

Development and Optimization of Nepafenac-Loaded Niosomal Gel for Topical Anti-Inflammatory Delivery: A Quality-by-Design Approach

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Abstract

Nepafenac, a class II non-steroidal anti-inflammatory drug (NSAID) under the biopharmaceutics classification system (BCS), has poor aqueous solubility and dose-limiting gastrointestinal toxicity with oral administration, hence limiting its effective clinical use in musculoskeletal conditions. So, this study was planned to develop, optimize, and characterize a nepafenac-loaded, niosome-based gel for sustained topical drug delivery to bypass its first-pass metabolism and systemic adverse effects. Niosomes were made with the non-ionic surfactant (Span 60) and cholesterol as bilayer-forming agents. The *in vitro* Span 60 and cholesterol level-dependent formulation of niosomal nepafenac was optimized using a two-factor, three-level (3) factorial design by employing the following independent variables, Span 60 (A) and cholesterol (B), and vesicle size (VS) and entrapment efficiency (EE) as the dependent variables. Preformulation studies of the drug included organoleptic evaluation, melting point, solubility analysis, UV spectrophotometry, and FTIR compatibility studies to establish identity, purity, and excipient compatibility. Nine niosome batches (F1–F9) were characterized for niosome particle morphology, size, polydispersity index (PDI), ZP, and EE. Both responses fitted quadratic models with high adjusted R² values of 0.8804 and 0.8516, respectively. The optimized niosome batch F5 showed a VS of 356.2 ± 10.2 nm, PDI of 0.298 ± 0.016, ZP of 31.6 ± 1.7 mV, and EE of 88.4 ± 1.9%. The Formulation F5 was loaded into Carbopol 934 gel base (at four levels of 1.0, 1.25, 1.5, and 1.75%w/v) and evaluated with the gel base G3 (1.5%w/v of polymer) exhibiting suitable viscosity (8450 ± 134 cPs), spreadability (10.8 ± 0.5 g cm/s), and drug content (97.6 ± 0.8%) using *in vitro* studies, G3 releases the nepafenac in a controlled manner to the tune of 76.8 ± 2.1% than the plain drug gel (96.8 ± 3.4%) after 12 h following Korsmeyer-Peppas model with non Fickian anomalous (n = 0.726) transport. DSC confirmed the amorphous state of the drug and intermolecular interactions within the niosomal bilayer, consistent with sustained drug release. The stability study of the optimized gel formulation for 6 months under accelerated storage indicates its stability. These niosomes are a novel vesicular carrier for sustained topical delivery of nepafenac and might be a potential drug delivery platform.

Keywords: Nepafenac; Niosomes; Topical gel; Factorial design; Entrapment efficiency; Sustained release

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1. Introduction

The use of non-steroidal anti-inflammatory drugs (NSAIDs) remains the mainstay of pharmacotherapy

for the control of inflammatory and pain processes occurring in osteoarthritis, rheumatoid arthritis, and

other musculoskeletal conditions. Nepafenac is an NSAID that has a specific ophthalmic indication for management of postoperative inflammation and pain in the eye following cataract surgery[1]. Nepafenac is a prodrug with good penetration into the cornea, which is biotransformed within the eye by several tissues, including the iris, ciliary body, and retina/choroid, to the active metabolite amfenac[2]. Nepafenac formulations for ophthalmic administration are mainly distinguished by their rapid penetration of the cornea and by their sustained anti-inflammatory activity. The general disadvantages of eye drops include a poor precorneal residence time, rapid removal from the eye via the lacrimal system, the need for repetitive applications, and diminished penetration of the drug through the cornea, all of which can result in a reduced bioavailability[3]. To overcome these problems, several ophthalmic drug delivery approaches such as in situ gels, nanoparticles, nanogels, nanoemulsions, liposomes, and mucoadhesive systems have been suggested in the existing literature to improve the residence time of drugs on the cornea, the absorption of drugs, and to deliver them with prolonged or controlled release properties. Furthermore, as a BCS Class II drug, nepafenac exhibits low aqueous solubility and high permeability, which results in a dissolution-rate-limited and sometimes erratic oral bioavailability[4].

These issues have prompted continued work into alternative delivery options for nepafenac that do not involve oral intake. The transdermal/topical route is an especially appealing one for musculoskeletal and joint conditions, given the localised nature of inflammation in many disorders[5]. Topical delivery also boasts several theoretical advantages over oral delivery: no utilization of the hepatic first-pass, implantation of drug directly in the diseased tissue, lowered systemic drug levels necessary to achieve an effect, increased patient compliance, no disruption to the gastrointestinal tract, and the ability to quickly and easily cease delivery if an adverse reaction is observed. Unfortunately, typical topical formulations with simple gel, cream, and ointment bases are limited by their inability to penetrate the skin barrier, maintain high drug levels locally for long periods, and require the repetitive application of their drug content to maintain benefits. These problems are compounded when the drug in question, such as nepafenac, is not readily water-soluble.

Vesicular drug delivery systems are now recognized as a rational approach to address these problems. The sponge-like non-ionic surfactant vesicles (niosomes-bilayer vesicles consisting of mixed sorbitan esters (e.g., Span series) and cholesterol) have gained considerable attention as an alternative to liposomes. This is because niosomes can entrap similar amounts

of both hydrophilic and lipophilic drugs in the aqueous core and lipid bilayer, respectively, are chemically more stable, cost less, and are easier to store than liposomes based on phospholipids (due to increased oxidation/hydrolysis stability of non-ionic surfactants vs. phospholipids)[6]. Topically, niosomes are thought to form a reservoir of drug in the uppermost skin layers, regulate drug discharge, increase the residence time at the application site, and, in most systems described, increase drug fluxes across the skin compared to simple topical vehicles[7]. Incorporation of niosome dispersions into a semisolid gel base further increases the adhesion, application, and spread of the formulation to the skin, as well as controlling the discharge of the entrapped drug to the skin over time[8].

However, the primary characteristics of niosomal systems- VS, size distribution, surface charge, and most importantly entrapment efficiency (EE) are complex functions of formulation parameters, which include the type and levels of the non-ionic surfactant and the drug-to-cholesterol ratio within the bilayer[9]. Cholesterol effects on bilayer integrity are well documented: it can alter bilayer rigidity, fluidity, and permeability, while surfactant chain length and HLB will alter bilayer self-assembly processes and stability. In the interest of effectively exploring the highly interactive and often non-linear relationships that exist among these formulation constituents, to optimise a quality formulation, Quality by Design (QbD) experimental strategies and multi-factorial experimental designs such as factorial designs and response-surface methodology can be applied. In comparison to traditional experimental strategies, these methodologies (Design of Experiments or DoE) are highly efficient, as they explore the effects of both individual formulation factors and their interactions, and operate within a statistically established optimization space to find a formulation comprising defined desirability parameters. With this information, the objective for the study described herein was to (i) characterise nepafenac using a series of preformulation studies such as organoleptic evaluation, melting point, solubility, development of the UV spectrophotometric method and drug-excipient compatibility studies based on Fourier transform infrared (FTIR) analysis; (ii) produce nepafenac-loaded niosomes using Span 60 and cholesterol using a statistically mechanistic framework known as the two-factor, three-level factorial design in which the effects of cholesterol and surfactant (Span 60) concentration on VS and EE are investigated; (iii) characterise the produced niosome formulation for its vesicle morphology, PS, ZP, EE and *in vitro* drug discharge; (iv) incorporate an optimized formulation into a gel base comprised of varying levels of Carbopol

934 and select a gel formulation with optimal rheological characteristics and drug discharge profile; (v) compare the *in vitro* drug discharge and discharge mechanism of the niosomally encapsulated drug versus the plain drug in gel formulation; and (vi) apply differential scanning calorimetric analysis in order to assess the thermal behaviour of an optimized formulation and to evaluate its physicochemical stability over a period of six months under accelerated storage conditions. All of the above points collectively define the entire scheme behind this research work that is to devise a statistically optimized, mechanistically supported niosomal gel formulation in order to sustain the topical delivery of nepafenac.

2. Materials and Methods

2.1 Materials

Nepafenac was obtained as a gift sample from Mankind Pharma, Bengaluru, and used as such. Span 60, cholesterol, carbopol 934, triethanolamine, chloroform, methanol, and phosphate-buffered saline (PBS) (pH 7.4) (AR grade) were purchased from SD Fine Chemicals. Double-distilled water was used in the study.

2.2 Preformulation Studies

2.2.1 Organoleptic characterization

Visual inspection of the nepafenac sample, including colour, physical form, and odour, and tasting using a taste assessment scale against the specifications detailed in the relevant pharmacopoeial monograph[10].

2.2.2 Melting point of nepafenac

Determination of the melting point of nepafenac was carried out 3 times using the open capillary method. The obtained melting point was compared to the official range to ensure drug purity and identity[11].

2.2.3 Solubility study

The solubility of nepafenac was determined by the shake-flask method in various aqueous and organic solvents such as purified water, 0.1 N HCl, pH 7.4 PBS, methanol, ethanol, acetone, and chloroform. Excess nepafenac was added to a specified amount of each solvent and was shaken to attain the equilibrium level of drug in the solvent; the mixture was then filtered, and the drug level was determined spectrophotometrically. The solvent–drug solution was then classified as to the apparent drug solubility according to the pharmacopoeial solubility criteria[12, 13].

2.2.4 Development of UV spectrophotometric technique

A stock solution was made using PBS (buffer pH 7.4) and was scanned within the UV region to find the λ_{max} . A calibration curve was made in the concentration span of 2-10 μ g/ml and linear regression analysis was applied to acquire the regression equation

and R^2 , respectively, for all the other quantitative determinations.

2.2.5 Differential Scanning Calorimetry (DSC)

Thermal behaviour of pure nepafenac, Span 60, cholesterol, and the optimized nepafenac-loaded niosomal formulation was studied by differential scanning calorimetry. Samples were heated in sealed pans over an appropriate temperature range at a controlled heating rate under an inert atmosphere, and the resulting thermograms were analysed for characteristic endothermic transitions, shifts in peak temperature, and evidence of drug–excipient interaction or changes in the crystalline state of the drug upon encapsulation.

2.2.6 FTIR spectroscopic compatibility study

The FTIR spectra of the pure drug, the separate excipients used (Span 60, cholesterol, Carbopol 934), and the physical mixtures of nepafenac with the separate excipients were obtained in the region of wavenumbers used for the interpretation of the peaks for identification of functional groups in organic molecules as a KBr pellet spectrum. These spectra were examined for characteristic nepafenac absorption bands and for any shift, broadening, or disappearance of bands in the physical mixture of ACE with the separate excipients.

2.3 Preparation of Nepafenac-Loaded Niosomes

The niosomes were made by the thin-film hydration method. The amount of all solid components, such as Span 60 and cholesterol, required for the preparation of all the different formulations was weighed according to the ratio of Span to cholesterol as recommended in the factorial design (Section 2.4). These were dissolved in a round-bottomed flask in the ratio of chloroform to methanol as required for all the different formulations, and the solvent was removed under vacuum by rotary evaporator at 60°C until a thin film was formed on the wall of the round-bottomed flask[14-16]. The film was hydrated with PBS (pH 7.4) containing a definite amount of nepafenac at 60°C with continuous rotation, thus obtaining a coarse multilamellar niosomal dispersion, which was subjected to probe sonication to reduce VS and size distribution and stored at 2 to 8°C.

2.4. Experimental Design and Optimization

A 3^2 factorial design was used to investigate the effects of the formulation variables on niosome properties and to optimize the formulation. Cholesterol (Factor A) (50-100 mg) and Span 60 (Factor B) (100-200) were considered as independent variables, and therefore, two quantitative factors were varied at 3 coded levels (1, 0, and +1). VS (Response 1) and EE (Response 2) were considered the dependent responses, which are considered the critical quality attributes for this formulation. In accordance with the design, nine experimental runs (formulations F1-F9) were created,

with formulation F1 as the reference point, and made as described in section 2.3 [17, 18].

2.5 Characterization of Niosomal Formulations

2.5.1 Morphological characterization

The morphology of all niosomal dispersions was visualized by optical microscopy at 40 magnification. The optimized formulations were further visualized by scanning electron microscopy (SEM) to analyze the vesicle shape and the surface morphology, aggregation, or no aggregation of optimized niosome formulations[19, 20].

2.5.2 Vesicle size, PDI and zeta potential

The mean VS, PDI, and zeta potential (ZP) of each formulation (F1-F9) were measured in triplicate by dynamic and electrophoretic light scattering, respectively, with a VS and ZP analyzer after adequate dilutions of niosomal dispersions with double-distilled water[21, 22].

2.5.3 Entrapment efficiency

The free drug (untrapped) was removed from the niosomes by dialysis/centrifugation. Its amount was determined by measuring the absorbance at λ_{max} . The total drug that was entrapped was expressed as a percentage of the total drug used[23, 24].

2.6 *In vitro* Drug Release from Niosomes

The *in vitro* discharge of nepafenac from the selected niosomal dispersions (F1, F3, F5, F7 and F9), representative of the design space, was performed using the dialysis bag diffusion technique. Niosomal dispersions and a plain nepafenac solution (used as a control) were loaded into a dialysis bag and carried in the diffusion medium (PBS pH 7.4) under continuous stirring at physiological temperature (32°C). The discharge medium samples were withdrawn at specific time points over 12 h and replaced with an equal volume of fresh medium, and estimated spectrophotometrically to find the cumulative drug release (CDR)[25, 26].

2.7 Selection of the Optimized Niosomal Formulation

F5 was selected as the best niosomal batch for loading in the gel base based on the overall merit, considering the VS, PDI, ZP, EE, and *in vitro* discharge characteristics, including the amount of VS, shape, and size distribution based on the desirability criteria derived from the factorial design (highly loaded vesicles with EE and narrow size distribution).

2.8 Preparation of Niosomal Gel

The following gels were made using various concentrations of Carbopol 934 (G1-G4) as gel bases. For this purpose, the quantity of Carbopol 934 required was dispersed in water with continuous stirring until gel formation occurred. The gelled mixture was allowed to hydrate completely, following which the dispersed gel was neutralized using triethanolamine to obtain the gel consistency. The most advantageous niosomally dispersed drug mixture (F5), in the form of a fixed dose (nepafenac), was then dispersed uniformly into each of the gel systems with gentle stirring to prevent the breakdown of the vesicles formed[27-29].

2.9 Evaluation of Niosomal Gel Formulations

Following the preparation of the G1-G4 gels, their physical appearance and homogeneity were checked visually, their pH determined using a calibrated digital pH meter, their drug content extracted and assayed spectrophotometrically, their viscosity determined at 10 rpm using a Brookfield-type viscometer, and their spreadability assessed by the parallel-plate method, the diameter of spread of a measured amount of gel under a given load over a specified time being recorded[30-32].

2.10 *In vitro* Release of the Optimized Niosomal Gel

The optimized niosomal gel (G3; as selected in section 3) and plain drug gel were tested for drug release *in vitro* employing the Franz diffusion cell having a dialysis membrane as a barrier and PBS pH 7.4 as the receiver medium kept at body temperature with constant stirring, and the samples from the receiver chamber were withdrawn at particular time intervals for 12 h and replaced with an equivalent amount of the fresh medium and analyzed using a spectrophotometer for calculating the CDR[33-35].

2.11 Drug Release Kinetics

The discharge data of niosomes and niosomal/plain gels were subjected to model fitting with various models- zero-order, first-order, Higuchi, Hixson-Crowell, and Korsmeyer-Peppas equations to determine the type and mechanism of drug discharge. The model showing the maximum coefficient of determination (R^2) was considered for each formulation. The release exponent (n) of the Korsmeyer-Peppas model was used to interpret Fickian and non-Fickian release transport.

2.12 Accelerated Stability Studies

The best niosomes loaded with gel G3 were packed in a well-closed container and subjected to accelerated stability testing as per ICH-based storage conditions

for a period of 6 months. The samples were collected at an interval of 1 month and examined for parameters like physical appearance, pH, viscosity, drug content, and CDR after 12 h [36, 37].

2.13 Statistical Analysis

Experiments were all repeated in triplicate, and the values are given as mean standard deviation (SD) unless mentioned otherwise. The analysis of variance associated with the factorial design was conducted using Design-Expert software and at a significance level of $p \leq 0.05$ [38].

3. Results

3.1 Preformulation Studies

Nepafenac was obtained as a white to off-white crystalline powder that was practically odourless, with a slightly bitter taste, which conforms to the official description, and attested to the identity of the drug substance. The melting point of nepafenac was observed to be 149-152 °C which was found to agree with the official melting point of 149-153 °C.

The solubility data indicated the following solubility profile of nepafenac in purified water (~23 µg/mL), 0.1 N HCl, acetone, and chloroform (free solubility), and good solubility in methanol and ethanol (Table 1). Its hydrophobic nature and poor aqueous solubility resulted in its BCS class II classification, which supported the basis for vesicular delivery to improve its actual dissolution and permeation characteristics.

Table 1. Solubility profile of nepafenac in various solvents

Solvent	Solubility
Purified water	Very slightly soluble (~23 µg/mL)
0.1 N HCl	Practically insoluble
PBS of pH 7.4	Slightly soluble (~45 µg/mL)
Methanol	Soluble
Ethanol	Soluble
Acetone	Freely soluble
Chloroform	Freely soluble

The spectrophotometric scan of nepafenac in PBS pH 7.4 revealed an absorbance maximum at 236 nm in comparison to the literature value. The calibration curve (obtained from 2 to 10 µg/mL) was linear ($R = 0.9998$, with an equation of $y = 0.0457 + 0.0080$), ensuring adherence to the Beer-Lambert law and indicating that the method was appropriate for use in quantifying nepafenac.

DSC analysis of pure nepafenac revealed a sharp endothermic peak at 180.53 °C corresponding to the melting of crystalline drug. Importantly, the

characteristic melting endotherm of crystalline nepafenac was shifted left (reduced melting point) seen in the niosomal formulation, thereby suggesting that the drug had transitioned from a crystalline to an amorphous/molecularly dispersed state in the vesicular bilayer (Figure 1). These observations highlight a strong interaction between the drug and the niosomal bilayer, which is in keeping with the high EE and sustained-release behavior exhibited by the optimized formulation.

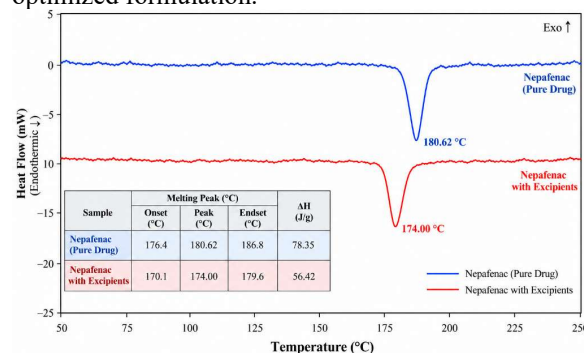


Fig.1. DSC thermogram of Nepafenac alone and its combination with the excipients

FTIR spectrum of pure nepafenac exhibited the major peaks at 3325 cm⁻¹ (N-H stretch-secondary amine), 2943 cm⁻¹ (aromatic C-H stretch), 1773 cm⁻¹ (ethyl, ester C=O stretch), 1728 cm⁻¹ (acid C=O), 1603 cm⁻¹ (aromatic C=C stretch), 1264 cm⁻¹ (ether C-O-C stretch) and 753 cm⁻¹ (C-Cl stretch) (Figure 2). When the FTIR spectrum of nepafenac was obtained with that of Span 60 and cholesterol mixture in physical blending form, all the characteristic absorption peaks of nepafenac were retained without any shift in intensity and broadening or disappearance of any peaks, which suggested no major physicochemical interaction was occurring and there was no incompatibility between the drug and the excipients used.

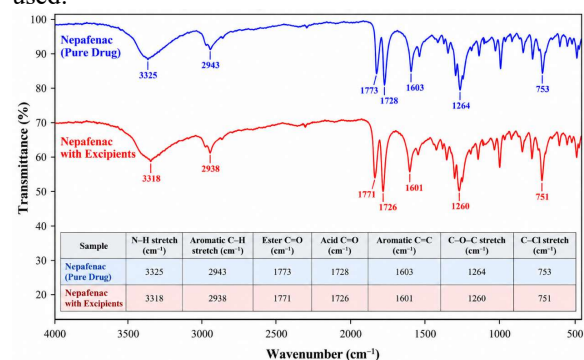


Fig.2. FTIR spectra of Nepafenac alone and its combination with the excipients

3.2 Evaluation of Niosomal Formulations

The optical microscopy revealed clearly separated spherical vesicles with a smooth surface, with

multilamellar vesicles more clearly seen in the formulations with high cholesterol content (F2, F6, and F8). SEM studies for the optimized formulation (F5) showed spherical vesicles that had clear boundaries and were uniform in shape, appeared intact, and were not fused together or associated, suggesting physical stability of the formulation as a colloidal dispersion.

For the nine niosomes were made (F1-F9), all vesicles had a size within the typical VS range (223.4±6.8 nm (F8) to 356.2±10.2 nm (F5)), showed PDI values indicating homogenous VS (0.298±0.016 (F5) to 0.459±0.042 (F8)), had a ZP indicating highly stable batches (21.9±2.6 mV (F8) to 31.6±1.7 mV (F5)), and high EE (69.8±1.4% (F8) to 88.4±1.9% (F5)) (Table 2). The F5 batch achieved the highest average EE, the most negative (most stable) ZP value, and the smallest average VS in this formulation space; F8 achieved the smallest average VS in this formulation space, likely correlating with the observations made here of an inverse relationship between average EE and average VS.

Table 2. Vesicle size, PDI, zeta potential, and entrapment efficiency of niosomal formulations (F1–F9)

Batch	Vesicle size (nm)	PDI	Zeta potential (mV)	EE (%)
F1	237.5 ± 6.8	0.381 ± 0.021	-22.4 ± 1.8	70.0 ± 1.5
F2	258.2 ± 7.4	0.412 ± 0.033	-24.1 ± 2.2	72.6 ± 1.8
F3	291.5 ± 8.9	0.345 ± 0.018	-27.3 ± 1.9	79.0 ± 2.1
F4	284.6 ± 8.2	0.376 ± 0.024	-25.7 ± 2.0	74.2 ± 2.0
F5	356.2 ± 10.2	0.298 ± 0.016	-31.6 ± 1.7	88.4 ± 1.9

F6	246.8 ± 6.5	0.436 ± 0.038	-23.8 ± 2.4	71.5 ± 1.6
F7	312.1 ± 9.1	0.322 ± 0.019	-28.4 ± 1.6	80.2 ± 2.2
F8	223.4 ± 6.8	0.459 ± 0.042	-21.9 ± 2.6	69.8 ± 1.4
F9	334.8 ± 10.2	0.318 ± 0.022	-29.1 ± 1.8	82.6 ± 2.0
Values in mean±SD; n=3				

3.3 Factorial Design Analysis

3.3.1 Response 1 (Vesicle size)

The quadratic model was determined to be the most appropriate sequential model for VS, with a good sequential p-value (0.0283) and a much improved adjusted R² (0.8804) as compared to the linear model (adjusted R² = 0.0334) and 2FI model (adjusted R² = 0.2273); a cubic model, although showing a higher adjusted R² (0.9920), was aliased. The ANOVA table determined that the quadratic model overall was significant (F = 12.78, p = 0.0310). Although not significant at the 5% level; the interaction term between AB (F = 19.54, p = 0.0215), the A quadratic term (F = 18.15, p = 0.0237) and the B quadratic term (F = 7.789, p = 0.0693) was significant; individual linear terms A (p = 0.0612), B (p = 0.0842) and the B term (p = 0.9012) was not. The fitted coded equation was:

$$VS = +246.80 + 16.25A + 14.15B - 34.73AB + 39.24A^2 + 1.24B^2$$

The positive linear A and B coefficients suggested VS may increase with higher individual levels of cholesterol and Span 60 by conventionally increasing bilayer thickness and rigidity. The negative AB interaction coefficient implied VS decreased when both cholesterol and Span 60 were increased concurrently relative to their additive effects, while the sizeable positive A coefficient implied significantly increased curvature at the higher levels of cholesterol. Diagnostic plots [normal probability of residuals, residuals versus run, predicted versus actual, and Cook's distance] showed the residuals were normally distributed, randomly spread without obvious trend, well correlated with the fitted model, and lacking any influential runs (all Cook's distances less than the critical value of 1.0). Contour and 3D response-surface

plots revealed a curved valley of decreasing VS with increasing both factors, with the most diminutive VS predicted at the intermediate concentrations and larger VS closer to the outer margins of the design space.

The study used response surface methodology to study the interaction effect of CHL (A) and Span 60 (B) on VS. Figure 3A: The contour plot shows the significant change in VS over the experimental region. The response measured was between about 220 and 300 nm. The regions with minimum VS of about 220-240 nm were observed at low to medium CHL (50-70 mg) and Span 60 (100-140 mg). With increasing CHL toward the higher region from 70 to 90-100 mg, the VS began increasing from 230-240 up to 280-300 nm. There was a general trend of increase in VS with increasing Span 60 >140 mg up to 160 mg, where the VS was between 250-260 nm, and further increase of Span 60 over 160 mg resulted in increased VS (280-300 nm). The 3D response surface graph in Figure 3B showed a curved surface with the minimum response surface for both factors at medium levels of both independent variables, and the shape of the surface indicated significant curvature with the influence of both linear and quadratic terms of the independent variables. Experimental points over the response surface indicated variation of VS at various combinations of the formulation variables.

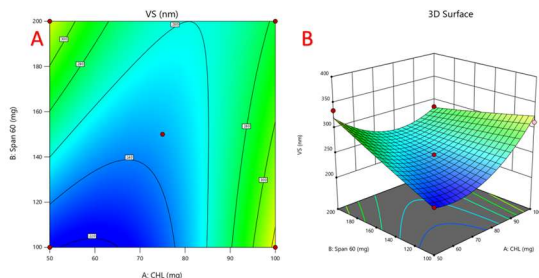


Fig.3. Impact of independent variables on the response (vesicle size): A) Contour plot B) 3D plots

3.3.2 Response 2 (EE)

For EE, the recommended model was again quadratic (sequential $p = 0.0280$, adjusted $R^2 = 0.8516$), better than linear (adjusted $R^2 = 0.1605$) and 2FI (adjusted $R^2 = 0.0340$), and the cubic was aliased; again, ANOVA showed the model to be significant ($p = 0.0424$, $F = 10.18$). The AB interaction ($p = 0.0322$, $F = 14.37$) and A term ($p = 0.0270$, $F = 16.47$) were significant, while other linear terms A ($p = 0.1173$) and B ($p = 0.2320$) and B term ($p = 0.8497$) were not. The coded equation obtained was:

$$EE = +71.50 + 1.91A + 1.31B - 4.70AB + 5.90A^2 - 0.3000B^2$$

The positive linear coefficients suggested that increasing either cholesterol or Span 60 individually would generally tend to increase EE, with cholesterol having the larger effect. The significant negative value of the AB interaction indicated an antagonistic

interaction when the two factors were combined, while the positive-A coefficient indicated a relatively strong non-linear effect (curvilinear relationship) of cholesterol on EE. Diagnostic plots again showed the residuals to be normally distributed and random, the regression to be well correlated with the EE values, and all runs to be appropriately influential. Contour and 3D response-surface plots indicated that EE was at a minimum at intermediate levels of cholesterol and low-to-moderate levels of Span 60, and increased toward the upper and outer ends of the design space, consistent with the significant curvature shown by the ANOVA (Figure4).

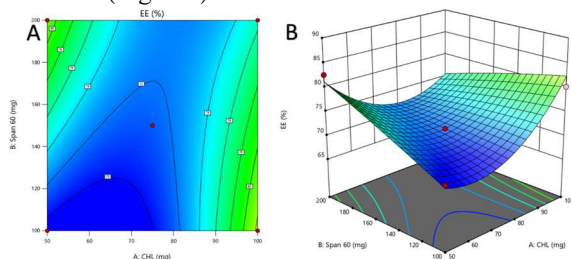


Fig.4. Impact of independent variables on the response (entrapment efficiency): A) Contour plot B) 3D plots

3.4 In vitro Drug Release from Niosomes

Among the various niosomal batch formulations, the results showed that the F5 formulation achieved sustained discharge (79.8%) of entrapped drug after 12 h as compared with a plain nepafenac solution, which achieved 97.1% discharge after 12 h (Table 3). The sustained drug discharge property of F5 could be due to the slightly greater EE and the more rigid bilayer of the formulation, resulting from the Span 60: cholesterol ratio. The drug discharge profile was similar to the ordinary diffusion of the plain drug, which was released rapidly to achieve 97.1% after 12 h.

Table 3. In vitro cumulative percentage drug discharge from representative niosomal formulations and plain drug solution

Time (h)	F1	F3	F5	F7	F9	Plain solution
0.5	8.2	9.4	6.8	9.0	9.8	21.4
1	14.6	16.8	12.4	15.9	17.6	36.2
2	24.8	27.6	21.3	26.4	29.1	54.8
3	33.2	36.4	28.9	34.8	38.6	66.3

4	41.6	45.2	36.2	43.1	47.8	74.5
6	54.3	58.6	47.8	56.4	61.3	84.2
8	65.8	70.4	59.3	68.2	73.6	90.6
10	74.9	80.1	70.1	77.9	83.4	94.3
12	82.6	87.3	79.8	85.4	88.9	97.1

3.5 Evaluation of Niosomal Gel Formulations

For gel development, F5 was used as the optimized niosotransfersomal batch because it was found to have the highest EE value, the lowest PDI, and the most negative ZP of the nine formulations screened. The four gels G1-G4 were all clear, homogeneous, and free from any visible particulate matter or phase separation, demonstrating the ability of the niosomes to be entrapped while maintaining vesicle integrity, as the presence of visible phase separation was observed in the subsequent gels G5-G7. The pH of the gel formulations was found to be within the acceptable range of 6.4-6.6, and drug content values were all above 93% (Table 4).

Increasing the concentration of Carbopol 934 resulted in an increase in viscosity of the gel, from 1840±82 cPs for G1 to 14,280±218 cPs for G4 (10 rpm), and a corresponding decrease in spreadability from 18.2±0.9 g cm/s for G1 to 6.4±0.4 g cm/s for G4. Although G1 showed high spreadability, its relatively low viscosity may not effectively retain the product on the skin, and G4, being highly viscous, may be difficult to apply due to its low spreadability. It was evident from the results obtained that G3 (1.5%w/v) carbopol 934 gel was the most promising formulation, since it had satisfactory parameters of viscosity (8450±134 cPs), spreadability (10.8±0.5 gcm/s), and drug content (97.6±0.8%).

Table 4. Physical evaluation of niosomal gel formulations (G1–G4)

Parameter	G1	G2	G3	G4
Appearance	Clear, slightly viscous	Clear, viscous gel	Clear, viscous gel	Clear, highly viscous gel

Colour	Pale white	Pale white	Pale white	Pale white
Homogeneity	Homogeneous	Homogeneous	Homogeneous	Homogeneous
Grittiness	Absent	Absent	Absent	Absent
pH	6.6 ± 0.08	6.5 ± 0.06	6.5 ± 0.07	6.4 ± 0.09
Drug content (%)	93.4 ± 1.2	96.8 ± 0.9	97.6 ± 0.8	97.2 ± 1.1
Viscosity (cPs @ 10 rpm)	1840 ± 82	4620 ± 96	8450 ± 134	14,280 ± 218
Spreadability (g·cm/s)	18.2 ± 0.9	14.6 ± 0.7	10.8 ± 0.5	6.4 ± 0.4

3.6 *In vitro* Drug Release from the Optimized Niosomal Gel versus Plain Drug Gel

The optimized niosomal gel (G3) showed significantly more prolonged and controlled drug discharge than the plain drug gel at all the time intervals studied ($p < 0.05$). The plain drug gel released about 96.8±3.4% of the drug in 12 h, while the niosomal gel released only about 76.8±2.1% of the drug (Table 5). The reduced ability of the drug to diffuse out of the vesicular lumen (bilayer membrane barrier) and diffuse through the Carbopol 934 gel matrix (diffusional barrier) was primarily responsible for the observed sustained action.

Table 5. *In vitro* cumulative percentage drug discharge from the optimized niosomal gel (G3) versus plain drug gel

Time (h)	G3 Niosomal gel (% CDR)	Plain drug gel (% CDR)
0.5	5.4 ± 0.4	18.2 ± 1.2
1	9.8 ± 0.6	31.4 ± 1.4
2	17.2 ± 0.8	48.6 ± 1.8
3	24.6 ± 1.0	60.3 ± 2.1

4	31.4 ± 1.1	69.8 ± 2.4
6	43.8 ± 1.4	79.6 ± 2.7
8	56.2 ± 1.7	86.4 ± 2.9
10	67.4 ± 1.8	92.3 ± 3.2
12	76.8 ± 2.1	96.8 ± 3.4

3.7 Drug Release Kinetics

Release data for niosomal gel (G3) and drug gel were fitted to zero-order, first-order, Higuchi, and Korsmeyer–Peppas models (Table 6). Niosomal gel was most closely fitted to the zero-order model ($R^2 = 0.9923$), which suggests a zero (zero-order) concentration-independent release rate of the drug in a gel formulation, and the Korsmeyer–Peppas model gave the best overall fit ($R^2 = 0.9948$) with a discharge exponent of $n = 0.726$ (between 0.5 and 1.0), consistent with a non-Fickian (anomalous) discharge mechanism driven by diffusion through the vesicular bilayer as well as gel matrix and relaxation of the polymeric network. Plain drug gel was most closely fitted to the first-order model ($R^2 = 0.9768$), with a Korsmeyer–Peppas exponent $n = 0.388$ (less than 0.5), as expected for simple Fickian diffusion driven by a concentration gradient through the gel matrix without a vesicle-regulating membrane.

Table 6. Release kinetic parameters for the niosomal gel (G3) and plain drug gel

Formulation	R^2 (Zero-order)	R^2 (First-order)	R^2 (Higuchi)	K–P (n value)
G3 — Niosomal gel	0.9923	0.9412	0.9876	0.9948 ($n = 0.726$)
Plain drug gel	0.9214	0.9768	0.9834	0.9862 ($n = 0.388$)

A more general kinetic evaluation (Table 7) carried out on representative niosomal dispersions, themselves, indicated that all formulations (F1, F3, F5, F7, F9) showed good correlation to the zero-order model together with good correlations to the first-order, Higuchi, and Hixson–Crowell models, which signify concentration-independent discharge and

concentration-dependent diffusion and surface-area alterations, respectively. The discharge rate of F1, F3, F5, F7, and F9 niosomal gel was represented by the Korsmeyer–Peppas equation, with all correlation coefficients in the non-Fickian range ($n = 0.695$ – 0.767). However, F8 plain drug gel has Fickian release ($n = 0.462$).

Table 7. Kinetic release parameters for niosomal formulations and plain drug gel

Formulation	R^2 Zero-order	R^2 First-order	R^2 Higuchi	R^2 Hixson–Crowell	R^2 Korsmeyer–Peppas	n	Release mechanism
F1	0.9750	0.9957	0.9953	0.9994	0.9971	0.72	Non-Fickian
F3	0.9714	0.9918	0.9948	0.9996	0.9967	0.69	Non-Fickian
F5	0.9896	0.9962	0.9956	0.9972	0.9989	0.76	Non-Fickian
F7	0.9753	0.9926	0.9954	0.9996	0.9976	0.70	Non-Fickian
F9	0.9633	0.9937	0.9941	0.9990	0.9950	0.69	Non-Fickian
Plain drug gel	0.8188	0.9970	0.9957	0.9968	0.9516	0.46	Fickian

3.8 Accelerated Stability Studies

The physical appearance of the optimized niosomal gel (G3) was unchanged, clear and pale white throughout the accelerated stability study (Table 8). PH was only slightly reduced from 6.50 ± 0.07 initially to 6.30 ± 0.13 after 6 months (estimates within the narrow range of 6.47–6.51 at intermediate time points). The viscosity decreased progressively from 8450 ± 134 cP initially to 7980 ± 205 cP; the drug content reduced slightly from $97.6 \pm 0.8\%$ initially to $94.0/97.1$ 1.8% at 6 months (estimates above 97% at all intermediate time points). The CDR after 12 h was retained unchanged at all time points, remaining within the narrow range of 76.1–76.9%. These data confirm that the optimized niosomal gel exhibited acceptable physical and functional stability under accelerated storage conditions.

Table 8. Accelerated stability data for the optimized niosomal gel (G3) over six months

Parameter	0 M	1 M	2 M	3 M	4 M	5 M	6 M
Appearance	Clear, pale white	Clear, pale white	Clear, pale white	Clear, pale white	Clear, pale white	Clear, pale white	Clear, pale white
pH	6.50 ± 0.07	6.51 ± 0.02	6.49 ± 0.04	6.48 ± 0.10	6.47 ± 0.11	6.48 ± 0.12	6.47 ± 0.13
Viscosity (cP)	8450 ± 134	8448 ± 48	8462 ± 62	8467 ± 76	8155 ± 85	8069 ± 94	8050 ± 05
Drug content (%)	97.6 ± 0.8	97.4 ± 1.0	97.3 ± 1.2	97.4 ± 1.4	97.5 ± 1.5	97.6 ± 1.6	97.8 ± 1.8
CDR at 12 h	76.8 ± 2.1	76.3 ± 2.3	76.4 ± 2.4	76.1 ± 2.6	76.9 ± 2.7	76.6 ± 6	76.2 ± 3.0

12 h (%)						±2.8	
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4. Discussion

The preformulation data gathered in the course of this work confirmed the identity, purity, and poor aqueous solubility profile typical of a BCS class II drug for the procured nepafenac, which exhibited an order of magnitude change in solubility between water and the least polar/most organic solvent tested. This overall solubility profile, coupled with the lack of any evidence of drug-excipient interactions seen via FTIR analysis, suggested the feasibility of, and suggested the rationale for, the development of a lipid-bilayer-based vesicular carrier such as a niosome in which the poorly water-soluble nepafenac could be encapsulated within, or at the bilayer surface of, the surfactant rather than relying on direct aqueous solubilisation.

The factorial optimization experiments identified straightforward, significant, and mechanistically interpretable relationships between the two formulation variables-cholesterol and Span 60 concentration-and both of the key quality attributes of the niosomal system[39]. Both VS and EE were best predicted by quadratic models with significant AB terms and A quadratic terms, meaning that neither surfactant nor cholesterol concentration alone could be used to accurately predict niosomal behaviour; instead, their combined, nonlinear effects dictated vesicle assembly. Cholesterol inserts into the surfactant bilayer, stiffening the membrane and decreasing bilayer permeability at modest concentration levels, but excessive cholesterol can destabilise the bilayer by disrupting the ordered packing of cholesterol-lipid mixtures, causing larger, more heterogeneous vesicles (1)-a finding consistent with the marked positive A (curvature) term seen for VS in this work. Also, the presence of the negative AB interaction terms for both responses indicates that co-modulation of cholesterol and Span 60 loading changed bilayer packing via a different mechanism than the changes driven by either factor by itself; probably through competing effects on the amount of component molecules within the bilayer and the local curvature of the bilayer, leading to fluctuations in the process of vesicle formation and closure[40].

The formulation (F5) having the highest EE, lowest PDI, and the most negative ZP represents a "sweet spot" of formulation whereby increasing the vesicle rigidity (via the cholesterol: Span 60 ratio) reduced nepafenac escape from the bilayer while concomitantly resulting in a more colloiddally stable dispersion (on account of its larger VS). The inverse trend between increasing VS and EE was what could be reasonably expected due to a larger amount of rigid bilayer accommodating a larger volume and greater

hydrophobic barrier for lipophilic and amphiphilic nepafenac molecules, but necessitating a larger variation in VS unless carefully optimized, and highlighting the benefit of factorial design in capturing effects on multiple dependent variables rather than simply optimizing one or two parameters in isolation[41].

The *in vitro* discharge data support this view that the significantly slower discharge of nepafenac from F5 than the plain drug solution, and the significantly further, markedly reduced drug discharge rate when F5 was incorporated into the Gel 934 base (G3) compared to the plain drug gel, clearly demonstrate an additive, bimodal - namely encapsulation within the vesicular bodies and entrapment within the gel matrix, respectively - zero-order discharge effect of the vesicular system[42].

G3 (1.5% w/v Carbopol 934) gel was found to be the most suitable base for this reason, showing the same apparent viscosity as G4 (2.0% w/v) but without the reduced spreadability that would reduce acceptability, as well as the lowest spreadability of any of the gels but without the risk of reduced retention at the application site seen with G1 (1.0% w/v), such that G3 appeared to be a good compromise between these two parameters and also contained the most drug[43].

The DSC results, independently orthogonal to the entrapment-efficiency and discharge data, also indicate that the high loadings of less soluble drug at the bilayer are consistent with molecular dispersions or amorphization of the drug and Niosome contents throughout the Niosome bilayer, as often observed with formulations that show an increase in apparent solubility and a more controlled, diffusion-limited discharge, as was also confirmed in this investigation[43].

Lastly, the short-term stability data showed that the optimized niosomal gel retained its original physico-rheological-functional characteristics for the 6-month study period, experiencing only a mild, progressive decrease in pH, viscosity, and drug content, with no net change in the drug-discharge behaviour over time. Such stability is the only feasible route to the development of a topical formulation in the future, and also shows that formation of the bilayer itself, as optimized in this work, would not result in significant internal reorganization, losses, or chemical degradation of the entrapped drug to facilitate delivery.

Some limitations of this work need to be considered. Firstly, the study was limited to *in vitro* formulation characterization and discharge testing, and the true validation of the benefits of superior retention and localized anti-inflammatory effects would need *ex vivo* skin permeation studies and *in vivo* pharmacodynamic and pharmacokinetic assessment. The study has

successfully identified the main formulation determinants using a 2-factor factorial study; however, other formulation factors (i.e., sonication parameters, the use of different surfactant chain lengths/HLB, and the Hydration Medium composition) were held fixed, which could potentially provide further scope for optimization[44-63].

The difference in VS may be due to the combined effect of CHL and Span 60 on vesicle formation, packing, as well as interfacial properties[34]. Since the response surface included the quadratic effects of both variables, it could be a reflection of the nonlinear behavior of VS with respect to the independent variables CHL and Span 60. An optimum composition of relatively balanced CHL and Span 60 would enable bilayer formation with ease from the combined effects of lipid and surfactant molecules, thus delivering relatively small vesicles[45]. Increasing the relative concentration of CHL above the optimum region would also imply an increase in the amount of lipid that is available for bilayer formation, thus inducing the formation of larger vesicles. Similarly, increasing the amount of Span 60 would mean an increase in the convex curvature, fluidity, as well as packing properties of the bilayer membrane. An increase in the amount of surfactant beyond the optimum would also bring about a change in the interfacial tension and packing of vesicular membranes, thus forming larger vesicles or contributing to the aggregation of vesicles[46]. The large curvature of the 3D surface also supports the nonlinear interactions of CHL and Span 60. Based on these responses, the two independent variables should not be assessed independently. The QbD and response surface shown here give an optimal composition region capable of generating vesicles of a desirable size.

5. Conclusion

The work has resulted in the successful fabrication and optimization of nepafenac-loaded sustained topical delivery niosomal gel under an interesting systematic, QbD-driven factorial design approach. This has laid a foundation for further *in vivo* clinical studies to establish its therapeutic benefits. The preformulation characterization has helped to identify the drug positivity, the purity, the solubility limitations, and the rationale behind vesicular entrapment, while the FTIR studies have demonstrated the stability of the drug in the presence of drug-excipients used for formulation. The experimental optimization using a 3-factor design has enabled a one-shot direct optimization of the system variables, particularly the span 60 and cholesterol levels. The final selection of formulation F5 was based on the balanced adequacy of the VS, the PDI, the ZP, and primarily the high EE% (88.4±1.9%). The inclusion of F5 into a carbopol-based gel system (glycerol and 0.25% of methylparaben as additives)

led to an acceptable formulation system with respect to the pH, the drug content, the viscosity, and the spreadability, and reflected a significantly prolonged nepafenac discharge (76.8% over 12 h) compared to the plain drug gel (96.8%) governed by a non-Fickian discharge mechanism owing to the resistance of both the polymeric gel system and the bilayer structure of the niosomes themselves. Differential scanning calorimetry has corroborated these drug incorporation findings by demonstrating drug amorphization and molecular association with the bilayer, and the accelerated stability studies have demonstrated the physical stability of the formulation when stored for 6 months. The results can, therefore, be summarized as a successful candidate: a mechanistically sound, statistically optimized nepafenac niosomal gel with the promising potential as a sustained anti-inflammatory agent, a localizing agent, and an effective localized topical gel for the treatment of a variety of acute or chronic inflammatory conditions. Further, the work can proceed to the study of *in vitro* permeation and *in vivo* efficacy testing.

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