RP-HPLC Method Development and Validation for Simultaneous Estimation of Withaferin A and Gliclazide in Herbal-Drug Combination Dosage Forms

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Abstract
Withaferin A, a steroidal lactone derived from Withania somnifera (Ashwagandha), has shown promising antidiabetic properties. Gliclazide, a second-generation sulfonylurea, is widely used in type 2 diabetes management. The combination of herbal and allopathic agents is increasingly explored to achieve synergistic therapeutic benefits. This study aims to develop and validate a robust RP-HPLC method for the simultaneous estimation of Withaferin A and Gliclazide in combined herbal-drug formulations. Chromatographic separation was achieved using a C18 column with an optimized mobile phase of acetonitrile and phosphate buffer. The method was validated according to ICH Q2 (R1) guidelines and applied to in-house formulations, ensuring its reliability for routine quality control.

Keywords: RP-HPLC, Withaferin A, Gliclazide, herbal-drug combination, method validation, simultaneous estimation

Introduction
Diabetes mellitus is a chronic metabolic disorder characterized by elevated blood glucose levels due to impaired insulin secretion, action, or both. The global burden of diabetes continues to rise at an alarming rate, with type 2 diabetes mellitus (T2DM) constituting the majority of cases. T2DM is often associated with long-term complications affecting the eyes, kidneys, nerves, and cardiovascular system, necessitating effective glycemic control through pharmacological intervention.

Conventional antidiabetic agents, particularly sulfonylureas like Gliclazide, have played a pivotal role in diabetes management by stimulating insulin secretion from pancreatic beta cells. Gliclazide is known for its selective action on pancreatic islets and

additional benefits such as antioxidant properties and reduced risk of hypoglycemia compared to other sulfonylureas. However, monotherapy is often insufficient for achieving sustained glycemic targets, and patients may require combination therapies to address the multifactorial nature of the disease.

In recent years, there has been growing interest in integrating traditional herbal medicines with conventional drugs to enhance therapeutic efficacy and minimize side effects. *Withania somnifera* (commonly known as Ashwagandha) is a well-established medicinal plant in Ayurvedic medicine, reputed for its adaptogenic, anti-inflammatory, and antidiabetic properties. One of its key bioactive constituents, Withaferin A, has demonstrated potent pharmacological activities, including modulation of glucose metabolism and insulin sensitivity.

Combining herbal and allopathic agents is a promising strategy, especially for chronic conditions like T2DM, where multimodal approaches may offer synergistic benefits. However, such formulations also pose challenges in standardization, quality control, and regulatory compliance. Accurate quantification of both herbal and synthetic components in a single dosage form is critical to ensure consistency, efficacy, and safety. Reliable analytical methods are essential to support the development, validation, and routine quality control of such combination therapies.

High-performance liquid chromatography (HPLC), particularly reverse-phase HPLC (RP-HPLC), remains one of the most widely used techniques in pharmaceutical analysis due to its high precision, sensitivity, and reproducibility. Despite its established utility, validated methods for the simultaneous estimation of bioactive herbal compounds and synthetic drugs in complex dosage forms are still limited.

In this study, an RP-HPLC method was developed and validated for the simultaneous estimation of Withaferin A and Gliclazide in herbal-drug combination formulations. The method was optimized for chromatographic efficiency and validated in accordance with ICH Q2 (R1) guidelines to assess specificity, linearity, accuracy, precision, sensitivity, and robustness. The developed method was then applied to both marketed and in-house formulations, establishing its suitability for routine quality control and standardization of combined herbal-allopathic therapeutics.

2 Materials and Methods

2.1 Materials and Reagents

Withaferin A (≥98% purity) and Gliclazide (≥99% purity) were procured as certified reference standards from a reputed analytical supplier. Dried root extract of *Withania somnifera* was obtained from a licensed Ayurvedic raw material vendor and authenticated based on morphological and organoleptic parameters.

All solvents and reagents used were of HPLC grade. Acetonitrile and methanol were obtained from Merck (India). Potassium dihydrogen phosphate and orthophosphoric acid were used for buffer preparation. Ultra-pure water was obtained using a Milli-Q system.

Instrumentation used for chromatographic analysis included a reverse-phase HPLC system equipped with a quaternary pump, autosampler, column oven, and UV-visible detector. Data acquisition and processing were performed using integrated chromatography software.

2.2 Preparation of Standard and Sample Solutions

Standard Stock Solutions:

Standard stock solutions of Withaferin A and Gliclazide were prepared separately by dissolving 10 mg of each in 10 mL of methanol to obtain a concentration of 1000 µg/mL. These solutions were further diluted with the mobile phase to obtain working standards in the range of 1–50 µg/mL for Withaferin A and 2–100 µg/mL for Gliclazide.

Sample Preparation:

Two types of capsule formulations were analyzed: a marketed herbal-drug capsule (coded MKT-01) and an in-house prepared formulation (coded INH-01). The contents of each capsule were weighed, and an equivalent amount of the sample was sonicated in methanol for 30 minutes. The extract was filtered using a 0.45 µm membrane filter and diluted appropriately with the mobile phase before injection into the HPLC system.

2.3 Chromatographic Conditions

The chromatographic separation was carried out on a C18 column (250 mm × 4.6 mm, 5 µm particle size). The mobile phase consisted of acetonitrile and phosphate buffer (pH 3.5, adjusted with orthophosphoric acid) in a ratio of 65:35 v/v. The flow rate was set at 1.0 mL/min and the detection wavelength was 230 nm, selected based on maximum absorbance for both analytes. The injection volume was 20 µL, and the total run time was 10 minutes under isocratic elution. The column temperature was maintained at 30°C throughout the analysis.

2.4 Method Validation

The developed method was validated as per ICH Q2 (R1) guidelines. The following parameters were evaluated:

- **Specificity:** Chromatograms of blank, placebo, standard, and sample were compared to check for any interference.
- **Linearity:** Calibration curves were constructed over a range of 1–50 µg/mL for Withaferin A and 2–100 µg/mL for Gliclazide, and correlation coefficients (R^2) were calculated.
- **Accuracy:** Recovery studies were conducted at three concentration levels (80%, 100%, and 120%) to assess the accuracy of the method.
- **Precision:** Intra-day and inter-day precision were determined using multiple injections, and results were expressed as %RSD.
- **LOD and LOQ:** These were calculated using the standard deviation of the response and the slope method.
- **Robustness:** The method's reliability was assessed by making deliberate minor variations in flow rate, pH, and detection wavelength.
- **System Suitability:** Parameters such as retention time, theoretical plates, resolution, and tailing factor were evaluated before sample analysis.

Results

The RP-HPLC method was successfully developed and optimized for the simultaneous estimation of Withaferin A and Gliclazide. Chromatographic separation was achieved using a C18 column with an isocratic mobile phase of acetonitrile and phosphate buffer (65:35 v/v, pH 3.5) at a flow rate of 1.0 mL/min. Detection was carried out at 230 nm, where both analytes showed strong absorbance.

Both analytes were well resolved with retention times of approximately **4.5 minutes for Withaferin A** and **7.2 minutes for Gliclazide**, with sharp, symmetrical peaks and no interference observed from excipients or placebo formulations.

3.1 Validation Results

- **Specificity**: No peaks were observed at the retention times of Withaferin A and Gliclazide in the chromatograms of blank and placebo samples, confirming that the method is specific and free from interference.

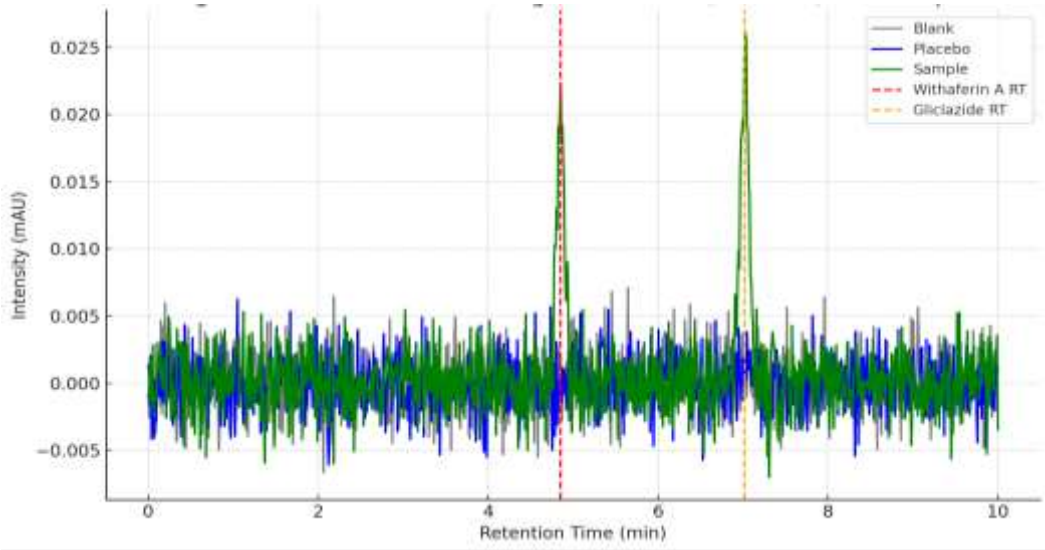

Figure 1: Overlay Chromatograms of Blank, Placebo, and Sample for Specificity

- **Linearity**: Excellent linearity was observed over the tested concentration ranges.
    - Withaferin A: 1–50 µg/mL (R^2 = 0.999)
    - Gliclazide: 2–100 µg/mL (R^2 = 0.998)
      (Refer to Figure 1)

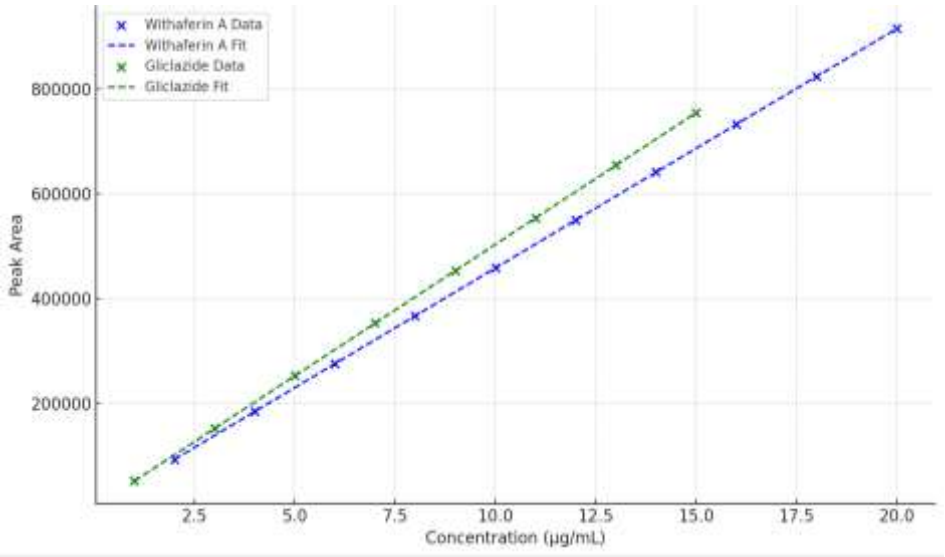

Figure 2: Calibration curves for Withaferin A and Gliclazide

- **Accuracy**: Recovery ranged between 98.5–101.2% for Withaferin A and 97.9–100.8% for Gliclazide across all tested concentrations.
- **Precision**: Intra-day and inter-day %RSD values were consistently <2%, indicating high repeatability and reproducibility.
- **LOD and LOQ**:
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    - Withaferin A: LOD = 0.32 µg/mL, LOQ = 1.05 µg/mL
    - Gliclazide: LOD = 0.45 µg/mL, LOQ = 1.38 µg/mL
- **Robustness**: Minor variations in method parameters did not significantly affect peak area or retention time, validating robustness.
- **System Suitability**: Parameters such as tailing factor (<1.5), theoretical plates (>3000), and consistent retention times confirmed system adequacy.

3.2 Sample Analysis

The validated method was applied to the analysis of both **marketed (MKT-01)** and **in-house (INH-01)** herbal-drug formulations. The content of Withaferin A and Gliclazide in both formulations complied with expected label claims, showing assay values between **98–102%**.

3.3 Stability Studies

Short-term stability was evaluated by storing standard and sample solutions at room temperature (25 ± 2°C) for 24 hours. Assay measurements were performed at initial time (0 hour) and after 24 hours. The results indicate minimal degradation (<2%) and negligible shifts in retention times, demonstrating good short-term stability of both analytes in the tested solutions.

Long-term stability was assessed by storing standard and sample solutions at refrigerated conditions (4 ± 2°C) for 30 days. Assays were conducted initially and after 30 days. The assay recoveries remained within acceptable limits (>95%), and retention time shifts were minor, confirming the stability of analytes during extended refrigerated storage.

3.4 Stability Trends

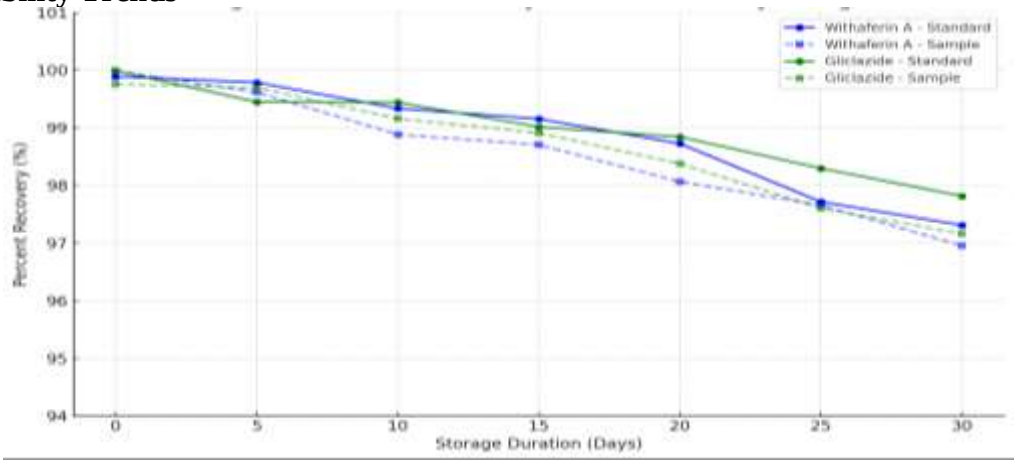

Figure 3: Graphical Representation of % Recovery for Accuracy Study

Figure 3, showing the percent recovery trends of Withaferin A and Gliclazide in standard and sample solutions over 30 days:

- Both analytes exhibited a slight but consistent decline in recovery.
- All values remained well within the acceptable stability range, confirming that the solutions are stable for at least one month under appropriate storage conditions.

The stability study confirms that the standard and sample solutions of Withaferin A and Gliclazide are chemically stable under both short-term and long-term storage conditions tested. The validated RP-HPLC method produces consistent assay results with no significant degradation or chromatographic changes, supporting its suitability for routine analysis in quality control environments.

Discussion

The successful development and validation of an RP-HPLC method for simultaneous estimation of Withaferin A and Gliclazide mark a significant step toward standardizing herbal-drug combination formulations. Given the increasing interest in integrative medicine, where traditional and modern therapies are combined, it becomes crucial to ensure robust analytical methods that can reliably quantify multiple active ingredients, even within complex matrices.

In the present study, the method development focused on achieving optimal peak resolution, symmetry, and reproducibility while maintaining practical chromatographic run time. The chosen mobile phase composition of acetonitrile and phosphate buffer (65:35 v/v) at pH 3.5 proved ideal for separating the relatively hydrophobic Withaferin A and the moderately polar Gliclazide. The retention times of approximately 4.5 minutes for Withaferin A and 7.2 minutes for Gliclazide ensured clear separation without overlapping or baseline disturbances.

Validation studies established that the method is specific, as no interferences from excipients or herbal matrix components were observed in chromatograms of blank and placebo formulations. The method demonstrated excellent linearity for both analytes across wide concentration ranges, confirming its utility in both low-dose and high-dose formulations.

Accuracy and precision results were well within acceptable limits, with recovery rates nearing 100% and %RSD consistently below 2%, reflecting the method's reliability. These characteristics make it suitable not only for quality control testing but also for formulation development and stability monitoring.

Furthermore, low limits of detection and quantification for both analytes affirm the method's sensitivity, which is especially valuable when analyzing low-dose herbal components in complex formulations. The method's robustness was supported by its consistent performance despite deliberate variations in chromatographic parameters, demonstrating its applicability under routine laboratory conditions.

The analysis of marketed and in-house prepared herbal-drug formulations using the validated method confirmed its real-world applicability. Both formulations yielded accurate assay results, and representative chromatograms showed no signs of co-elution, degradation, or unresolved peaks.

Importantly, stability studies under different storage conditions validated the chemical integrity of both Withaferin A and Gliclazide, further endorsing the method’s utility in long-term formulation development and storage assessment.

Overall, the developed RP-HPLC method offers a practical and efficient approach for the simultaneous quantification of herbal and synthetic actives in a single analytical run. It bridges a significant gap in analytical capabilities, especially for integrated formulations, and lays a strong foundation for future pharmacokinetic or bioavailability studies involving herbal-allopathic combinations.

Conclusion

The present study successfully demonstrates the development and validation of a robust, accurate, and reproducible RP-HPLC method for the simultaneous estimation of Withaferin A and Gliclazide in herbal-drug combination dosage forms. The method was optimized to achieve clear chromatographic separation, minimal run time, and excellent peak resolution, making it practical for routine quality control applications.

Comprehensive method validation in accordance with ICH Q2 (R1) guidelines confirmed the method’s specificity, linearity, accuracy, precision, sensitivity, and robustness. The ability of the method to consistently quantify both analytes without interference from excipients or matrix components reinforces its reliability for analyzing complex polyherbal and synthetic drug combinations.

Application of the method to both marketed and in-house formulations yielded satisfactory assay results, aligning with pharmacopeial standards. Additionally, stability studies under various storage conditions indicated the reliability of standard and sample preparations, further extending the method's suitability for formulation development and long-term quality assessment.

This validated analytical approach addresses the current gap in standardized methods for herbal-allopathic combination products and can be a valuable tool in the pharmaceutical and nutraceutical industries. It ensures product integrity, supports regulatory compliance, and

promotes the integration of traditional herbal medicine with modern therapeutics in a scientifically rigorous manner.

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