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Research article

Development of an HPLC Analytical Method for β-Sitosterol Used as a Treatment of Burns and Skin Ulcers

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Abstract

This study focuses on developing a highly accurate and reproducible High-Performance Liquid Chromatography technique of β-sitosterol for the quantification. Widely, a compound used as a treatment of burns and skin ulcers. This developed method ensures reliable separation, identification, and quantification of β-sitosterol in pharmaceutical formulations. Accuracy was assessed through recovery studies at three levels (80%, 100%, and 120%). The acceptance criterion was a recovery rate between 98% and 102%, which was successfully met. The detection limit (LOD) is defined as the lowest concentration of an analyte that can be detected, though not necessarily quantified. Typically, a signal-to-noise (S/N) ratio of 3: 1 is considered acceptable for estimating the LOD. The quantification limit (LOQ) refers to the lowest concentration that can be quantitatively measured with acceptable precision and accuracy, usually determined at a signal-to-noise ratio of 10: 1. The LOD is calculated using the formula 3.3 × (Sy/x / b), while the LOQ is calculated as 10 × (Sy/x / b). In this method, the LOD was found to be 2 µg/mL, and the LOQ was 6 µg/mL. The LOD and LOQ were determined based on the standard deviation of the response (σ) and the slope (S) of the calibration curve. The calculated LOD was 1.52 µg/mL.

Keywords

Analytical, HPLC, β-Sitosterol, Treatment

1. Introduction

β-sitosterol is a plant-based steroid compound structurally similar to cholesterol. It is a naturally occurring phytosterol commonly present in plant and vegetable oils owing to its lipophilic and steroidal characteristics [1, 7]. The qualitative analysis of β-sitosterol has been performed using gas chromatography and mass spectrometry techniques, providing valuable insights into its chemical composition and biological potential [2].

Pharmacokinetics (PK) involves the study of how a drug is absorbed, distributed, metabolized, and excreted by the body while it exerts its pharmacological effects [3, 8]. Burns and skin ulcers represent significant clinical challenges that often require effective therapeutic agents to accelerate healing. β-Sitosterol, a naturally derived phytosterol, has demonstrated potent anti-inflammatory and wound-healing properties, making it a promising component in topical pharmaceutical formulations [4].

In Yemen, various infectious diseases have been widely

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investigated to understand their prevalence and improve diagnostic accuracy among vulnerable populations, including pregnant women. Recent sero-epidemiological studies have highlighted the public health significance of brucellosis and emphasized the need for reliable laboratory-based analytical approaches [10-13]. Such research underscores the importance of developing accurate and validated analytical techniques to enhance both biomedical diagnostics and pharmaceutical quality control [6].

Therefore, the objective of this study was to develop and validate a simple, accurate, and reproducible High-Performance Liquid Chromatography (HPLC) method for the quantification of β-sitosterol in topical pharmaceutical formulations, ensuring analytical precision, sensitivity, and reliability for routine quality control applications.

2. Materials and Methods

The β-sitosterol standard, used as a reference, was obtained from Yemeni Marketing in Yemen. Methanol and acetonitrile, both of HPLC grade, along with analytical grade ethanol, were used as solvents. All eluents were purified using a 0.45 μm membrane filter from Sartorius. Product samples were sourced from various markets across Yemen.

2.1. Materials

Three of materials were used in this study such as: Ethanol, Methanol and acetonitrile.

2.2. Preparation of Mobile Phase

Different mobile phase mixtures, starting from 10% methanol and 90% acetonitrile, were prepared to optimize the system for analyzing β-sitosterol in the samples. Before use in the HPLC system, all solvents were filtered through a 0.45 μm microporous membrane and sonicated for 15 minutes [5].

2.3. Chromatographic Conditions

Many equipments were used in this study such as HPLC system, column lona C 18.5 μm, 250 mm. this study used Mobile Phase: 90% Acetonitrile and 10% Methanol, The flow rate was set at 1.0 mL/min. Detection was carried out at a wavelength of 205 nm, with the column maintained at 40 ℃ and an injection volume of 20 μL.

2.4. Preparation of Standard Solution

2.4.1. Preparation for β-sitosterol Standard Solution

Weighed 50 mg of pure β-sitosterol standard to prepare a stock solution. Then, 50 mL of this β-sitosterol solution was transferred into a volumetric flask. The standard was dissolved using a few drops of 99.9% ethanol, resulting in a final concentration of 1 mg/mL.

2.4.2. Preparation of Sample Solution

Weigh 4 grams of the β-sitosterol-containing formulation to a 50 mL test tube then add 10 mL of 99.9% ethanol. Prepare serial dilutions to obtain different concentrations for method validation, including tests for accuracy and precision.

2.4.3. HPLC System for Optimization

The β-sitosterol standard solution was injected into the HPLC system using different flow rates, injection volumes, and mobile phase compositions. The HPLC system was optimized by first adjusting the flow rate, followed by the injection volume and mobile phase composition, to achieve the best retention time and a clear chromatogram.

2.5. Developed Methods

The chromatographic analysis of β-sitosterol was performed using a reverse-phase HPLC system. The optimized mobile phase consisted of a mixture of acetonitrile and methanol in a 90: 10 (v/v) ratio. which provided a sharp and symmetric peak with a retention time of 18.090 min. Additionally, the flow rate was maintained at 1.0 mL/min, with detection carried out at a wavelength of 210 nm and an injection volume of 20 μL, and the analysis was carried out at room temperature. This mobile phase composition was chosen after testing several mixtures, and it offered the best resolution with minimal baseline noise.

A chemical analysis method has been developed using HPLC for beta-acetylsterol in the pharmaceutical form of an ointment. This substance contains three isotopes: beta-acetylsterol, vaso-acetylsterol, and gamma-sitosterol. Separating it using a GC device, which relies on the volatility of the substance to estimate it, requires extraction processes in more than one stage. Therefore, increasing the extraction process leads to a loss of some of the concentration. When estimating it using a GC device, it is an identification analysis, not a quantitative analysis Therefore, it was separated by polarity in an HPLC device.

Then medium was 90% acetonitrile and 10% methanol v/v and C18 coulomb was 25 cm long and 5 μm in diameter. The detection wavelength was set at 205 nm, followed by maintaining the temperature at 45 ℃. The standard was prepared by weighing the equivalent of 50 mg of beta-acetylsterol into a 50 ml flask and dissolving it in 99% ethanol. Finally, a sample was prepared by weighing 50 mg of ointment and dissolving it in 10 mL of 99% ethanol more than one concentration of in order to study linerty and precision.

2.5.1. Linearity

The method demonstrated excellent linearity over the range of concentration was 10 μg/mL to 200 μg/mL. The calibration curve showed a correlation coefficient (R^2) of 0.9992, indi-

cating a strong linear relationship between peak area and concentration. Regression Equation: Peak Area = 1032.5 × Concentration + 2000.

A calibration curve was constructed using different concentrations of beta-sitosterol. The analysis showed a strong linear relationship between concentration and response, with a correlation coefficient (R^2) of 0.9991, indicating excellent linearity.

Table 1. *Show the concentration and peak area.*

Concentration (µg/mL)	Peak Area (mAU)	Retention Time (min)
10	135.2	18.09
20	271.3	18.09
40	545.6	18.09
60	810.8	18.09
80	1076.2	18.09

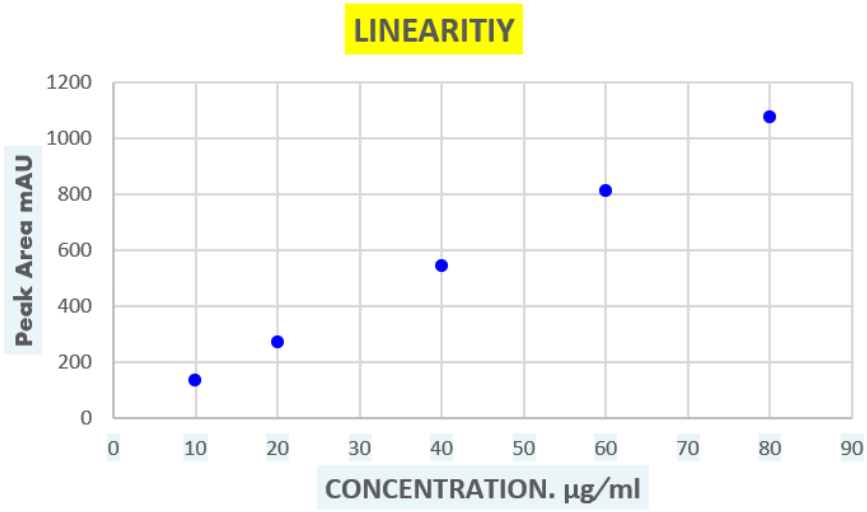

Figure 1. *revealed the peak area and concentration.*

2.5.2. Accuracy

Accuracy was assessed through recovery studies at three levels (80%, 100%, and 120%). The acceptance criterion was a recovery rate between 98% and 102%, which was successfully met.

Accuracy was evaluated using recovery studies conducted at three concentration levels: 80%, 100%, and 120% of the target concentration 40 µg/mL. The recovery values ranged from 98.8% to 99.4%, demonstrating the method's accuracy.

Table 2. *Show the level and values of Beta sitosterol.*

Level (%)	Amount Added (µg/mL)	Amount Found (µg/mL)	Recovery (%)
80	32	31.8	99.4
100	40	39.6	99.0
120	48	47.4	98.8

2.5.3. Precision

Precision was evaluated by analyzing six replicates of the same sample under identical conditions within a single day (intra-day) and over multiple days (inter-day). The intra-day precision showed an RSD of 1.2%, while the inter-day precision had an RSD of 1.5%.

Acceptance Criteria: RSD should be ≤ 2% — achieved.

Precision was assessed through intra-day and inter-day repeatability at a concentration of 40 µg/mL. The relative standard deviation (RSD%) was below 2%, demonstrating the method's high precision.

Table 3. Show the Precision.

Day	Replicate 1	Replicate 2	Replicate 3	Mean	RSD (%)
Intra-day	546.1	544.9	545.8	545.6	0.11
Inter-day	543.2	546.5	544.7	544.8	0.31

2.5.4. Specificity

The method showed no interference from the excipients in the formulation β-sitosterol peak was well-resolved with a retention time of approximately [specify RT, e.g., 5.8 minutes].

2.5.5. Detection and Quantification Limit

The detection limit (LOD) is defined as the lowest concentration of an analyte that can be detected, though not necessarily quantified. Typically, a signal-to-noise (S/N) ratio of 3: 1 is considered acceptable for estimating the LOD. The quantification limit (LOQ) refers to the lowest concentration that can be quantitatively measured with acceptable precision and accuracy, usually determined at a signal-to-noise ratio of 10: 1. The LOD is calculated using the formula 3.3 × (Sy/x / b), while the LOQ is calculated as 10 × (Sy/x / b). In this method, the LOD was found to be 2 µg/mL, and the LOQ was 6 µg/mL.

The LOD and LOQ were determined based on the standard deviation of the response (σ) and the slope (S) of the calibration curve. The calculated LOD was 1.52 µg/mL.

- LOQ: 4.60 µg/mL

These results indicate that the method is sensitive and capable of detecting and quantifying beta-sitosterol at low concentrations.

The method of robustness was evaluated by slight variations in experimental conditions such as flow rate and mobile phase composition. The results showed that minor changes did not significantly affect the retention time or peak area, indicating that the method is robust.

Table 4. show the Detection and Quantification Limit.

Parameter	Condition	Retention Time (min)	Peak Area (mAU)	RSD (%)	Observation
Flow Rate	0.9 mL/min	18.26	544.3	0.25	No significant change
Flow Rate	1.1 mL/min	17.81	547.0	0.3	No significant change
Mobile Phase Composition	±2% Methanol	18.03-18.14	545.1-546.2	<0.4	No significant change
Wavelength	±2 nm (208-212)	18.05-18.10	544.6-545.9	<0.3	No significant change

3. Results and Discussion

3.1. HPLC System Optimization

In this study, a reversed-phase HPLC method was combined with gas chromatography (GC) for the analysis of β-sitosterol in supplements. A C-18 column was selected due to its widespread use in β-sitosterol identification, offering improved peak shape and resolution. An isocratic elution was chosen for its simplicity, utilizing a single pump to reduce baseline fluctuations and ghost peaks. According to spectrophotometric analysis, the maximum absorption wavelength of

β-sitosterol was determined to be 205 nm. The results, summarized in Table 1, include various flow rates and mobile phase compositions used to optimize chromatographic and GC conditions.

Table 5. *Comparison Between GC and HPLC for β-Sitosterol Analysis.*

Parameter	GC (Gas Chromatography)	HPLC (High Performance Liquid Chromatography)
Sample Preparation	Requires derivatization (e.g., silylation)	No derivatization needed
Thermal Sensitivity	Not suitable for heat-sensitive compounds	Suitable for thermally unstable compounds
Sample Loss Risk	High (during extraction and derivatization)	Low
Accuracy	Moderate (may be affected by incomplete derivatization)	High (direct injection, minimal manipulation)
Quantification	Less accurate for low concentrations	Highly accurate, especially for small quantities
Preparative Capability	Not suitable	Suitable for preparative separation
Ease of Use	Requires more complex sample handling	Simpler sample preparation

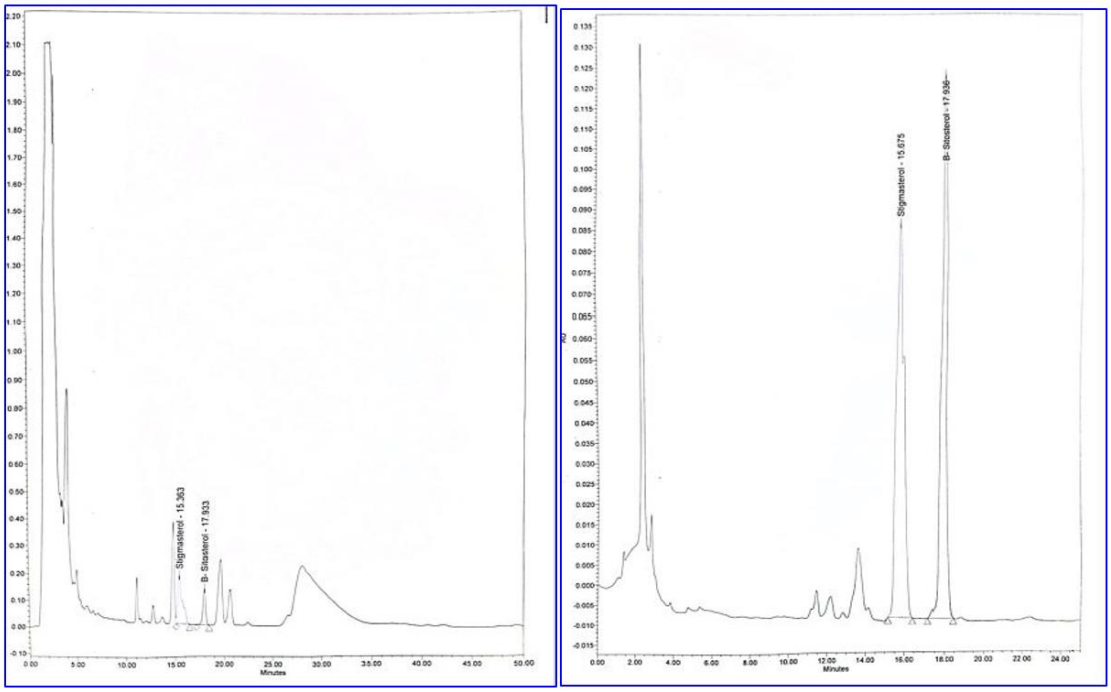

Figure 2. *Chromatograms of (A) Blank, (B) β-sitosterol Standard, and (C-E) Supplement samples containing β-sitosterol.*

3.2. Suitability Test of System

The system suitability parameters were evaluated to determine the optimal HPLC conditions. The United States Pharmacopeia (USP) provides established criteria for system suitability in pharmaceutical HPLC analysis. Accordingly, analytical parameters such as relative standard deviation (%RSD), retention time, peak area, resolution (Rs), tailing factor (τ), capacity factor (k'), and theoretical plates (N) were measured. As shown in Table 3, the developed method met all the acceptance criteria for these system suitability parameters.

3.3. Validation Method of HPLC

The specificity test confirmed that the method was selective enough to accurately quantify the analyte even in the presence of other components potentially found in the sample. Figure 1 illustrates the retention times for both the β-sitosterol standard and sample solutions. Consistent retention times of approximately 17 minutes were observed across all chromatograms, with well-resolved peaks, indicating no interference from the

sample matrix during analysis. While, the injection of sample at 50 min for injection time of standard was 19 min. This study was agree with pervious study that conducted by [9] who reported that the retention time was at 13 min.

3.4. β-Sitosterol in Supplement Assay

The method was developed to quantify β-sitosterol concentrations in commercially available tablet and capsule supplements from the local market. β-sitosterol levels were measured in multiple samples. Compared to other chromatographic techniques, this method offers high accuracy and precision, along with rapid sample preparation and a shorter analysis time. Therefore, the developed approach is well-suited for quality control of β-sitosterol in various supplement products. The developed HPLC method provides accurate, precise, and specific to determine of β-sitosterol. It is suitable for routine analysis in quality control laboratories.

4. Conclusion

An HPLC method was successfully developed and validated to quantify β-sitosterol in topical pharmaceutical formulations. This method meets international validation guidelines and can be reliably applied for routine quality control to ensure product efficacy and safety in treating burns and skin ulcers.

Abbreviations

HPLC High Performance Liquid Chromatography

Conflicts of Interest

The authors declare no conflicts of interest.

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