mobile phase was filtered through 0.45 µm Millipore filter and degassed. The flow rate was adjusted to 1.0 ml/min. Injection volume was adjusted to 20 µl and detection was made at 226 nm.

- **System suitability:** The system suitability test was performed to ensure that the chromatographic assay was suitable to the analysis intended. Chromatographic parameters including peak area, retention time, theoretical plates and tailing factor were measured and the standard deviation (RSD) for each parameter was determined.

- **Validation parameters:** The method was validated according to ICH guideline for linearity, precision, accuracy, selectivity, limit of detection and limit of quantification**.** Linear regression analysis was used to calculate the slope, intercept, and coefficient of determination/regression coefficient (r^2) for calibration plot. Evaluation was on the basis of peak area.

  To study precision of analytical methods, three different concentrations of marker compound solution in triplicates were injected on three different times within the same day and repeating the same on three different days to record intra-day and inter-day variations in the results.

  The accuracy of the proposed method was determined by a recovery study, carried out by adding known quantity of standard markers in the formulation extract. The samples were spiked with two different amounts of standard compounds prior to extraction.

  The selectivity of the respective method was determined by comparing the retention time and absorbance/reflectance spectrum of the standard compound and the corresponding peak obtained from the extract of the formulation.

  The UV-Visible spectra of marker compound were compared with its counterpart in formulation at three different positions, the peak start, peak center, and peak end. The LOD and LOQ were calculated based on response at the signal to noise ratio 3:1 and 10:1 respectively.

  LOD and LOQ were experimentally verified by diluting the known concentration of standard until the average response for six replicates.

RESULTS AND DISCUSSION:

Selection and Optimization of chromatographic condition: To optimize the RP-HPLC parameters, several mobile phase compositions were tried. A satisfactory separation and good peak symmetry for Andrographolide (Fig. 2) was obtained with a mobile phase Acetonitrile: 0.1% ortho Phosphoric acid (40:60, v/v) at a flow rate of 1.0 ml/min to get better reproducibility and repeatability. Selecting 226 nm as the detection wavelength resulted in an acceptable responses and enable the detection of compound under investigation.

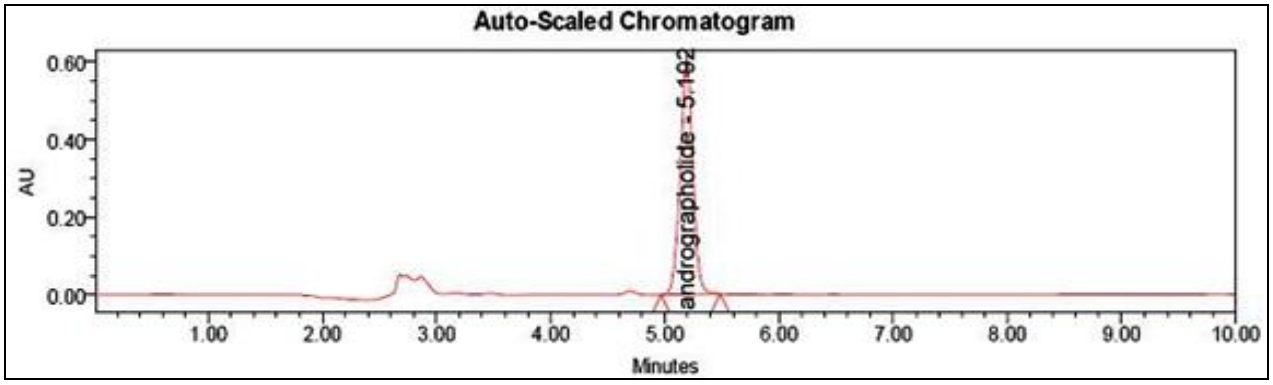

FIG. 2: CHROMATOGRAM OF STANDARD ANDROGRAPHOLIDE AT 226 nm

System suitability: The analytical results obtained by the method developed are only valid if the defined system suitability criteria are fulfilled. In this investigation, the experimental result (**Table 1**) indicates that the chromatographic system was suitable for intended analysis. A standard solution containing 40 µg ml^{-1} of Andrographolide was injected six times.

The retention time for the reference compound was 5.19 minutes. The RSD values for peak area and retention time i.e. 0.79% and 0.41 %, respectively, suggested the reproducibility for these parameters. The good peak symmetry of Andrographolide was indicated by value 0.985 for the parameter tailing factor. Mean value with RSD 1.08 % for theoretical plates suggested the good efficiency of column.

TABLE 1: SYSTEM SUITABILITY PARAMETERS

Sr. No.	Parameters	Data obtained [a]	RSD
1	Peak area	2054628	0.79
2	Retention Time (min)	5.19	0.41
3	Theoretical plates	9985	1.08
4	Tailing factor	0.985	0.28

a = Mean of six replicates

Validation of the Proposed Method: The proposed method was validated for the determination of Andrographolide in commercial polyherbal formulation using following parameters:

a. Calibration: The marker compound in the formulation was quantified using a calibration curve established with five dilutions of the standard at concentrations ranging from 40-80 µg ml^{-1}. The corresponding peak area in formulation was plotted against the concentrations of the standard injected (**Fig. 3**). Peak identification was achieved by comparison of both the retention time (RT) and UV absorption spectrum with those obtained for standard.

b. Linearity: Linear regression analysis was used to calculate the slope, intercept, and coefficient of determination/regression coefficient (r2) for calibration plot. Linearity was determined by using five concentrations of the standard solution. The calibration curve was obtained by plotting the area versus the concentrations of the standard solution. Response was linear in the concentration ranges investigated (**Fig. 3, Table 2**).

c. Range: Range is the interval between upper and lower concentration (amount) of analyte in sample for which it has been demonstrated that the analytical method has suitable level of precision accuracy and linearity.

The linear response was observed at 226 nm over a range of 40-80 ppm for the standard compound (**Fig. 3, Table 2**).

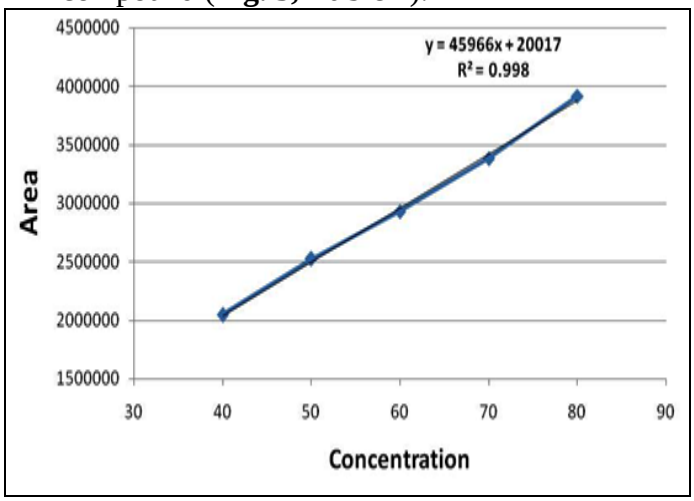

FIG. 3: CALIBRATION CURVE OF ANDOGRAPHO-LIDE AT 226 nm

TABLE 2: RESULTS OF PRECISION, LINEAR REGRESSION ANALYSIS AND THEIR CORRELATION COEFFICIENT FOR QUANTITATIVE ANALYSIS OF MARKER COMPOUND

Parameters	Andrographolide
Concentration range [µg ml^{-1}]	40- 80
Regression equation	y = 45996x+20017
Correlation Coefficient (r2)	0.998
Amount of marker compound in Toxiroak premix [%] (w/w)[a]	0.0525 ± 0.0020
Precision - RSD [%]	
Intraday 1	0.611
Interday 3	0.926

y = peak area response; x = amount of marker compound; [a] = Mean ± SD, n=6

d. Precision: Three different concentrations of marker compound solution in triplicates were injected on three different times within the same day and repeating the same on three different days to record intra-day and inter-day variations in the results. The low %RSD values of Intraday (0.611) and Interday (0.926) for marker compound reveals that the proposed method is precise (Table 2).

e. Limit of Detection (LOD) and Limit of Quantification (LOQ): For determination of limits of detection and quantification different dilutions of the marker compound i.e. Andrographolide were injected with mobile phase as blank and determined on the basis of signal to noise ratio 3:1 and 10:1 respectively. The LOD and LOQ for the standard compound were found to be 0.06 and 0.2 µg ml^{-1} respectively.

f. Selectivity: The retention time of standard and the same compound in the formulation was 5.19 ±0.002 minute. The UV-Vis spectrum of marker compound was compared with its counterpart in formulation at three different positions, the peak start, peak center, and peak end. There was good correlation between spectra obtained at each of the three positions. The Andrographolide peak was, therefore, not masked by any peak of other compound present in the formulation (**Figures 4 & 5**), which was indicative of peak purity.

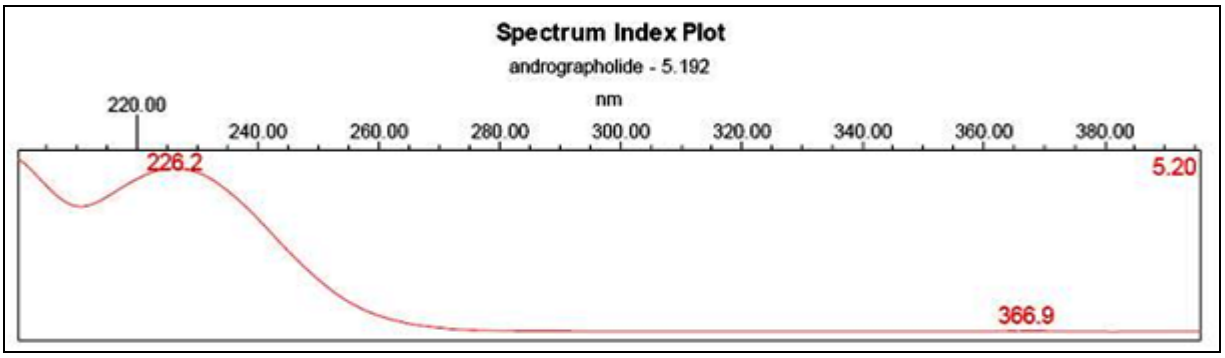

FIG. 4: SPECTRUM INDEX PLOT OF STANDARD ANDROGRAPHOLIDE

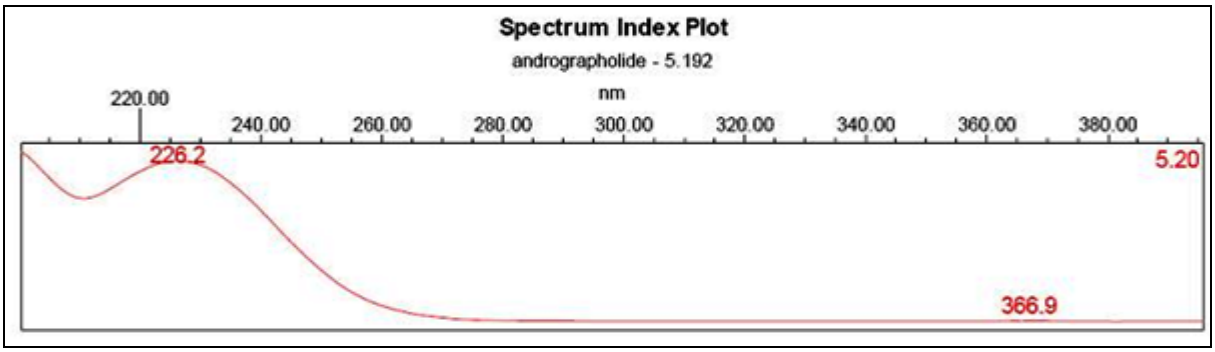

FIG. 5: SPECTRUM INDEX PLOT OF ANDOGRAPHOLIDE IN FORMULATION

g. Accuracy: Recovery experiments were conducted to check for the presence of positive or negative interferences from other ingredients/excipients present in the formulation and to study the accuracy of the method. Recovery was determined by the standard addition method. Andrographolide standard were added to the formulation at two different concentrations, extraction and analysis was performed as described above. Recovery was calculated for each standard at each concentration. The summary of the results and average mean of recovery data for each level of marker was within accepted range as shown in **Table 3**. The low value of relative standard deviation indicates that the proposed method is accurate.

TABLE 3: RESULTS FROM DETERMINATION OF RECOVERY

Parameter	Andrographolide		
Initial concentration in formulation [mg g^{-1}]	0.525	0.525	0.525
Concentration added [mg g^{-1}]	0	0.263	0.525
Total concentration [mg g^{-1}]	0.525	0.788	1.05
Concentration found [mg g^{-1}]	0.520	0.776	0.998
RSD [%] (n=7)	0.45	0.60	0.48
Recovery [%]	99.04	98.47	95.04
Mean recovery [%]		97.51	

h. Quantification of marker compound: The overlaid chromatogram (**Fig. 6)** of polyherbal formulation showed clear resolution of the marker compound from other ingredients present and was quantified with respect to standard compound. The average content of Andrographolide in formulation was found to be 0.052 %.

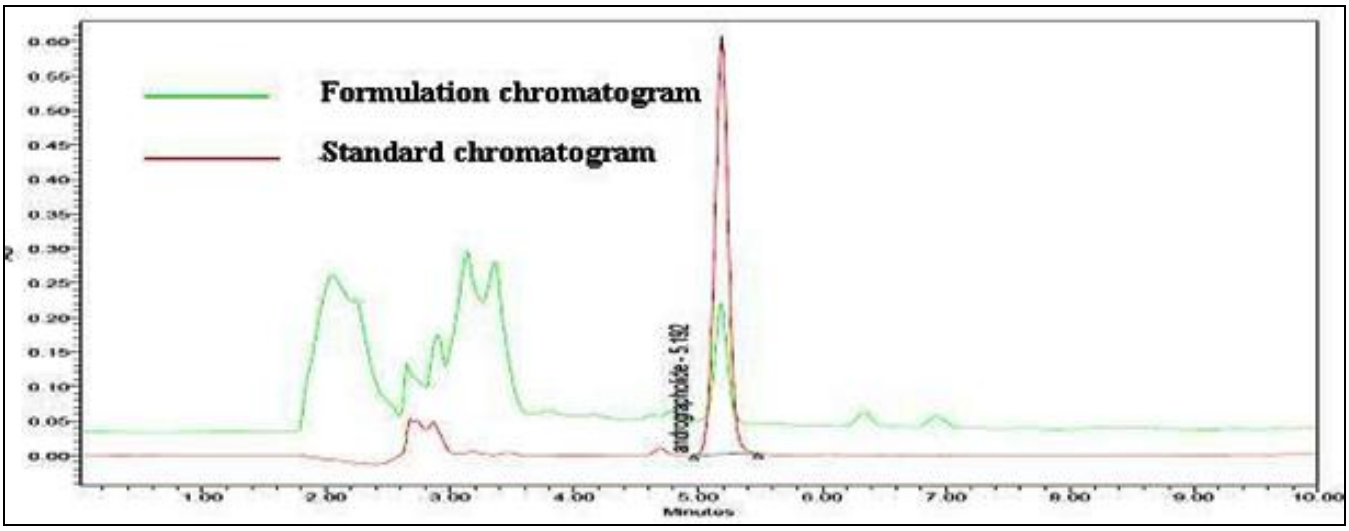

FIG. 6: OVERLAID CHROMATOGRAM OF THE STANDARD ANDROGRAPHOLIDE AND THE COMPOUND PRESENT IN FORMULATION

CONCLUSION: Toxiroak premix- a mycotoxin inhibitor effectively binds the toxins in the GI tract and helps in bio-neutralization of the toxins in liver. Presence of herbs like *Andrographis paniculata* helps in increasing the activity of liver drug metabolizing enzymes leading to detoxification of mycotoxins. To ensure the batch to batch consistency in term of biological efficacy and chemical profiling, the newly developed HPLC method shall help in the quantification of bioactive compound andrographolide in the formulation *vis a vis* to authenticate the optimum quantity of herb i.e . *Andrographis paniculata* added in turn.

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