DEVELOPMENT AND VALIDATION OF NOVEL REVERSE-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR SIMULTANEOUS ESTIMATION OF ANDROGRAPHOlide AND ALOE-EMODIN

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INTRODUCTION
Ayurvedic medicines are polyherbal formulations and every herb consists of an array of chemical constituents.

Plants are considered as a conventional source for a large number of phytochemicals [1]. Ayurvedic medicines are polyherbal formulations and each herb consists of various chemical constituents [2]. The quality assessment of herbal formulations is vital to justify their acceptability in the modern system of medicine. The production and primary processing of herbal substance influences the quality of the active pharmaceutical ingredient [3]. Standardization of polyherbal formulations and the quantitative determination of markers in any polyherbal formulation is very challenging [4]. Standardization of herbal products can be achieved if they are evaluated using sophisticated techniques such as ultraviolet (UV)-visible, infrared, thin-layer chromatography, high-performance liquid chromatography (HPLC), high-performance thin-layer chromatography, gas chromatography-mass spectrometry, liquid chromatography-mass spectrometer, atomic absorption spectrometer, and other such methods [5]. The literature survey reveals that various methods were developed for estimation of andrographolide and aloe-emodin alone or in combination with other markers [6-12], but no such HPLC analysis method for simultaneous estimation of andrographolide and aloe-emodin is reported.

This paper presents the development of a novel reverse-phase HPLC (RP-HPLC) method for the simultaneous estimation of andrographolide and aloe-emodin. The developed and validated method was applied for the standardization of marketed formulation for these two markers. The selected formulation is a well-known formulation and is indicated against various complications such as jaundice, liver disorder, indigestion, hepatotoxicity, liver enlargement, gastroenteritis, fungal infections, gastritis, and other conditions [13]. This tablet consists of various medicinal plants namely, Bhringraj (Eclipta alba), Revandchini (Rheum emodi), Sarapunkha (Tephrosia purpurea), Kalmegh (Andrographis paniculata), Kasni (Cichorium intybus), Giloy (Tinospora cordifolia), Haretaeki (Terminalia chebula), and Bhumyamliaki (Phyllanthus niruri). Andrographolide from A. paniculata is reported to possess abortifacient, anti-inflammatory, antibacterial, antipyretic, antithrombotic, antiviral, antineoplastic, cardioprotective, choleretic, digestive, expectorant, hepatoprotective, hypoglycemic, immune enhancement, laxative, and sedative activity [1-4]. Andrographolide is effective against liver damage caused by paracetamol or galactosamine. It also played a hepatoprotective role by reducing a lipid peroxidation product malondialdehyde [15]. aloe-emodin from R. emodi possess antioxidant, antimicrobial, antifungal, anticancer, antiulcer, antifluidicidal, hemostatic, antiinflammatory, and immune-enhancing activity [16]. Aloe-emodin also possesses multiple antiproliferative and anticarcinogenic properties in a host of cancer cell lines [17].

METHODS

Instrument
RP-HPLC Shimadzu LC Prominence-ii 2030 model consisting of UV detector and autosampler was employed for the method development and validation. Software used was Lab Solution. UV-visible spectrophotometer was used for obtaining maximum wavelength (λ max) of the compounds of interest.
Standards and reagents
Andrographolide and aloe-emodin standards were obtained from Yucca Enterprises, Mumbai, Maharashtra, India. Marketed formulation of Livfit Tablet of Alembic Pharmaceuticals Ltd. was procured from the local market of Mumbai, Maharashtra, India. All the chemicals used were of HPLC grade, which were procured from Thermo Fisher Scientific, India Pvt. Ltd., Powai, Mumbai.

Chromatographic conditions
HPLC (Shimadzu, Prominence-C-r 2030 model) with Lab Solution software was employed in this method. Prontosil C18 (250×4.6 mm, 5μ) column was used for analysis. The mobile phase was acetonitrile: 0.05% orthophosphoric acid (45:55), at the flow rate of 1.0 ml/min and the injection volume was kept 10 μl. The column temperature was set at 28°C. Andrographolide and aloe-emodin were detected at 225 nm using a UV detector.

Selection of wavelength
Standard solutions of andrographolide and aloe-emodin were prepared and scanned by a UV spectrophotometer. The range of detection was kept from 200 to 400 nm and the overlay spectra of andrographolide and aloe-emodin obtained are shown in Fig. 1. 225 nm was selected as the detection wavelength for the analysis of andrographolide and aloe-emodin as both the markers showed appreciable absorption at 225 nm.

Preparation of standard solutions
Hundred mg of each marker (andrographolide and aloe-emodin) was transferred individually in two volumetric flasks of 100 ml and the volume was made up with methanol to obtain solutions of 1000 µg/ml. These were used as stock solutions and were used after suitable dilutions.

Preparation of working solutions
Working solutions were prepared from the standard solution of markers. A combined solution of markers having a concentration of 100 µg/ml was prepared from the stock solution. This was further diluted to get dilutions of 0.5, 1, 5, 10, 20, 50, and 60 µg/ml which were used to construct a calibration curve.

Preparation of sample solution
Ten tablets were triturated and about 2 g powder was weighed and was subjected to reflux using methanol as extracting solvent. Triturated powder was transferred into the 100 ml round bottom flask and 100 ml methanol was added to it and was placed in a heating mantle and extraction was continued for 20 min. The solution was further filtered using the Whatman Filter Paper to get a clear solution and the volume was made up to 100 ml using methanol. This solution was sonicated before injection.

RESULTS AND DISCUSSION

Method development
A series of trials was carried out using various mobile phases such as acetonitrile: Phosphate buffer (60:40) having different pH, acetonitrile: 0.1% orthophosphoric acid (50:50), acetonitrile: 0.05% orthophosphoric acid (40:60) to develop RP-HPLC method for simultaneous estimation of andrographolide, and aloe-emodin in the marketed formulation. Finally, acetonitrile: 0.05% orthophosphoric acid (45:55) was selected as the mobile phase based on better peak resolution and peak symmetry. Prontosil C18 column (250×4.6 mm, 5 μ) was used for analysis and injection volume was kept 10 μl. The flow rate was 1.0 ml/min and the run time was 15 min. The column temperature was set at 28°C and the detection was carried out at 225 nm. The retention time (RT) of andrographolide and aloe-emodin obtained was found to be 4.57±0.2 min and 12.29±0.2 min, respectively. Chromatograms of standard and sample of andrographolide and aloe-emodin are shown in Figs. 2 and 3. The optimized chromatographic conditions are tabulated in Table 1.

Method validation
The developed method was validated for parameters such as linearity, specificity, precision, accuracy, robustness, and solution stability as per the International Conference on Harmonization (ICH) guidelines [18].

Specificity
It is performed to ensure the identification, purity testing, and quantification of marker compound from the ayurvedic formulation under analysis. Specificity was confirmed by comparing the RTs and UV spectra of the standards with the component obtained in chromatograms of the extract of tablets. The developed method was found to be specific as there was no interference of any other constituents at the RTs of both markers andrographolide and aloe-emodin as depicted in Figs. 2 and 3.

Linearity
Linearity was evaluated by analyzing the plot area as a function of the concentration of analyte. Andrographolide and aloe-emodin showed a linear response in the concentration range of 0.5–60 µg/ml. The linearity was constructed by plotting peak area versus concentration of analyte. The linearity was validated by the high value of correlation coefficients (r²) 0.9992 and 0.999 for andrographolide and aloe-emodin, respectively, which meets the acceptance criteria for method validation. The results are tabulated in Table 2 and plots obtained are given in Figs. 4 and 5.

![Fig. 1: Ultraviolet overlap spectrum of andrographolide and aloe-emodin](image-url)
Limit of detection (LOD)
The LOD of an individual analytical method is the lowest amount of analyte in a sample which can be detected but not necessarily quantified as an exact value. The LOD is expressed as LOD=3.3 σ/S, where σ=standard deviation of intercepts of the calibration curve and S is the Slope of the calibration curve.

Limit of quantification (LOQ)
LOQ is a parameter of quantitative assays for low levels of compounds (markers) in extracts. The LOQ is expressed as LOQ=10 σ/S.

LOD and LOQ of andrographolide were found to be 0.14 and 0.44 µg/ml, respectively, and that of aloe-emodin was found to be 0.13 and 0.40 µg/ml, respectively. A low LOD and LOQ value indicates that the method is sensitive.

Quantification of markers
The amount of andrographolide and aloe-emodin present in the formulation was calculated using linear regression analysis. Quantification of the markers was done by performing HPLC analysis of test solutions. The area obtained for each of the markers from formulation was extrapolated on the calibration curve of the respective marker. The results are shown in Table 3.

Precision
The system precision was carried out by injecting six injections of standards of andrographolide and aloe-emodin and method precision was performed by injecting a sample of the same concentration 6 times. The percent relative standard deviation (%RSD) was calculated from the area obtained from the chromatogram. The standard analysis of the results proved that %RSD of the peak areas obtained was <2%; hence, the developed method was found to be precise. The data of precision are tabulated in Tables 4 and 5.

Accuracy (recovery)
Recovery of andrographolide and aloe-emodin from formulation was checked by spiking a known quantity of standards at three concentration levels.

Table 1: Optimized chromatographic conditions for andrographolide and aloe-emodin

| Parameters       | Optimized conditions                                      |
|------------------|----------------------------------------------------------|
| Column           | Prontosil C18, (250×4.6 mm, 5 μ)                          |
| Mobile phase     | Acetonitrile: 0.05% orthophosphoric acid (45:55)          |
| Detector         | UV detector                                              |
| Detection wavelength | 225 nm                                               |
| Column temperature | 28°C                                                 |
| Injection volume | 10 µl                                                   |
| Flowrate         | 11.0 ml/min                                              |
| Run time         | 15 min                                                   |
| Retention time   | 4.57 and 12.29 min                                       |

UV: Ultraviolet

Fig. 2: High-performance liquid chromatography chromatogram of a standard mixture of andrographolide and aloe-emodin obtained using optimized conditions

Fig. 3: Chromatogram of extract of marketed formulation

LOQ and LOQ of andrographolide were found to be 0.14 and 0.44 µg/ml, respectively, and that of aloe-emodin was found to be 0.13 and 0.40 µg/ml, respectively. A low LOD and LOQ value indicates that the method is sensitive.
Table 2: Linear regression data obtained from calibration curves of andrographolide and aloe-emodin

| Concentration (µg/ml) | Area (Andrographolide) | Area (Aloe-emodin) |
|-----------------------|------------------------|--------------------|
| 0.5                   | 709,805                | 30,416             |
| 1                     | 202,898                | 62,888             |
| 5                     | 357,499                | 157,427            |
| 10                    | 731,227                | 329,612            |
| 20                    | 1,433,500              | 656,922            |
| 50                    | 3,619,588              | 1,612,455          |
| 60                    | 4,232,730              | 2,018,190          |
| Slope                 | 70,024                 | 32,900             |
| Intercept             | 61,238                 | 68,464             |
| Correlation           | 0.9992                 | 0.999              |

Fig. 4: Calibration curve of andrographolide

y = 70024x + 61238
R² = 0.9992

Fig. 5: Calibration curve of aloe-emodin

y = 32900x + 6846.4
R² = 0.999

Table 3: Analysis of markers in formulation

| Markers          | %w/w content |
|------------------|--------------|
| Andrographolide  | 0.0023       |
| Aloe-emodin      | 0.0115       |

Table 4: System precision results

| S. no. | Andrographolide (10 µg/ml) | Aloe-emodin (20 µg/ml) |
|--------|-----------------------------|------------------------|
| Peak area | 741,227                     | 656,922                |
| 1.      | 741,708                     | 654,925                |
| 2.      | 744,624                     | 649,835                |
| 3.      | 749,309                     | 653,309                |
| 4.      | 741,889                     | 652,233                |
| 5.      | 741,264                     | 652,590                |
| Mean±SD | 743,37 ± 3188                | 653,30 ± 2425           |
| %RSD    | 0.43                        | 0.37                   |

%RSD: Percentage relative standard deviation, SD: Standard deviation

Table 5: Method precision results

| S. no. | Andrographolide | Aloe-emodin |
|--------|-----------------|-------------|
| Peak area | 125,903         | 158,946     |
| 2.      | 124,982         | 157,982     |
| 3.      | 125,467         | 158,268     |
| 4.      | 125,948         | 158,923     |
| 5.      | 125,120         | 157,689     |
| 6.      | 123,832         | 159,893     |
| Mean±SD | 125,209±781     | 158,617±802|
| %RSD    | 0.62            | 0.51        |

%RSD: Percentage relative standard deviation, SD: Standard deviation

levels (i.e., 80%, 100%, and 120% of the quantified amount) to the test samples in triplicate using HPLC. This way, accuracy was performed and calculated for nine determinations over a specified range and mean recovery was calculated. The acceptance limit for percent recovery ranges from 98 to 102%. The mean % recovery was found to be within the range, which indicates that the method is accurate. The percentage of recovery results is tabulated in Tables 6 and 7.
A novel HPLC method was developed and validated for the simultaneous estimation of andrographolide and aloe-emodin. This method was validated according to the ICH Q2 (R1) guidelines in the terms of linearity, precision, LOD, LOQ, accuracy, and robustness. The developed method is simple, linear, robust, precise, and accurate for the determination of andrographolide and aloe-emodin and can be used for the analysis of both the markers in the formulation. Peaks of both the markers were sharp and well resolved. Both the markers were quantified from formulation under study. Hence, the proposed method can be applied for routine qualitative and quantitative analysis of andrographolide and aloe-emodin in an ayurvedic formulation containing these phytoconstituents.

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AUTHORS’ CONTRIBUTIONS
All the authors have contributed equally in performing analysis and writing the manuscript.

CONFLICTS OF INTEREST
The authors declare that there are no conflicts of interest in this research work.

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