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Quality by Design (QbD) Assisted Formulation and Development of Microsphere Containing Beta-Sitosterol by Design of Expert (DoE) Approach

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Abstract

The present study focuses on the Quality by Design (QbD)–assisted formulation and optimization of beta-sitosterol-loaded microspheres using a Design of Experiments (DoE) approach. Beta-sitosterol, a bioactive phytosterol with proven therapeutic potential, suffers from poor aqueous solubility and limited bioavailability. To overcome these limitations, microspheres were developed using the emulsion solvent evaporation technique with Eudragit RS100 as the polymer. A Box-Behnken design was employed to systematically evaluate the effect of critical formulation variables, namely polymer concentration, surfactant concentration, and stirring time, on particle size and entrapment efficiency. The optimized microsphere formulation exhibited nanosized particles with high drug entrapment efficiency and controlled drug release behavior. Preformulation, physicochemical characterization, in vitro release, antimicrobial activity, and stability studies confirmed the suitability and stability of the developed system. Overall, the QbD–DoE approach proved effective in developing a robust and reproducible beta-sitosterol microsphere formulation with improved delivery characteristics and potential therapeutic applicability.

Keywords; Beta-Sitosterol, Bioactive Phytosterol, Antimicrobial Activity, Microsphere Formulation, Physicochemical Characterization.

INTRODUCTION

Quality by Design (QbD) has emerged as a systematic and science-based approach in pharmaceutical development that emphasizes predefined objectives, product understanding, and process control. Promoted by regulatory bodies such as the FDA and ICH, QbD integrates risk management and statistical tools like Design of Experiments (DoE) to ensure consistent product quality. Unlike traditional trial-and-error methods, QbD enables identification of critical material attributes and process parameters, leading to robust formulations and reduced development costs (Montgomery 2017).

Microspheres are widely explored drug delivery systems due to their ability to enhance drug stability, control release profiles, and improve bioavailability. These systems consist of drugs uniformly dispersed within polymeric matrices and can be tailored for targeted, sustained, or site-specific delivery (Yang et al., 2016; Virmani et al., 2017). Depending on their composition and functionality, microspheres are classified into bioadhesive (Alawd et al., 2021), magnetic (Farah, 2016), floating (Ishak, 2015), polymeric (Prajapati et al., 2015), and radioactive types (Dhadde et al., 2021), each offering distinct therapeutic advantages. Their formulation methods, such as emulsion techniques, polymerization, spray drying, solvent evaporation, and ionotropic gelation, allow flexibility in encapsulating drugs with diverse physicochemical properties.

Beta-sitosterol is a naturally occurring phytosterol known for its anti-inflammatory, antioxidant, cholesterol-lowering, and anticancer activities. Despite its therapeutic potential, its clinical application is limited by poor aqueous solubility and low bioavailability. Encapsulation of beta-sitosterol into microspheres offers a promising strategy to overcome these limitations by enhancing solubility, protecting the drug from degradation, and enabling controlled release.

In this context, the integration of QbD principles with DoE provides a rational framework for optimizing beta-sitosterol-loaded microsphere formulations. By systematically evaluating formulation and process variables such as polymer concentration, surfactant level, and stirring time, it becomes possible to achieve desired particle size, high drug entrapment efficiency, and reproducible performance. Thus, the present research focuses on developing and optimizing a microsphere-based delivery system for beta-sitosterol using a QbD-assisted DoE approach to enhance its therapeutic effectiveness and ensure regulatory compliance.

DRUG PROFILE

Beta-Sitosterol

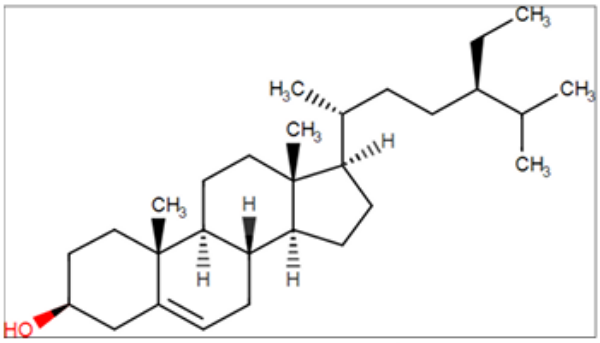

Figure 1: Beta-Sitosterol

- **Generic Name**-Beta-Sitosterol
- **Molecular weight:** 414.718
- **Chemical Formula:** C29H50O
- **Melting Point:** 140°C
- **IUPAC name:** (3S)-17-(5-ethyl-6-methylheptan-2-yl)-10,13-dimethyl 2,3,4,7,8,9,11,12,14, 15,16,17 dodecahydro-1H-cyclopenta [a]phenanthren-3-ol
- **Synonyms:** Azuprostat, Cupreol, Nimbosterol, Sitosterol, Triastonal

Description-

Sitosterolisa member of the class of phytosterols that is stigmast-5-enesubstituted bya beta- hydroxy group at position 3. It has a role as a sterol methyltransferase inhibitor, an anticholesteremic drug, an antioxidant, a plant metabolite and a mouse metabolite. It is a 3 beta-sterol, a stigmastane sterol, a 3 beta-hydroxy-Delta (5)-steroid, a C29-steroid and a member of phytosterols. It derives from a hydride of a stigmastane.

(i) **Biosynthesis of β-Sitosterol:** Biosynthesis of the phytosterols is regulating during membrane biogenesis. The literature showed that β-sitosterol is biologically synthesized from both mevalonate and deoxyxylulose pathways.

(ii) **Natural occurrences and food:** It is found in vegetable oil, nuts, avocados, and derived prepared foods such as salad dressings. Ola visual garvensis, a species of marine annelid, predominantly incorporate sitosterol into their cell membranes instead of cholesterol, though cholesterol is also present in said membranes.

(iii) **Dosing:** In adults, beta-sitosterol has most often been used in doses of 3-4 grams by mouth daily for upto 3 months. It's also been used at a lower dose of 60 -130 mg daily for up to 18 months. In children, beta-sitosterol has most often been used in doses of 6-12 grams by mouth daily for up to 3 months. Beta-sitosterol is also available in ointments.

(iv) **Physical properties:** Physical properties revealed that β-sitosterol sugar esters had enhanced water solubility (3.0–8.0 mM at 35 °C), reduced crystallinity, and high wett ability. Their lyotropic liquid crystal properties were observed by polarized light microscopy. Furthermore, β-sitosterol sugar esters could be hydrolyzed into β-sitosteroladipate under simulated intestinal conditions at a low rate (2.83–18.14%). Most β-sitosterol sugar esters probably entered into intestinal bile salt micelles with ester bonds intact and showed upto10 - fold higher in vitro bioaccessibility than free β-sitosterol in non-fat systems.

MATERIAL AND METHOD

The Materials and Methods section of a study provides a detailed yet concise description of how the research was conducted, ensuring the work is reproducible and scientifically valid. It typically includes the list of materials such as drugs, chemicals, polymers, and instruments used, along with their sources. This section outlines the step-by-step procedures followed during formulation,

characterization, and analysis, including experimental design methods like Design of Experiments (DoE) if applicable. It also describes the techniques used for evaluating the formulation, such as particle size analysis, drug entrapment efficiency, in vitro release studies, and statistical tools used for data interpretation. Overall, this section serves as a blueprint of the experimental work carried out in the study.

Selection of drug and chemical

Table 1: list of chemical

S. No.	Name of Chemicals	Company
1	Beta-Sitosterol	Herbo Nutra Extract Pvt. Ltd.
2	Methanol	Rankem
3	Ethanol	Merck India Ltd. Mumbai, India
4	Acetonitrile	Merck India Ltd. Mumbai, India
5	Ethyl cellulose	Sigma eldrich
6.	Carbopol 934	Sulab
7.	Triethanolamine	Loba
8.	Propylene glycol	Merck
9.	Methyl paraben	Merck
10.	Distilled water	Vizag Chemicals

Table 2: List of glassware's

S. No	List of Glassware's	Type/Company
1.	Test tubes	Borocil
2.	Petri dishes	Borocil
3.	Glass rod	Borocil
4.	Beaker	Borocil
5.	Conical flask	Borocil
6.	Measuring cylinder	Borocil
7.	glass slide	Borocil
8.	Volumetric flask	Borocil

Table 3: List of instruments used

S. No.	Name of Instrument	Company
1	Electronic Weighing Balance	A & D Company HR 200
2	pH meter	Remi Motors, India.
3	Magnetic Stirrer	MC Dalal & amp; Co India
4	UV Visible Spectrophotometer	Shimadzu 1700
5	Stability Chamber	Inlab Equipments Madras PVT (LTD)

Preformulation studies of Beta-Sitosterol

Preformulation studies of Beta-Sitosterol are a crucial initial step in developing a stable and efficient topical drug delivery system. These early investigations focus on assessing key physicochemical properties, including organoleptic characteristics, solubility in different solvents, pH determination, melting point analysis, and UV spectroscopic analysis to determine the absorption maximum (λmax). Drug–excipient compatibility was assessed using Fourier Transform Infrared (FT-IR) spectroscopy. Understanding these parameters is essential for guiding the selection of suitable formulation components and ensuring optimal drug performance. Such data not only aid in predicting the behavior of Beta-Sitosterol within the formulation but also help in enhancing its bioavailability, therapeutic effectiveness, and overall shelf life. Ultimately, the goal of preformulation studies is to provide a scientific basis for designing a robust and effective topical delivery system for Beta-Sitosterol (Srinivasan et al., 2025).

Preparation of Microsphere containing Beta-Sitosterol

Table 4: Composition of Microsphere Formulation

S. No.	Run	Polymer (Eudragit RS100) (mg)	Surfactant (Tween 80) (%)	Stirring time (hrs)	Particle size (nm)	Entrapment efficiency (%)
1	MS 1	300	0.3	1	727.3	92.6
2	MS 2	300	0.3	3	181.9	89.1
3	MS 3	300	0.5	2	446.7	81.6
4	MS 4	175	0.1	3	191.6	73.2
5	MS 5	50	0.1	2	332.8	66.8
6	MS 6	175	0.1	1	622.1	74.5
7	MS 7	50	0.3	3	260.6	60
8	MS 8	50	0.3	1	359.3	60.3
9	MS 9	175	0.5	3	253.6	79.8
10	MS 10	50	0.5	2	253	62.3
11	MS 11	300	0.1	2	433.9	90.3
12	MS 12	175	0.5	1	420.2	68.5

Microsphere formulations using Eudragit RS100 as a carrier polymer were prepared using emulsion solvent evaporation technique. Desired quantity of Eudragit RS100 polymer was dissolved in 10 ml of chloroform to form a homogenous polymer solution. Calculated quantity of drug was added to this polymer solution and mixed thoroughly. The resulting mixture was then added to 250 ml of aqueous mucilage of sodium CMC (0.5%) containing 1% v/v tween 80, while stirring at 1000 rpm for emulsification. Chloroform was removed by evaporation during continuous stirring at room temperature for 3 h to produce spherical microspheres. Chloroform was used as the polymer solvent, aqueous mucilage of sodium CMC as microencapsulating vehicle and tween 80 as dispersing agent. During 3 h of stirring period, chloroform was completely removed by evaporation. Microspheres were collected by vacuum filtration, washed repeatedly with distilled water and petroleum ether and dried at room temperature for 24 h to get free flowing microspheres.

Independent and Dependent variables

Table 5: Independent and Dependent variables

Sr. no.	Independent variables	Dependent variables
1	(W1) Polymer Eudragit RS100 (mg)	(X1) Particle size (nm)
2	(W2) Surfactant Tween 80 (%)	(X2) Entrapment efficiency EE (%)
3	(W3) Stirring time (hrs)	

Evaluation parameter of Beta-Sitosterol loaded Microsphere formulation

Beta-Sitosterol-Loaded Microsphere`s Physical appearance

The physical properties of the Beta-Sitosterol-loaded microsphere formulation were thoroughly evaluated to ensure formulation quality and stability. Visual inspection was carried out to assess the shape, color uniformity, and overall appearance of the microspheres. The dispersion was examined for clarity, noting any turbidity or signs of cloudiness that might indicate instability. Surface characteristics were observed to identify any aggregation or irregularities in particle texture (Penjuri et al., 2016).

Particle Size of Beta-Sitosterol-Loaded Microsphere

The particle size of the Beta-Sitosterol-loaded microsphere formulation was measured using dynamic light scattering (DLS) with a particle size analyzer. For accurate results, a small volume of the microsphere suspension was diluted with distilled water to achieve optimal light scattering conditions. The diluted sample was placed in a clean, dust-free cuvette and analyzed at room temperature. The instrument reported the average particle size along with the polydispersity index (PDI), which reflects the uniformity of particle size distribution. These parameters are essential for assessing the formulation's physical stability and its suitability for topical or transdermal application (Abbas et al., 2018).

Zeta Potential of Beta-Sitosterol-Loaded Microsphere

The zeta potential of the Beta-Sitosterol-loaded microsphere formulation was analyzed using a Malvern Zetasizer (Malvern Instruments). A properly diluted microsphere suspension was placed into a specialized zeta potential cell for testing. Laser Doppler velocimetry was employed to measure the electrophoretic mobility, from which the zeta potential values were calculated. These measurements offered valuable information about the surface charge and colloidal stability of the formulation key factors that affect both the physical stability of the microspheres and their behavior in biological environments (Seema et al., 2015).

Drug Entrapment Efficiency of Beta-Sitosterol-Loaded Microsphere

To evaluate the entrapment efficiency of Beta-Sitosterol in the microsphere formulation, 10 mL of the Beta-Sitosterol-loaded microsphere suspension was combined with 5 mL of ethanol in a volumetric flask. The mixture was vortexed thoroughly for one minute to ensure complete disruption of the microspheres and release of the encapsulated drug. The solution was then diluted to a final volume of 10 mL using an appropriate solvent. After filtration to remove any undissolved particles, the drug concentration was determined using UV-Visible spectrophotometry. This data was used to calculate the entrapment efficiency, indicating the percentage of Beta-Sitosterol successfully incorporated into the microspheres. The entrapment efficiency was determined using the following formula:

$$\text{Entrapment efficiency \%} = \frac{\text{Total drug conc.} - \text{Supernatant drug conc.}}{\text{Total drug conc.}} \times 100$$

This method provides an accurate estimation of how much Beta-Sitosterol was successfully encapsulated within the Microsphere (Krishna et al., 2021).

In Vitro Drug Release Study of Beta-Sitosterol-Loaded Microsphere

The in vitro release behavior of Beta-Sitosterol from the Microsphere formulation was assessed using the dialysis bag diffusion method. A defined quantity of the Beta-Sitosterol-loaded Microsphere suspension was introduced into a pre-soaked dialysis membrane, which was then suspended in 100 mL of phosphate buffer solution (pH 7.4) contained in a beaker. The setup was maintained at 37 ± 2 °C with constant stirring at 100 rpm using a magnetic stirrer to mimic physiological conditions.

At predetermined time intervals, 2 mL aliquots were withdrawn from the release medium and immediately replaced with an equal volume of fresh buffer to maintain sink conditions. The collected samples were suitably diluted and analyzed using a UV-Visible spectrophotometer at a wavelength of 427 nm to quantify the amount of Beta-Sitosterol released over time. The resulting release data were then subjected to various kinetic modeling approaches (e.g., zero- order, first-order, Higuchi, and Korsmeyer-Peppas models) to better understand the mechanism governing drug release from the Microsphere system.

- **Zero-order model:** Drug release occurs at a constant rate, independent of concentration.
- **First-order model:** Release rate depends on the remaining drug concentration.
- **Higuchi model:** Drug release follows a diffusion mechanism proportional to the square root of time.
- **Korsmeyer-Peppas model:** A log-log plot used to analyze the drug release mechanism from the polymeric Microsphere system.

Antimicrobial Activity (Well Diffusion Assay)

(i) Preparation of Nutrient Agar Media

A total of 2.8 grams of nutrient media was accurately weighed and dissolved in 100 mL of distilled water. The pH of the prepared medium was measured before sterilization to ensure suitability for microbial growth. The solution was then sterilized using an autoclave at 121 °C and 15 psi pressure for 15 minutes. Following sterilization, the nutrient medium was aseptically poured into sterile Petri dishes and placed in a laminar airflow cabinet to allow the agar to solidify under sterile conditions.

(ii) Well Diffusion Assay

To evaluate the antibacterial activity of the Beta-Sitosterol-loaded Microsphere against Escherichia coli and Staphylococcus aureus, a well diffusion assay was employed. A bacterial suspension of S. aureus was standardized to approximately 10^8 CFU/mL and agitated on a shaker to ensure homogeneity. Likewise, an overnight culture of E. coli was adjusted to match the 0.5 McFarland standards. For both strains, 100 µL of the inoculum was evenly spread on the surface of freshly prepared and solidified sterile nutrient agar plates using sterile cotton swabs to create a uniform bacterial lawn.

Wells were punched into the agar using a sterile cork borer, and each well was filled with a fixed volume of the Beta-Sitosterol-loaded Microsphere. A blank Microsphere (lacking the active drug) served as the negative control, while a standard antibiotic disc or solution was used as the positive control. The plates were kept at room temperature for 30 minutes to allow pre-diffusion of the test formulations, followed by incubation at 37 °C for 24 hours.

Post-incubation, the zones of inhibition around each well were measured in millimeters (mm) to assess the antimicrobial efficacy of the formulation against both gram-positive (S. aureus) and gram-negative (E. coli) bacteria (Athanassiadis et al., 2009).

Stability Studies

The Microsphere formulation was sealed and subjected to accelerated stability testing in accordance with ICH guidelines. Samples were stored under two defined environmental conditions: 25 ± 2 °C with 60 ± 5% relative humidity (RH) and 40 ± 2 °C with 70 ± 5% RH for a period of three months. Stability assessments were conducted at predetermined intervals days 30, 45, 60, and 90 to monitor any changes in critical parameters, including pH and viscosity. These evaluations were essential for determining the physical stability of the formulation and ensuring that its quality, consistency, and performance remained unaffected under stress storage conditions (Mundada and Borate 2025).

RESULTS AND DISCUSSION

Pre-formulation study of drug

Organoleptic properties

Based on the observations, the organoleptic evaluation of Beta-Sitosterol revealed characteristics consistent with its known physical profile. The drug exhibited a white to off-white color, which aligns with standard descriptions and indicates the absence of any visible impurities or degradation. Its odorless or neutral scent further confirms the chemical stability of the compound and suggests that no volatile degradation products or contaminants are present.

The appearance was noted as a white, waxy powder, and the physical state was described similarly, reinforcing the material's expected consistency. These organoleptic properties are important not only for quality assurance but also for ensuring formulation compatibility and patient acceptability, particularly in topical and transdermal applications. Overall, the results confirm the purity and integrity of Beta-Sitosterol, supporting its suitability for further formulation into microsphere-based drug delivery systems.

Solubility study

The solubility profile of Beta-Sitosterol, highlights its poor aqueous solubility, with the drug being practically insoluble in water. This low solubility poses a significant challenge for formulation development, particularly for oral or topical systems where dissolution rate impacts bioavailability. However, Beta-Sitosterol demonstrated good solubility in ethanol and chloroform, indicating its lipophilic nature. It was found to be sparingly soluble in methanol and moderately soluble in DMSO, which are common solvents used during analytical and formulation processes. These findings suggest that organic solvents or lipid-based carriers may be more suitable for enhancing the solubility and delivery of Beta-Sitosterol. The data obtained from the solubility study is essential for guiding the selection of appropriate solvents, excipients, and delivery systems, especially in the development of microsphere formulations aimed at improving the drug's bioavailability and therapeutic efficacy.

pH determination

The observed pH of Beta-Sitosterol was 5.8, which falls within the acceptable reference range of 5.4 to 7.2. This slightly acidic pH indicates good compatibility with topical formulations, as it aligns well with the natural pH of human skin, thereby minimizing the risk of irritation upon application. Maintaining the drug's pH within this range is also important for ensuring chemical stability and solubility, as extreme pH values could lead to degradation or reduced efficacy. Overall, the pH result supports the suitability of Beta-Sitosterol for incorporation into topical microsphere-based delivery systems.

Melting point

The observed melting point of Beta-Sitosterol was 138°C, which lies within the reported reference range of 136–140°C. This confirms the purity and identity of the drug, as deviations from the expected melting point can indicate the presence of impurities or structural changes. A consistent melting point also reflects good thermal stability, which is essential during microsphere formulation, where processes like drying or encapsulation may involve exposure to heat. Therefore, the result supports the suitability of Beta-Sitosterol for further pharmaceutical development, particularly in temperature-sensitive delivery systems such as microspheres.

Lambda max of Beta-Sitostero

Double beam UV visible spectrophotometer (Shimadzu-1700) was used to determine the lambda max (absorption maxima) of a substance. he UV absorption maximum (λmax) for Beta-Sitosterol was recorded at 204.0 nm. This wavelength represents the point at which the drug exhibits maximum absorbance in the UV range, making it the most suitable for quantitative analysis using UV-Visible spectrophotometry. Identifying λmax is crucial for developing accurate and reliable analytical methods, ensuring precise measurement of drug concentration during formulation and evaluation processes. The absorption at 204.0 nm indicates that Beta-Sitosterol contains microsphere capable of absorbing in the lower UV region, which is typical for compounds with limited conjugation or aliphatic structures.

Calibration curve of Beta-Sitosterol

Table 6: Calibration curve Beta-Sitosterols

Concentration (µg/ml)	Absorbance
5	0.087
10	0.179
15	0.281
20	0.368
25	0.473
30	0.583
Mean	0.3285
SD	0.184466
%RSD	56.154

Based on the data presented in Table 6, the calibration curve of Beta-Sitosterol was constructed using standard solutions at concentrations ranging from 5 to 30 µg/mL. The absorbance values showed a progressive increase with concentration, indicating a direct correlation and adherence to Beer-Lambert's law within this range. The mean absorbance was calculated as 0.3285, with a standard deviation (SD) of 0.184466. However, the percentage relative standard deviation (%RSD) was found to be 56.154, which is significantly high and suggests considerable variability among the absorbance readings. The drug's reaction was linear in the concentration range studied, with the linear regression equation $y = 0.0197x + 0.0164$ and a correlation coefficient $R^2 = 0.9988$.

Functional group identified by Infra-Red spectroscopy

Table 7: Interpretation of IR spectrum of Beta-Sitosterol

S. No.	Peak obtained	Reference peak	Functional group	Name of functional group
1	3449.10	3500-3400	N-H stretching	Primary amine
2	2929.48	3000-2840	C-H stretching	Alkane
3	1655.83	1658-1648	C=C stretching	Alkene
4	1399.87	1440-1395	O-H bending	Carboxylic acid
5	1054.62	1085-1050	C-O stretching	Primary alcohol

The IR spectrum interpretation of Beta-Sitosterol, as presented in Table 7, confirms the Based on Table 7, the FT-IR spectral interpretation of Beta-Sitosterol confirmed the presence of key functional groups consistent with its known chemical structure. A broad peak at 3449.10 cm^{-1} corresponds to N-H stretching, indicative of a primary amine group. The absorption at 2929.48 cm^{-1} matches the C-H stretching range for alkanes, confirming the presence of saturated hydrocarbon chains. A peak at 1655.83 cm^{-1} reflects C=C stretching, characteristic of alkene functionalities. The O-H bending vibration observed at 1399.87 cm^{-1} suggests the presence of carboxylic acid groups, while the C-O stretching peak at 1054.62 cm^{-1} aligns with primary alcohols. These findings confirm the presence of essential functional groups in Beta-Sitosterol and suggest no major structural degradation, supporting its purity and compatibility for further formulation development.

Effect of formulation variables on particle size (ANOVA for 2FI model)

Response 1: particle size

Table 8: Response 1: particle size (ANOVA for 2FI model)

Source	Sum of Squares	Mean Square	F-value	p-value	
Model	3.100E+05	51668.24	177.78	< 0.0001	significant
A-Polymer	42646.60	42646.60	146.74	< 0.0001	
B-Surfactant	5350.95	5350.95	18.41	0.0078	
C-Stirring time	1.926E+05	1.926E+05	662.61	< 0.0001	
AB	2143.69	2143.69	7.38	0.0420	
AC	49885.22	49885.22	171.65	< 0.0001	
BC	17410.80	17410.80	59.91	0.0006	
Residual	1453.13	290.63			
Cor Total	3.115E+05				

Factor coding is Coded.

Sum of squares is Type III - Partial

The Model F-value of 177.78 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, B, C, AB, AC, BC are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

Table 9: Coefficients in Terms of Coded Factors

Factor	Coefficient Estimate	Standard Error	95% CI Low	95% CI High	VIF
Intercept	373.58	4.92	360.93	386.23	
A-Polymer	73.01	6.03	57.52	88.51	1.0000
B-Surfactant	-25.86	6.03	-41.36	-10.37	1.0000
C-Stirring time	-155.15	6.03	-170.64	-139.66	1.0000
AB	23.15	8.52	1.24	45.06	1.0000
AC	-111.68	8.52	-133.59	-89.76	1.0000
BC	65.97	8.52	44.06	87.89	1.0000

The coefficient estimate represents the expected change in response per unit change in factor value when all remaining factors are held constant. The intercept in an

orthogonal design is the overall average response of all the runs. The coefficients are adjustments around that average based on the factor settings. When the factors are orthogonal the VIFs are 1; VIFs greater than 1 indicate multi-colinearity, the higher the VIF the more severe the correlation of factors. As a rough rule, VIFs less than 10 are tolerable.

Predicted value and actual value of all formulations

Table 10: Predicted value and actual value of all Microsphere formulations

S. No.	Formulations	Actual Value	Predicted Value
1	MS 1	727.30	713.42
2	MS 2	181.90	179.77
3	MS 3	446.70	443.88
4	MS 4	191.60	178.32
5	MS 5	332.80	349.58
6	MS 6	622.10	620.57
7	MS 7	260.60	257.10
8	MS 8	359.30	344.05
9	MS 9	253.60	258.55
10	MS 10	253.00	251.56
11	MS 11	433.90	449.31
12	MS 12	420.20	436.90

Upon fitting the formulation results into different models based on the experimental design, the linear model emerged as statistically significant for particle size. This was indicated by an F-value of 177.78 and a highly significant p-value of 0.0500, confirming the model's reliability in predicting the response. The derived model equation for particle size (Particle size, T1) is as follows:

Particle size (W1) = +373.58 Intercept +73.01AW1 -25.86 B W2 -155.15 C W3 +23.15 AB2

W1 -111.68 AC2 W2+65.97 BC2 W3 Here, A represents polymer concentration, B is surfactant level, and C is stirring time. The response surface plot (as illustrated in the corresponding figure) visually demonstrates the individual and combined effects of these formulation variables on Particle size, showing a strong influence particularly from stirring time, which had a markedly negative effect on particle size.

Effect of formulation variables on Entrapment efficiency

Table 11: Response 2: Entrapment efficiency (Fit Summary)

Source	Sequential p-value	Adjusted R^2	Predicted R^2	
Linear	0.0002	0.8713	0.7894	Suggested
2FI	0.5405	0.8614	0.6372	
Quadratic	0.9204	0.7815	0.0465	Aliased

ANOVA for linear model

Response 2: EE (ANOVA Linear)

Table 12: Response 2: Entrapment efficiency

Source	Sum of Squares	Mean Square	F-value	p-value	
Model	1381.85	460.62	25.83	0.0002	significant
A-Polymer	1357.20	1357.20	76.10	< 0.0001	
B-Surfactant	19.85	19.85	1.11	0.3223	
C-Stirring time	4.81	4.81	0.2694	0.6178	
Residual	142.68	17.84			
Cor Total	1524.54				

Factor coding is Coded.

Sum of squares is Type III - Partial

The Model F-value of 25.83 implies the model is significant. There is only a 0.02% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. In this case A is a significant model term. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

Table 13: Coefficients in Terms of Coded Factors

Factor	Coefficient Estimate	Standard Error	95% CI Low	95% CI High	VIF
Intercept	74.92	1.22	72.11	77.73	
A-Polymer	13.02	1.49	9.58	16.47	1.0000

B-Surfactant	-1.58	1.49	-5.02	1.87	1.0000
C-Stirring time	0.7750	1.49	-2.67	4.22	1.0000

The coefficient estimate represents the expected change in response per unit change in factor value when all remaining factors are held constant. The intercept in an orthogonal design is the overall average response of all the runs. The coefficients are adjustments around that average based on the factor settings. When the factors are orthogonal the VIFs are 1; VIFs greater than 1 indicate multi-colinearity, the higher the VIF the more severe the correlation of factors. As a rough rule, VIFs less than 10 are tolerable.

Predicted and actual value of Entrapment efficiency

Table 14: Predicted and actual value of Entrapment efficiency

S. No.	Formulations	Actual Value	Predicted Value
1	MS 1	92.60	87.17
2	MS 2	89.10	88.72
3	MS 3	81.60	86.37
4	MS 4	73.20	77.27
5	MS 5	66.80	63.47
6	MS 6	74.50	75.72
7	MS 7	60.00	62.67
8	MS 8	60.30	61.12
9	MS 9	79.80	74.12
10	MS 10	62.30	60.32
11	MS 11	90.30	89.52
12	MS 12	68.50	72.57

The analysis of formulation data using various model types indicated that the linear model was the most suitable for predicting entrapment efficiency. This model demonstrated statistical significance, with an F-value of 25.83 and a p-value of 0.0500, confirming its adequacy in explaining the observed variations. The resulting polynomial equation for entrapment efficiency (EE, X2) is: (X2) = +74.92 Intercept +13.02A X1 -1.58 B X2 +0.7750 X3 in this equation, A represents polymer concentration, B denotes surfactant level, and C corresponds to stirring time. The 3D response surface plot (as illustrated in the figure below) provides a visual depiction of how these formulation variables affect entrapment efficiency. The plot highlights the significant positive effect of stirring time (C) and the complex interactions among the variables. Based on this design, the data were effectively analyzed and optimized to identify the ideal formulation conditions for maximizing entrapment efficiency.

Optimized formula of Microsphere formulation

The Box–Behnken design was utilized to statistically optimize the formulation variables and evaluate their main, interaction, and quadratic effects on two key responses: partiicle size (X1) and entrapment efficiency (X2). This three-factor, three-level design, developed under the framework of Response Surface Methodology (RSM), involved a total of 12 experimental runs, including three centre points performed in triplicate to ensure experimental reliability and model validity. The outcomes revealed that Particle size across the formulations ranged from 449.308 nm to 375.8 nm, while entrapment efficiency varied between 89.517% and 91.43 %, indicating that the selected variables had a significant impact on the formulation characteristics.

All response data obtained from the 12 experimental formulations were analyzed using Design Expert software (Version 12.0.1.0, Stat-Ease Inc.). The data were fitted to various model types, including linear and quadratic models, to determine the most appropriate predictive model for the response variables. Among the tested models, the linear model was identified as the best fit for both Particle size (X1) and entrapment efficiency (X2), based on statistical significance and model adequacy parameters. Two-dimensional contour plots for both responses are depicted in Figure 15, while the corresponding three-dimensional response surface plots are illustrated in Figure 16. These 3D surface plots clearly demonstrate the interactions between formulation variables and allow for better visualization of the influence exerted by two independent factors on each response parameter.

Table 15: Final Composition of optimized Microsphere formulation as per Design of experiment approach

S. No	Run	Formulation	Polymer Eudragit RS100 (mg)	Surfactant Tween 80 (mg)	Particle size (nm)	Entrapment efficiency (%)	Stirring time (Hrs)
1	12	MS	300.000	0.100	449.308	89.517	2.000

Characterization of optimized Microsphere formulation

Physical Appearance

Table 16: Physical Appearance of Beta-Sitosterol loaded Microspheres

Parameter	Observation
Color	White or off-white
Odor	Characteristic, slightly fatty or oily
Appearance	Small, spherical particles
State	Solid, spherical particles

Particle size

Table 17: Particle size of microsphere formulation

S. No	Formulation	Particle size (Predicted value)	Particle size (Actual value)
1.	Microsphere	449.308 nm	375.8 nm

As presented in Table 17, the particle size of the Beta-Sitosterol-loaded microsphere formulation was evaluated in both predicted and actual terms. The predicted particle size was 449.308 nm, while the actual measured size was 375.8 nm. This indicates that the actual formulation resulted in smaller particles than expected, which may be attributed to factors such as more efficient emulsification, proper stirring conditions, or optimized surfactant and polymer concentrations. The reduction in particle size is favorable, as smaller microspheres can enhance drug release, skin penetration, and formulation stability, making the formulation more suitable for topical or transdermal applications.

Zeta potential

Table 18: Zeta potential of microsphere formulation

S. No	Formulation	Zeta potential
1.	Microsphere	-20.0 mV

As shown in Table 29, the zeta potential of the Beta-Sitosterol-loaded microsphere formulation was recorded at -20.0 mV. This negative surface charge indicates moderate electrostatic stability, as particles with zeta potential values greater than ±30 mV are generally considered highly stable. While -20.0 mV suggests that the microspheres may have a tendency toward limited aggregation, it still reflects an acceptable level of colloidal stability for short- to medium-term storage. The negative charge may also influence the interaction with skin surfaces, potentially aiding in adhesion and improving topical delivery performance.

Entrapment efficacy

Table 19: Entrapment efficacy of microsphere formulation

S. No.	Formulations	Entrapment efficacy (Predicted value)	Entrapment efficacy (Actual value)
1.	Microsphere	89.517 %	91.43 %

As shown in Table 30, the entrapment efficiency of the Beta-Sitosterol-loaded microsphere formulation was found to be 91.43% (actual), slightly higher than the predicted value of 89.517%. This positive deviation indicates that the formulation process was effective in maximizing drug encapsulation within the microspheres. The high entrapment efficiency suggests efficient interaction between Beta-Sitosterol and the polymer matrix, likely due to optimized concentrations of polymer and surfactant, as well as appropriate stirring conditions. Such a high level of drug loading enhances the therapeutic potential of the microspheres and supports their use in controlled or sustained drug delivery systems.

In Vitro drug release study

Table 20: In-vitro drug release studies

Time (Hr)	cumulative % drug released	% drug remaining	Square root time	log Cumu % drug remaining	log time	log Cumu % drug released
0	0	100	0.000	2.000	0.000	0.000
2	22.14	77.86	1.414	1.891	0.301	1.345
4	31.26	68.74	2.000	1.837	0.602	1.495
6	43.30	56.7	2.449	1.754	0.778	1.636
9	54.29	45.71	3.000	1.660	0.954	1.735
12	64.37	35.63	3.464	1.552	1.079	1.809
14	77.34	22.66	3.742	1.355	1.146	1.888
16	86.41	13.59	4.000	1.133	1.204	1.937
18	95.46	4.54	4.243	0.657	1.255	1.980

Table 21: Correlation value (R2 value)

Formulation	Model	Kinetic parameter values
Microsphere Formulation	Zero Order	$R^2 = 0.990$
	First Order	$R^2 = 0.884$
	Higuchi	$R^2 = 0.845$
	Korsmeyerpeppas	$R^2 = 0.810$

The in vitro drug release study demonstrated a sustained and progressive release of the drug over 18 hours. Initially, a cumulative release of 22.14% was observed at 2 hours, which gradually increased to 95.46% by the end of 18 hours, indicating efficient and prolonged drug release behavior. The release kinetics were analyzed using various mathematical models to determine the mechanism of drug release. The correlation coefficient (R^2) values obtained for different models revealed that the release profile best fit the Zero Order model ($R^2 = 0.990$), suggesting a constant release rate independent of drug concentration. In comparison, the First Order ($R^2 = 0.884$), Higuchi ($R^2 = 0.845$), and Korsmeyer-Peppas ($R^2 = 0.810$) models showed lower correlation values, indicating less suitability. The strong linearity in the zero- order plot further confirms that the release mechanism follows a controlled release pattern, which is desirable for maintaining consistent therapeutic levels over time.

Antimicrobial Activity (Well Diffusion Assay)

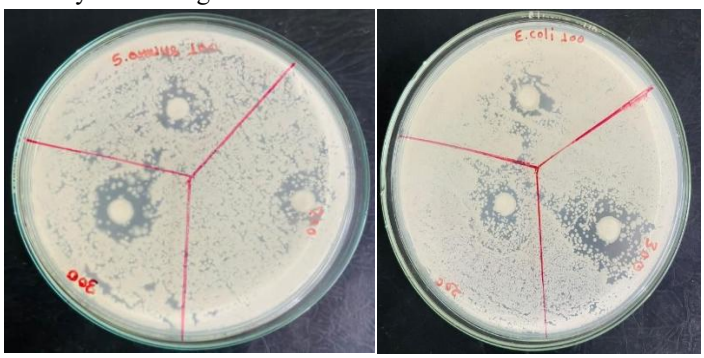

Figure 2: In-vitro antimicrobial activity of S. aureus and E. coli

Table 22: Antimicrobial activity of Beta-Sitosterol loaded microsphere formulation

S. No	Sample name	Zone of Inhibition (mm) *S. aureus*	Zone of Inhibition (mm) *E. coli*
1	F1 (Control)	0.0	0.0
2	F2 (1mg/ml)	3.1	4.9
3	F3 (1.5 mg/ml)	6.8	9.5

As shown in Table 22, the antimicrobial activity of the Beta-Sitosterol-loaded microsphere formulation was evaluated against Staphylococcus aureus and Escherichia coli. The control sample (F1) showed no zone of inhibition, confirming the absence of antimicrobial effect without the active ingredient. In contrast, F2 (1 mg/mL) exhibited modest inhibition zones of 3.1 mm for S. aureus and 4.9 mm for E. coli, indicating initial antibacterial activity. Notably, F3 (1.5 mg/mL) demonstrated a significant increase in activity, with inhibition zones of 6.8 mm and 9.5 mm against S. aureus and E. coli, respectively. These results suggest a concentration-dependent antimicrobial effect, highlighting the potential of Beta-Sitosterol- loaded microspheres as an effective agent for managing microbial infections, particularly in topical therapeutic applications.

Stability study

Table 23: Stability Study of optimized microsphere formulation

S. No	Time (Days)	$25^0C \pm 2\ ^0C$ and 60 ± 5% RH		$40^0C \pm 2\ ^0C$ and 70 ±5% RH	
		Particle size (nm)	Entrapment efficiency (%)	Particle size (nm)	Entrapment efficiency (%)
1.	0	375.8 nm	91.43 %	375.8 nm	91.43 %

2.	30	379.5 nm	91.45 %	376.4 nm	91.39 %
3.	45	371.3nm	91.40 %	377.9 nm	91.44 %
3.	60	379.4 nm	91.47 %	371.8 nm	91.47 %
4.	90	373.7 nm	91.39 %	378.2 nm	91.49 %

Table 23 presents the results of a stability study conducted on the optimized Beta-Sitosterol- loaded microsphere formulation under two storage conditions: 25 ± 2°C / 60 ± 5% RH and 40 ± 2°C / 70 ± 5% RH over a 90-day period. The particle size and entrapment efficiency remained relatively stable throughout the study, with only minor fluctuations observed. At room temperature, the particle size varied slightly from 375.8 nm to 373.7 nm, and entrapment efficiency remained within a narrow range of 91.39% to 91.47%. Similarly, under accelerated conditions, the particle size ranged from 375.8 nm to 378.2 nm, while entrapment efficiency fluctuated minimally between 91.39% and 91.49%.

These results indicate that the formulation maintains its physical stability and drug loading capacity under both normal and stressed storage conditions. The minimal changes in key parameters suggest that the microsphere formulation is stable for at least 90 days, supporting its suitability for long-term storage and commercial development.

CONCLUSION

The results of this study successfully demonstrate the feasibility of formulating Beta- Sitosterol into stable and efficient microspheres suitable for topical or transdermal delivery. Pre-formulation studies confirmed the physicochemical stability and purity of Beta-Sitosterol, which was essential for ensuring consistent drug performance throughout formulation development. The Box–Behnken design approach effectively optimized the microsphere formulation by identifying the significant roles of polymer concentration, surfactant percentage, and stirring time in determining critical quality attributes such as particle size and drug entrapment efficiency. The optimized microspheres displayed favorable characteristics, including a small particle size (375.8 nm), high entrapment efficiency (91.43%), and moderate zeta potential (-20.0 mV), which contribute to improved drug bioavailability, stability, and targeted delivery potential. The antimicrobial assessment confirmed that the formulation retains biological efficacy, with enhanced antibacterial effects observed at higher concentrations. Furthermore, the stability studies affirmed the robustness of the optimized formulation over 90 days under both normal and accelerated storage conditions, highlighting its potential for commercial development. Collectively, these findings underscore the suitability of the Beta-Sitosterol-loaded microsphere system as a promising delivery platform for enhancing the therapeutic performance of lipophilic drugs, particularly in the context of sustained and localized treatment strategies.

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