

Analytical Method Development and Validation of Simultaneous Estimation and Percentage Recovery of Natural Active Constitutes (Beta Sitosterol and Andrographolide)

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ABSTRACT: The study aimed to develop and validate a simple, precise, and reliable UV spectrophotometric method for simultaneous estimation of Beta Sitosterol and Andrographolide in pharmaceutical formulations. Organoleptic properties and melting points (140 °C and 231 °C) confirmed purity and identity. Both drugs were soluble in ethanol, which was selected as the solvent. FTIR analysis verified functional groups, while UV analysis showed λ_{max} at 211 nm and 225 nm with an isobestic point at 215 nm. Calibration curves (5–25 $\mu\text{g/mL}$) exhibited good linearity ($r^2 > 0.98$), following Beer–Lambert law. Validation studies demonstrated high precision, robustness, and ruggedness with %RSD < 2%. Low detection and quantification limits indicated good sensitivity. The method complied with ICH Q2(R1) guidelines and was found suitable, cost-effective, and reliable for routine pharmaceutical analysis.

KEYWORDS: Beta sitosterol, Andrographolide, UV spectrophotometry, Method validation.

I. INTRODUCTION

Spectroscopy is the study of the interaction between electromagnetic radiation and matter, and it serves as one of the most important analytical techniques in modern science. When radiation such as ultraviolet, visible, or infrared light interacts with a substance, the atoms and molecules present in that substance absorb or emit energy at specific wavelengths. This interaction produces a characteristic spectrum that is unique for each element or compound, often referred to as its “spectral fingerprint.” By analyzing this spectrum, it becomes possible to identify substances and determine their composition and concentration with high accuracy.

The fundamental principle of spectroscopy is based on the absorption, emission, or scattering of electromagnetic radiation by matter. When light passes through a sample, certain wavelengths are absorbed while others are transmitted or reflected. The absorbed energy causes transitions in the electronic, vibrational, or rotational states of molecules. These transitions are recorded using instruments known as spectrometers, which measure the intensity of radiation as a function of wavelength. The resulting spectrum provides valuable information about the structural and chemical properties of the substance. One of the key laws governing spectroscopic analysis is the Beer–Lambert law, which states that the absorbance of light is directly proportional to the concentration of the absorbing species and the path length of the sample.

There are several types of spectroscopy, each based on different regions of the electromagnetic spectrum and different types of molecular interactions. Infrared (IR) spectroscopy is widely used for identifying functional groups in organic compounds by analyzing vibrational transitions. Ultraviolet-visible (UV-Vis) spectroscopy measures the absorption of light in the UV and visible regions and is commonly used for quantitative analysis of compounds. Nuclear Magnetic Resonance (NMR) spectroscopy provides detailed information about molecular structure by studying the behavior of atomic nuclei in a magnetic field. Raman spectroscopy is based on the scattering of light and is useful for studying vibrational, rotational, and other low-frequency modes of molecules. Mass spectrometry, although slightly different from optical spectroscopy, is often included due to its ability to determine molecular

weight and structure by analyzing ions based on their mass-to-charge ratio.

Spectroscopy has a wide range of applications across various scientific fields. In pharmaceutical sciences, it is extensively used for drug identification, purity testing, and quantitative estimation of active pharmaceutical ingredients. It plays a crucial role in quality control and ensures that pharmaceutical products meet regulatory standards. In environmental science, spectroscopic techniques are used to detect pollutants in air, water, and soil, enabling effective monitoring and control of environmental contamination. In the biomedical field, spectroscopy aids in disease diagnosis, analysis of biological molecules such as proteins and nucleic acids, and medical imaging techniques like MRI. Additionally, spectroscopy is used in astronomy to study the composition, temperature, and movement of celestial bodies by analyzing the light they emit or absorb.

One of the major advantages of spectroscopy is that it is a non-destructive technique, meaning that the sample remains intact after analysis and can be used for further testing if

required. It is also highly sensitive, capable of detecting substances even at very low concentrations. The technique is relatively fast and requires minimal sample preparation, making it both time-efficient and cost-effective. Furthermore, spectroscopy is versatile, as it can be applied to solids, liquids, and gases, and it allows for both qualitative and quantitative analysis.

In addition to its advantages, spectroscopy also plays an essential role in research and development. It helps scientists understand molecular interactions, reaction mechanisms, and material properties. Modern advancements, including the integration of computational methods and artificial intelligence, have further enhanced the capabilities of spectroscopic techniques, allowing for faster data analysis and improved accuracy.

Overall, spectroscopy is a powerful and indispensable analytical tool that provides detailed insights into the composition and structure of substances. Its wide applicability, accuracy, and efficiency make it a cornerstone technique in fields such as chemistry, pharmacy, environmental science, and biomedical research.

II. REVIEW OF LITERATURE

Mevada et al. (2025) developed a simple and precise UV-Vis spectrophotometric method for simultaneous estimation of Andrographolide and Apocynin, showing excellent linearity, accuracy, and low LOD/LOQ values.

Penzel et al. (2025) proposed a turbidity compensation method in UV/Vis spectroscopy that significantly improved accuracy in turbid samples by reducing measurement errors.

Fadhillah et al. (2025) investigated bioactive compounds of *Andrographis paniculata* and demonstrated its potential anti-cardiac hypertrophy effect through anti-inflammatory and antioxidant pathways.

Selvam et al. (2025) studied andrographolide for antibacterial and anti-dengue activity, confirming its potential through in vitro and molecular docking studies.

Aljohar et al. (2025) developed a rapid and sensitive HPLC method for β -sitosterol estimation with short analysis time and good validation parameters.

Gomathi et al. (2024) isolated β -sitosterol and reported significant antioxidant and antidiabetic activities, indicating its therapeutic potential.

Afirosa et al. (2024) compared UV-Vis, HPLC, and qHNMR methods for andrographolide estimation and suggested cost-effective analytical approaches.

Dewi et al. (2024) used UV-Vis spectroscopy with chemometric analysis for rapid and efficient identification of rice compounds with high accuracy.

Patidar et al. (2024) developed and validated an RP-HPLC method for simultaneous estimation of β -sitosterol and quercetin with good precision and accuracy.

Gudasi et al. (2024) established an HPTLC method using DoE approach for simultaneous estimation of triacontane and β -sitosterol, showing robustness and reliability.

Chakraborty et al. (2023) developed an RP-HPLC method for simultaneous quantification of linoleic acid and β -sitosterol with good linearity and reproducibility.

Sharma et al. (2023) validated an HPTLC method for β -sitosterol estimation in plant extracts, showing accuracy and reproducibility.

Jana et al. (2023) developed validated RP-HPLC and HPTLC methods for quantification of

andrographolide in plant extracts as per ICH guidelines.

Sundhani et al. (2022) studied herb–drug interactions of andrographolide using RP-HPLC, confirming its effect on pharmacokinetic parameters.

Khonsa et al. (2022) developed a simple and accurate HPLC method for β -sitosterol estimation in supplements with high precision.

D’Souza et al. (2022) established an RP-HPLC method for simultaneous estimation of β -sitosterol and lupeol in plant extracts using QbD approach.

Patel et al. (2021) developed an LC-MS/MS method for simultaneous quantification of phytoconstituents including β -sitosterol with high sensitivity and accuracy.

Saxena et al. (2021) reported a TLC-based method for estimation of β -sitosterol along with other compounds in medicinal plants.

III. RESEARCH ENVISAGED

The study focused on developing and validating a UV-visible spectrophotometric method for simultaneous estimation of beta-sitosterol and andrographolide, two important bioactive compounds. Due to spectral overlap and structural complexity, a simple and cost-effective combined analytical method was required.

Pre-formulation studies were conducted to evaluate physicochemical properties, and a suitable solvent system was selected. The λ_{max} of both compounds was determined, followed by method development

IV. DRUG PROFILE

Beta-sitosterol:

Beta-sitosterol is a phytosterol with chemical formula $C_{29}H_{50}O$ and molecular weight 414.7. It is a white, waxy solid widely found in plant sources such as vegetable oils, nuts, and avocados. It shows similarity to cholesterol in structure and is soluble in organic solvents like alcohol. It acts by reducing cholesterol absorption, regulating inflammation, and inducing apoptosis through various cellular pathways. It is mainly used for lowering LDL cholesterol and managing benign prostatic hyperplasia (BPH), along with potential antioxidant and anticancer effects.

De et al. (2021) developed an RP-HPLC method for simultaneous estimation of andrographolide and curcumin in nanoformulations.

Raj et al. (2020) studied β -sitosterol-based nanoparticles and demonstrated their cytotoxic potential against cancer cells.

Shamsi et al. (2020) investigated drug-protein interaction using spectroscopic techniques, confirming strong binding affinity.

Ihsan et al. (2020) developed a selective HPLC method for andrographolide estimation in formulations with good linearity and precision.

Pancham et al. (2019) validated a UV spectrophotometric method for andrographolide with good accuracy and precision.

Nweze et al. (2019) isolated β -sitosterol and confirmed its antibacterial activity against pathogens.

Alvarez et al. (2019) evaluated β -sitosterol and phytosterols for anticancer activity, showing significant effects on cancer cell lines.

for their simultaneous estimation.

The method was validated as per ICH Q2(R1) guidelines, including parameters such as linearity, precision, accuracy, robustness, ruggedness, LOD, and LOQ. Percentage recovery studies confirmed its reliability.

Overall, the developed method was simple, reproducible, and suitable for routine quality control analysis of formulations containing beta-sitosterol and andrographolide.

Andrographolide:

Andrographolide is a labdane diterpenoid compound with chemical formula $C_{20}H_{30}O_5$ and molecular weight 350.45, obtained from *Andrographis paniculata*. It appears as a crystalline compound with a melting point of 229–232 °C. It possesses anti-inflammatory, antiviral, and anticancer properties. Its mechanism involves inhibition of inflammatory mediators such as TNF- α , IL-1 β , and NF- κ B pathways. It is commonly used in the treatment of respiratory infections, osteoarthritis, tonsillitis, and inflammatory bowel diseases.

V. MATERIAL AND METHODS

Various chemicals such as ethyl acetate, acetone, methanol, ethanol, chloroform, and DMSO were used in the study. Standard laboratory glassware including volumetric flasks, pipettes, beakers, and conical flasks were utilized. Instruments such as UV-Visible spectrophotometer, FTIR, digital pH meter, melting point apparatus, weighing balance, and ultrasonic bath were employed for analysis and method development.

Preliminary studies were carried out to evaluate physicochemical properties of the drugs. Organoleptic properties such as color, odor, and physical state were observed. Melting point determination was performed to assess purity and identity of the compounds. Solubility studies were conducted in different solvents, and ethanol was selected as the most suitable solvent. The pH of drug solutions was also determined to ensure stability and compatibility.

Identification of the compounds was confirmed using FTIR spectroscopy by analyzing characteristic functional groups. For method development, UV spectrophotometric analysis was performed by scanning drug solutions in the range of 200–800 nm to determine λ_{\max} . Standard stock solutions (1000 $\mu\text{g/mL}$) were prepared and further diluted to obtain working concentrations of 5–25 $\mu\text{g/mL}$. Calibration curves were constructed by plotting absorbance against concentration.

Simultaneous estimation of beta-sitosterol and andrographolide was carried out using the simultaneous equation (Vierordt) method, where absorbances were measured at respective λ_{\max} values and concentrations were calculated using absorptivity coefficients.

Method validation was performed according to ICH Q2(R1) guidelines. Linearity was established over the selected concentration range. Precision was evaluated through repeatability, intraday, and interday studies. Robustness was assessed by varying temperature conditions, while ruggedness was determined using different analysts. Sensitivity of the method was expressed in terms of LOD and LOQ. Accuracy was confirmed by percentage recovery studies at 50%, 100%, and 150% levels. Overall, the developed method was found to be reliable and suitable for routine analysis.

VI. RESULTS AND DISCUSSION

6.1 Preliminary Studies

6.1.1 Organoleptic Properties

The organoleptic evaluation of both drugs indicated good purity and quality. Beta-sitosterol was

observed as a white to off-white, odorless, waxy solid, while andrographolide appeared as a white, odorless crystalline powder. These characteristics were consistent with standard reported descriptions, confirming the authenticity of both compounds.

6.1.2 Melting Point Determination

The observed melting point of beta-sitosterol was found to be 140°C, which falls within the standard range of 136–142°C. Similarly, andrographolide showed a melting point of 231°C, consistent with the reported range of 229–235°C. The close agreement with standard values confirms the purity and identity of both compounds.

6.1.3 pH Determination

The pH of beta-sitosterol was found to be 6.3, while andrographolide exhibited a pH of 5.25. Both values fall within their respective acceptable ranges, indicating good stability and suitability for pharmaceutical applications.

6.1.4 Solubility Studies

Both drugs showed higher solubility in organic solvents compared to water. Beta-sitosterol was freely soluble in methanol and soluble in ethanol, chloroform, and DMSO, but insoluble in water. Similarly, andrographolide was freely soluble in methanol and DMSO, soluble in ethanol, slightly soluble in chloroform, and insoluble in water. Based on these findings, ethanol was selected as the suitable solvent for further analysis.

6.2 FTIR Analysis

The FTIR spectra of both compounds confirmed their structural identity.

The spectrum of andrographolide showed characteristic peaks corresponding to O–H stretching, C–H stretching, and C=O stretching, confirming the presence of alcohol and ester groups.

Similarly, the FTIR spectrum of beta-sitosterol exhibited peaks corresponding to O–H stretching, C–H stretching, and C=C stretching, indicating the presence of hydroxyl and alkene groups.

These findings confirm the structural integrity and purity of both compounds.

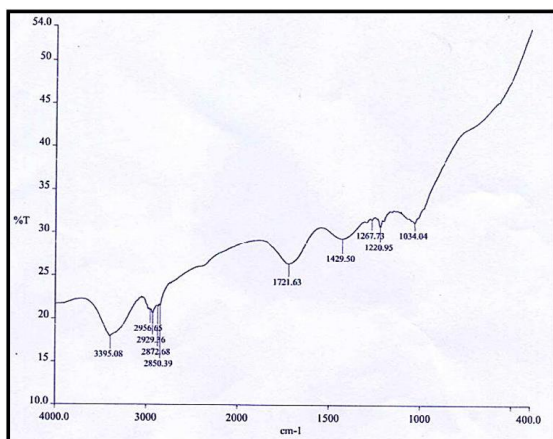


Figure: FTIR of pure Andrographolide drug

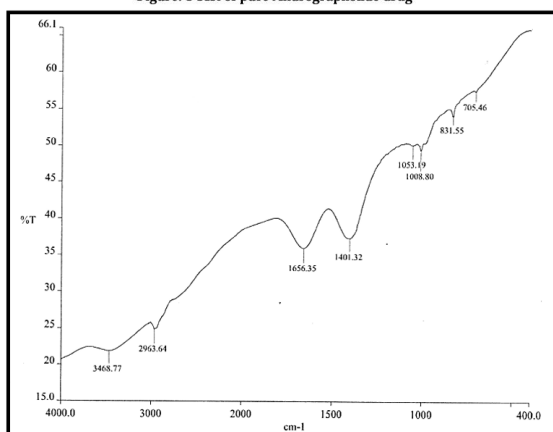


Figure: FTIR of pure Beta Sitosterol drug

6.3 Method Development by UV Spectroscopy

The UV spectra of both drugs were recorded in the range of 200–800 nm.

Beta-sitosterol exhibited a maximum absorbance (λ_{max}) at 211 nm, whereas andrographolide showed λ_{max} at 225 nm.

The overlay spectrum (Figure 5) revealed an isobestic point at 215 nm, indicating that both drugs can be simultaneously estimated without interference. This confirms the suitability of the developed UV spectrophotometric method.

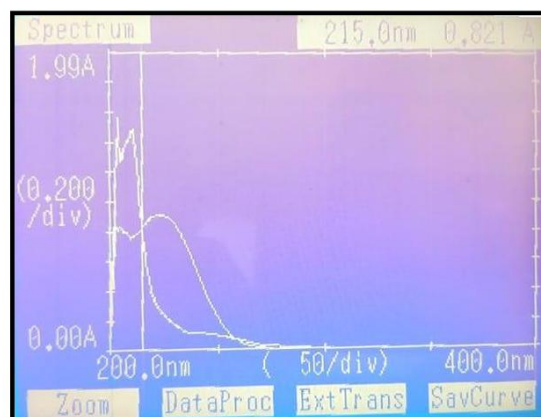


Figure 5: Overlay of Beta Sitosterol and Andrographolide showing isobestic point.

6.4 Simultaneous Equation Method

The simultaneous equation (Vierordt's) method was successfully applied for the estimation of both drugs. Absorbance values were recorded at 211 nm and 225 nm, and both drugs followed Beer-Lambert law in the concentration range of 5–25 $\mu\text{g/mL}$.

The calculated absorptivity values were consistent across concentrations, demonstrating the reliability of the method. The calibration curves showed good linearity with correlation coefficients close to unity, confirming the accuracy of the method.

6.5 Method Validation

6.5.1 Linearity

The calibration curves for both drugs showed a linear relationship between absorbance and concentration within the range of 5–25 $\mu\text{g/mL}$.

Beta-sitosterol showed a correlation coefficient (R^2) of 0.9856, while andrographolide exhibited a higher R^2 value of 0.997 (Figure 6 and Figure 7), indicating excellent linearity.

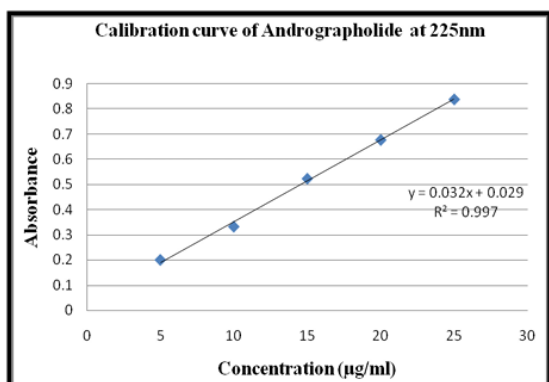


Figure 6: Calibration curve of Andrographolide at 225nm

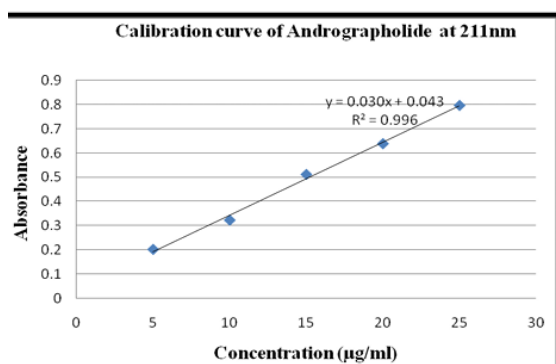


Figure 7: Calibration curve of Andrographolide at 211.0nm

6.5.2 Precision

The method demonstrated excellent precision with %RSD values below 2%.

Repeatability studies showed %RSD values of 0.606% for beta-sitosterol and 0.226% for andrographolide.

Intraday and interday precision studies also showed low variability, confirming the reproducibility and consistency of the method.

6.5.3 Ruggedness

The method was found to be rugged, as %RSD values obtained by different analysts were below 2%. This indicates that the method is reliable under varied experimental conditions.

6.5.4 Robustness

The robustness study showed that slight variations in temperature (25°C and 45°C) did not significantly affect the results. The %RSD values remained within acceptable limits, confirming the stability of the method.

6.5.5 LOD and LOQ

The LOD and LOQ values indicated good sensitivity of the method.

For beta-sitosterol, LOD and LOQ were 0.324 µg/mL and 0.983 µg/mL, respectively.

For andrographolide, LOD and LOQ were 0.206 µg/mL and 0.625 µg/mL, respectively.

7.6 Percentage Recovery

The accuracy of the method was confirmed through recovery studies.

Beta-sitosterol showed recovery values in the range of 92.47% to 98.21%, while andrographolide showed recovery between 89.47% and 93.98%.

The %RSD values were within acceptable limits, confirming the accuracy and reliability of the method for routine analysis.

VII. CONCLUSION

The present study was carried out to develop and validate a simple, precise, and reliable UV spectrophotometric method for the simultaneous estimation of beta-sitosterol and andrographolide. Organoleptic evaluation and melting point determination confirmed the purity and identity of both compounds. Solubility studies indicated ethanol as a suitable solvent for analysis. FTIR analysis verified the presence of characteristic functional groups, confirming structural integrity, while UV spectroscopic analysis showed λ_{max} at 211 nm for beta-sitosterol and 225 nm for andrographolide, with an isobestic point at 215 nm.

Calibration curves constructed in the range of 5–25 µg/mL exhibited good linearity ($R^2 > 0.98$), confirming adherence to Beer–Lambert law. The method showed excellent precision, ruggedness, and robustness, with %RSD values below 2%. Low LOD and LOQ values indicated good sensitivity, and recovery studies confirmed the accuracy of the method.

Overall, the developed method was found to be simple, accurate, precise, reproducible, and cost-effective. It is suitable for routine quality control and simultaneous estimation of beta-sitosterol and andrographolide in pharmaceutical formulations.

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