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ORIGINAL ARTICLE

Formulation and Evaluation of Butenafine Hydrochloride-Incorporated Solid Lipid Nanoparticles as Novel Excipients for the Treatment of Superficial Fungal Infections

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ABSTRACT

Objectives: The objective of the present study was to develop natural excipient-based solid lipid nanoparticles (SLN) of butenafine hydrochloride (BUTE) using a modified solvent emulsification technique and to evaluate the competence of *aloe vera* nanolipidgel in enhancing the penetration of BUTE.

Materials and Methods: BUTE-SLNs were prepared using a 2^3 factorial design to correlate the effect of formulation components on the BUTE-SLN. Particle size, polydispersity index (PDI), zeta potential, entrapment performance, and drug loading were assessed in the formed SLNs. The fabricated BUTE-SLN was evaluated for transmission electron microscopy, fourier transform infrared spectroscopy, differential scanning calorimetry, and X-ray diffraction study studies and revealed the encapsulation of BUTE in lipid in the amorphous state. BUTE-SLN-based *aloe vera* gel was formulated and evaluated compared with the marketed product with respect to primary skin irritation, hydration, skin permeation, and antifungal activity.

Results: The BUTE-SLN *aloe vera* gel, optimized for its formulation, features excellent slip properties and controlled drug release. DSC and XRD studies confirm its amorphous nature with effective drug entrapment. The gel provides enhanced skin deposition, improved antifungal activity, and reduced irritation. This makes it a cost-effective and innovative alternative to traditional dosage forms. BUTE-SLN promisingly showed no irritation, higher hydrating potential, slow and sustained release, and enhanced antifungal activity. With an aim to target deeper skin strata, minimize the side effects of drugs and symptomatic impact of fungal infection, and shorten the duration of therapy, BUTE-SLN was successfully prepared. The mean particle size and PDI were 261.25 ± 2.38 nm and 0.268 ± 0.01, respectively.

Conclusion: BUTE-SLN gel offers improved topical delivery of BUTE with significantly higher compatibility and antifungal activity than the marketed formulation.

Keywords: Modified solvent emulsification technique, factorial design, butenafine, *aloe vera* gel, natural origin lipid, surfactant, *in vitro* antifungal activity.

INTRODUCTION

Fungal infections are one of the most common skin diseases in the global population. According to an unpublished survey by the International Foundation of Dermatology, superficial mycosis was usually reported as one of the three most common diseases among the community patterns of skin diseases in nine different countries across the world.[1] Human skin has favorable conditions forthe growth of dermatophytes[1]. Dermatophyte fungi

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invade the stratum corneum. Dermatophytes areenriched with keratinolytic, proteolytic and lipolytic activity.[2] Dermatophytes also contain serine proteinases that play a major role in breaching the skin barrier. Invasion of the skin involves two basic mechanisms-Colonization and Host-parasite interaction. During the colonization phase, the host begins to respond immunologically, and the first detectable immune response is cell-mediated immunity (CMI), which is characterized in colonized skin by an intense inflammatory process. During the host-parasite interaction phase, CMI produces most of the pathology as an acute inflammatory type of dermatophytosis, which results in erythema and edema of the dermis and epidermis leadingto breaching epidermal integrity.[3]

Current medications for superficial fungal infections include various antifungal agents. Oral toxicity of antifungal drugs and the treatment of fungal infections in the stratum corneumfocus on topical delivery of antifungal agents. Topical treatment has several advantages over oral and systemic delivery. Still, it has some pitfalls, such as side effects, diffusion of the drug across biological tissues, drug and biological cell interactions, and residence time of conventional dosage forms. Novel colloidal drug delivery systems have been developed to overcome the limitations of the conventional route. Among the various colloidal carriers solid lipid nanoparticles (SLN) have shown promising healing abilities against skin infections.

Butenafine hydrochloride (BUTE) is a synthetic allylamine antifungal agent. The suggested mode of action is that inhibits the enzyme squalene monooxygenase, which is responsible for converting squalene to 2,3-oxide squalene. Hence, it interferes with the biosynthesis of ergosterol, resulting in increased cellular permeability and leakage of cellular contents. Blockage of squalene monooxygenase causes squalene accumulation and leads to a fungicidal effect.[4,5] The observed side effects of BUTE include contact dermatitis, erythema, irritation, burning, and itching at the site of application.[6] To mitigate the side effects of BUTE and the symptomatic effects of fungal infection, such as inflammation, stinging, and itching, a possible combination of BUTE and agents with anti-inflammatory activity can be applied. Topical steroids provide rapid symptomatic relief but are associated with many steroid-related complications like atrophy, purpura, and rosacea.[7] Hence, to provide better therapeutic and pharmacological effects for fungal infection, the present workaimed to formulate BUTE-SLN having natural lipid and surfactant incorporatedinto *aloe vera* gel, which has in-built anti-inflammatory, antioxidant, and healing properties that showed synergistic effects. The present research work can be used to develop natural excipient-based SLN of BUTE using a modified solvent emulsification technique and to evaluate the competence of *aloe vera* nanolipidgels for the enhancement of the penetration of BUTE.

MATERIALS AND METHODS

Materials

Butenafine was a kind gift from Cipla Pvt. Ltd., Mumbai (India). Olivem 1000 as a lipid and Olivem 300 as a surfactant were generous gifts from Chemhouse Marketing, Mumbai. D-α tocopheryl polyethylene glycol succinate (TPGS) was purchased from BASF India Ltd., Mumbai. Stearyl amine was purchased from Sigma-Aldrich GmbH (St. Louis, MO) and other chemicals, such as stearic acid, glyceryl monostearate, tween 80, acetone, dimethyl sulfoxide, and poloxamer 188, were of analytical grade and purchased from Merck Chemical Company (Mumbai, India).

Optimization of surfactant, solid lipid, and organic solvent concentrations/effects of variables

To determine the influence of the concentration of different ingredients on the properties of butenafine-loaded solid lipid nanoparticles (BUTE-SLN), 3-factor 2-level factorial experimental design was employed using Design Expert software 8.0.1 (Stat-Ease, Inc., Minneapolis, MN, USA). On the basis of the solubility study and pre-optimization study, various variables at different levels were selected. The effects of three independent variables concentration of surfactant Olivem 300 (X_1), concentration of lipid Olivem 1000 (X_2), and concentration of organic solvent (X_3), were determined on the formulation of nanoparticle dispersions in terms of five responses: particle size (Y_1), polydispersity index (PDI) (Y_2), zeta potential (ZP) (Y_3), percent entrapment efficiency (EE) (Y_4), and percent drug loading (DL) (Y_5). All eight possible combinations of the three factors at two different levels were evaluated for each response. All nine experimental runs, different independent variables with two levels [*i.e.*, low (-1) and high (+1)], and obtained responses are presented in Table 1. Using the Design Expert software, response surface plots were generated to identify the influence of significant variables.

Preparation of BUTE-SLN

A Simple and easily accessible method was adopted to prepare BUTE-SLN. A modified solvent emulsification technique was used, and a BUTE-SLN dispersion was prepared for the solubility and compatibility studies of lipids, drugs, and excipients. BUTE-SLN is composed of the natural excipients from the natural stearin fraction of olive as lipid Olivem 1000 and surfactant Olivem 300. BUTE-SLN was prepared by dissolving butenafine in acetone and dimethyl sulfoxide (DMSO) (1:1) and then mixing it with stearyl amine and lipid Olivem 1000 at 80 °C. The water phase containing TPGS and surfactant Olivem 300 was maintained at 80 °C, similar to the lipid phase. The lipid phase was added to the aqueous phase with constant stirring at 2000 rpm for 1 hour followed by sudden cooling of the dispersion.

Table 1. Independent variables and their levels of 23 factorial design for formulation of BUTE-SLN

Factors	Levels	
	Low (-1)	High (+1)
Surfactant concentration % (X1)	2	4
Concentration of lipid % (X2)	3	5
Concentration of organic solvent % (X3)	0.5	1.5

BUTE-SLN: Butenafine hydrochloride-solid lipid nanoparticles

Physicochemical characterization of BUTE-SLN dispersion

Particle size analysis (PS) and PDI

The particle size in the nanometric range can be determined by the concept of dynamic lightscattering. The particle size and PDI of the optimized BUTE-SLN were determined by photon correlation spectroscopy (PCS) using a Zeta sizer (Nano ZS 90, Malvern Instruments, UK) at 20 °C using 900 scattering optics.[8,9]

ZP

The ZP of the SLN dispersion was determined using a Zetasizer (Nano ZS 90, Malvern Instruments, UK) at 2 °C with an electric field strength of 23 V/m 10, 11, 12. The BUTE-SLN samples were diluted with double-distilled water.

EE and DL

EE% of butenafine in BUTE-SLN can be calculated by determining the concentration of butenafine in the supernatant of the SLN dispersion. An ultracentrifuge was used to determine the concentration of un-entrapped butenafine in an aqueous BUTE-SLN dispersion. 1.5 mL of BUTE-SLN dispersion was filled in Eppendorf tubes, and the speed of cooling ultracentrifuge (Remi Instruments Ltd., Mumbai, India) was maintained at 60,000 rpm for 45 minutes at 4 °C. An ultraviolet (UV)-visible spectrophotometer (UV 1700, Shimadzu, Japan) was used to measure the concentration of butenafine in the aqueous phase at a wavelength of 224 nm. Equations (Eq.). (1) and (2) were used to measure the EE and DL percent values, respectively.[10-13]

$$\% EE = \frac{\text{Total mass of Bute} - \text{Total mass of Bute in Supernatant}}{Total\ mass\ of\ Drug} \times 100 \quad \text{Eq. 1}$$

$$\% DL = \frac{\text{Total mass of Bute} - \text{Total mass of Bute in Supernatant}}{Total\ mass\ of\ Lipid} \times 100 \quad \text{Eq. 2}$$

Transmission electron microscopy study (TEM)

Particle size, shape, and morphology were studied using TEM. In this study, the BUTE-SLN dispersion was mixed with water, and a drop of it was mounted on a copper grid coated with a thin film of carbon, which was then dried for 45 minute. A transmission electron microscope Philips CM200 (Philips, Netherlands) was used for scanning.

Screening of cryoprotectant

BUTE-SLN dispersion was freeze-dried by using mannitol as a cryoprotectant. The frozen (-25 °C) dispersions containing 5, 7.5 and 10% mannitol were lyophilized at 0.25 barfor 24 hours, and the temperature was increased from 15 °C to 0 °C. In the final step, drying was carried out for 2 hours at +15 °C and 0.01 bar and then stored at 4 °C. BUTE-SLN was further characterized for differential scanning calorimetry (DSC), X-ray diffraction (XRD) study, and fourier transform infrared spectroscopy (FTIR) studies.[14,15]

Characterization of lyophilized BUTE-SLN

DSC

The thermal behavior and interaction between the drug and its additives were studied using DSC. In a differential scanning calorimeter (DSC 1 STARe System, Mettler-Toledo, Switzerland), an empty standard aluminum pan was used as a reference, and scans wererecorded at a heating rate of 10 °C/minute in the range of 30 °C-300 °C. DSC studies were carried out on Butenafine pure drug, bulk lipid Olivem 1000, and lyophilized BUTE-SLNs of the optimized batch.

XRD study

XRD analysis was performed to study the spacing of the lattice planes in the crystals. The crystal structures of butenafine, pure lipid, and butenafine in SLN were determined by XRD analysis. A Philips PAN analytical expert PRO X-ray diffractometer (1780) was used for the study. A Cu-Ka radiation source was used, and the scanning rate was 2°/minute. XRD measurements were performed on butenafine pure drug, bulk lipid Olivem 1000, and lyophilized BUTE-SLNs from the optimized batch.

FTIR study

A Jasco FTIR spectrophotometer (Perkin Elmer Jasco FTIR-401, Japan) was used to determine the compatibility between excipients. FTIR studies were carried out on pure lipid Olivem 1000, butenafine, and SLN loaded with butenafine. The samples were examined in transmission mode over awavenumber range of 4000 to 400 cm^{-1}, using about 1-2 mg of sample mixed with dry potassium bromide.

Preparation of BUTE-SLN into semisolid formulation

BUTE-SLNs were formulated using *aloe vera* gel as a semisolid base. *Aloe vera* gel was formulated with the *aloe vera* extract of *Aloe barbadensis* along with highly pure and GRASS-grade stabilizers, antioxidants, and preservatives. After centrifugation of the dispersion, drug contentwas determined, and a BUTE-SLN dispersion equivalent to 0.1 g butenafine was added to *aloe vera* gel to prepare 1% gel.

Evaluation of BUTE-SLN gel

Rheological measurement

Viscosity

The rheological behavior and characteristic flow properties of formulated *aloe vera* gels incorporated with SLN dispersion were studied. A cone and plate rheometer (Brookfield viscometer AP 2000+2) was used to determine the effects of shear stress and shear rate on the SLN gels. To determinethe influence of storage temperature and the nature of the lipid matrix on the rheological behavior of BUTE-SLN-based *aloe vera* gel was investigated by recording the variation in shear stress at a pre-defined shear rate from 0 s-1 to 1000 s-1 as an up curve and 1000 s-1 to 0 s-1 as a down curve at three different temperatures (5 °C, 25 °C and 40 °C).

Spreadability

The BUTE-SLN-enriched gel was evaluated on the basis of its rheological pattern, film-forming ability, degree of consistency, and spreadability. Spreadability is an important parameter for assessing topical dosage forms because it represents the stability and particle-particle interaction. This study includes the use of a wooden block and a glass slide apparatus in which one rectangular wooden block is fixed with a glass slide. In the same apparatus, another movable glass slide was attached with a thread to pan through a pulley. The time was noted to separate the upper movable slide from the fixed slide was noted when a BUTE-SLN gel was placed between the slides. Spreadability was quantitatively determined as the ratio of time required to completely separate two glass slides according to the weight of the sample. Spreadability was computed using formulas.[16,17]

$$S = \frac{M \times L}{T}$$

Where,

S = spreadability

M = weight of the upper glass slide

L = glass slide length

T = time taken to separate the slides from each other

Occlusive study

An *in vitro* occlusion study was performed to determine the efficiency of BUTE-SLN gel in preventing water loss from the surface of the test assembly, which simulates transepidermal water loss from the skin. In this study, pre-weighed beakers of approximately the exact dimensions with 50 mL capacity were filled with 25 mL purified water and covered with Whatman Microfiber Filters 9.0 cm. For a comparative study, one beaker surface was spread with 0.25 g of BUTE-SLN gel, the other with plain gel without BUTE-SLN, and one beaker without any application was kept as a reference. All beakers were stored at 32 °C and 60 ± 5% relative humidity for 72 hours. The occlusion factor, F, was calculated for the formulation according to formula.[18]

$$F = 100 \times \frac{A - B}{A}$$

Where,

Here, A represents water loss in the absence of a sample

B represents water loss in the presence of the sample

Ex vivo skin hydration study

The *ex vivo* skin hydrating effect of BUTE-SLN was studied and compared with the conventional cream. The BUTE-SLN *aloe vera* gel and the marketed cream were applied to the prepared human cadaver skin. The skin samples were isolated after 24 hours of gel and cream application. The skin was vertically sliced using a microtome, stained with carbol fuchsin, and observed under an optical microscope to determine stratum corneum. An optical microscope equipped with an image analyzer was used to obtain the photomicrographs (Magnus MLX).[19]

In vitro drug diffusion study

In vitro drug release of BUTE-SLN was studied in Franz diffusion cells using a cellulose acetate membrane. Phosphate buffer (pH 7.4) and methanol (60:40) were used as the diffusion medium, andthe membrane was soaked in the diffusion medium for 30 minutes before placing the sample. Phosphate buffer (pH 7.4) and methanol (60:40) (10 mL) were placed in the receptor compartment of Franz diffusioncells. The diffusion medium in the receptor compartment was continuously stirred using a magnetic bar while maintaining the temperature at 37 °C. The experiment was started with the application of 0.5 g of BUTE-SLN gel on the surface of the cellulose acetate membrane from the donor compartment side. Sampling was performed after 0, 1, 2, 5, 7, 9, 12, and 24 hours, and a fresh diffusion medium was addedwith each withdrawal of the sample. The samples were diluted and analyzed by UV spectrophotometry at 224 nm.

Drug permeation

In vitro skin penetration and permeation experiments were performed using Franz diffusion cellson pig ear skin. Among rodents, the most relevant animal model for human skin is the pig. Pig ear skin has been proven thave similar histological and biochemical properties, vascular anatomy, and stratum corneum contents to human skin, thereby giving comparable results to human skin. Fresh pig ears were obtained from a local slaughter house. Pig ears were washed with distilled water, and subcutaneous adipose tissue was removed. The skin was placed between the donor and receptor compartments of Franz diffusion cells, exposing the dermal side to the receptor compartment and stratum corneum in contact with the donor compartment. Sink conditions were maintained in the receptor compartment using diffusion fluid. The cell temperature was maintained at 37.0 ± 0.1 °C. The BUTE-SLN gel equivalent to 1% of the drug was applied on the membrane in the donor compartment, ensuring close contact with the skin. Sampling was performed at 0, 1, 2, 5, 7, 9, 12, and 24 hours. At each point, 1 mL aliquots were drawn from the receiver compartment, and an equivalent volume of the receptor fluid was simultaneously replaced. The amount of butenafine in the diffusion medium was determined by UV irradiation. A graph of cumulative percent drug release vs. time and another graph of the amount of BUTE diffused per unit area (Q/A) vs. time (h) were plotted. Excess BUTE amount found on the surfaceof the skin and in the entire dosing area was determined. The amount of unabsorbed drug on the skin was also quantified.[20,21]

In vivo skin retention study

BUTE-SLN gel (0.25 g) was applied to the shaved skin of albino rats. After 24 hours, the animals were humanely sacrificed, and their skin was collected. The applied formulation was removed, the stratum corneum layer was stripped away with adhesive tape, and the epidermis layer was differentiated using the heat separation technique. The presence of butenafine in different skin stratawas extracted and quantitatively determined.[22]

Skin irritation study

The primary skin irritation of the SLN-based butenafine gel was evaluated using an acute skin irritation test according to OECD

guidelines #404. The protocol for the study was approved by the Pravara Rural College of Pharmacy (approval number: 2013714-543, date: 07.01.2014). 2.5-3.0 kg healthy male New Zealand white rabbits were used in the study. Animals were categorized into four groups: positive control (formalin), plain butenafine gel, marketed formulation, and BUTE-SLN gel. 0.5 g of each test sample was applied to the hair-free shaved area of the skin, *i.e.*, right side of the trunk (approximately 6 cm^2), and covered with a gauze patch. The left side of the trunk was kept as an untreated skin area to serve as a control. After 1 hour of exposure to the test sample, the rabbits were observed for signs of erythema, edema, and irritation. The degree of irritation, erythema, and edema were assessed and scored according to the Grading of skin reactions according to the OECD guidelines. Data were recorded at interval of 24 hours, 48 hours and 72 hours after patch removal.[23]

Antifungal activity

An in vitro antifungal activity study was conducted against *Candida albicans* using a modified agar diffusion method. Fresh *C. albicans* cultures were prepared and then incubated in the dark at 37 ± 2 °C for 48 hours. The *in vitro* antifungal activity of blank gel, BUTE-SLN gel, plain butenafine gel, and the marketed formulations was studied. The formulations were mixed with potato dextrose agar and poured into a spherical ditch made using a sterile borer on an agar plate under sterile conditions. The plates were dried and incubated for 48 hours at 37 ± 2 °C. At the end of the incubation, the inhibition zone was measured.[23-26]

Statistical analysis

The effect of various independant variables on dependant variables of BUTE-SLN formulations was optimized using 23 factorial design and analyzed statistically using Design-Expert software (Version 8.0.1., Stat-Ease Inc., and Minneapolis, MN). The multiple linear regression analysis was used to generate polynomial models including interaction and regression equations for all response variable. Statistical significance of the suitable model selected by software were analyzed by analysis of variance (ANOVA) based on p value and regression analysis. The 3D response graphs were constructed using Design-Expert software. The model was validated using numerical optimization. The observed responses were coincided well with the predicted values given by optimization technique.

RESULTS

Effects of different variables on the preparation of BUTE-SLN and the optimization of the surfactant, solid lipid, and organic solvent concentrations.

A 2^3 factorial design was used to determine the effects of various variables on the optimization of the BUTE-SLN. The

Table 2. 2^3 factorial design for formulation of BUTE-SLN

Run code	Concentration of lipid % (X1)	Concentration of surfactant % (X2)	Concentration of organic solvent % (X4)
1	2	5	1.5
2	4	5	0.5
3	4	3	0.5
4	2	3	1.5
5	2	3	0.5
6	4	5	1.5
7	4	3	1.5
8	2	5	0.5

BUTE-SLN: Butenafine hydrochloride-solid lipid nanoparticles

Table 3. Observed responses of eight experimental formulations (2^3 factorial design)

Run code	Particle size nm	PDI*	ZP (mV)	% EE*	% DL*
1	271 ± 2.74	0.368 ± 0.014	20.2 ± 0.32	91.2 ± 1.82	18.33 ± 0.79
2	258 ± 3.61	0.267 ± 0.009	24.9 ± 0.41	91.65 ± 1.69	18.24 ± 0.86
3	231 ± 2.79	0.254 ± 0.012	17.8 ± 0.43	88.95 ± 2.17	21.05 ± 0.35
4	250 ± 3.65	0.324 ± 0.009	12.3 ± 0.22	88.29 ± 1.73	22.86 ± 0.46
5	245 ± 4.12	0.241 ± 0.005	11.7 ± 0.16	87.45 ± 2.26	21.33 ± 0.78
6	278 ± 3.19	0.369 ± 0.01	22.4 ± 0.26	89.25 ± 1.58	15.2 ± 0.31
7	239 ± 2.37	0.33 ± 0.009	17.23 ± 0.39	88.41 ± 1.42	22.16 ± 0.91
8	269 ± 4.26	0.264 ± 0.009	21.5 ± 0.31	90.46 ± 2.36	15.05 ± 0.58

*Each value represents as mean ± standard deviation
PDI: Polydispersity index, ZP: Zeta potential, EE: Entrapment efficiency, DL: Drug loading

eight formulations were obtained with a possible combination of surfactant concentration X_1, concentration of lipid X2 and concentration of organic solvent X3 as independent variables with low and high levels, as shown in Tables 1, 2. The responses Y1, Y2, Y3, Y4, and Y5 were found to be in the range of 231-278 nm, 0.241-0.369, 11.7-24.9 mV, 87.45-91.65% and 15.05-22.86% respectively as shown in Table 3. The observed responses were fitted to various models using the Design Expert software. The dependent variables show that the models were important for all measured responses, as determined by the negligible lack of fit ($p < 0.05$) ANOVA results. It was observed that independent variables X_1, X_2, and X_3 had positive as well as negative influences on selected dependent variables such as particle size, PDI, ZP, EE, and DL (see Eq. 3 to 7).

$$\text{Particle size}=+255.625-3.125*X_1+14.375*X_2+3.875*X_3+3.125*X_1X_2+2.125*X_1X_3 \quad \text{Eq. 3}$$

$$\text{Polydispersity index}=+0.302125+0.002875*X_1+0.014875*X_2+0.045625*X_3-0.001125*X_1X_3+0.005875*X_2X_3 \quad \text{Eq. 4}$$

$$\text{Zeta Potential}=+18.5038+2.07875*X_1+3.74625*X_2-0.47125*X_3-0.67875*X_1X_2+0.47875*X_2X_3 \quad \text{Eq. 5}$$

$$\text{Entrapment Efficiency}=+89.7075-0.17*X_1+1.1825*X_2+0.3575*X_3-0.565*X_1X_3-0.2975*X_2X_3 \quad \text{Eq. 6}$$

$$\text{Drug Loading}=+18.8625-0.21*X_1-1.7075*X_2-1.585*X_3+0.175*X_1X_2 \quad \text{Eq. 7}$$

As per the ANOVA analysis, near-100% desirability was found to be at predicted values of 264 nm for Y_1, 0.270 for Y_2, 24.60 mV for Y_3, 91.225% for Y_4, and 18.705% for Y_5 with a surfactant concentration of 4%, concentration of lipid 5% and concentration of organic solvent 0.5%. To confirm and reproduce the predicted model, the optimized values of the independent variables were applied, and the observed responses were measured, as shown in Table 4. The observed values of Y_1, Y_2, Y_3, Y_4, and Y_5 were 261.25 nm, 0.268, 23.98 mV, 91.35%, and 19.692%, respectively, which were in close agreement with the predicted values, demonstrating the reliability and reproducibility of this method. The effects of various independent variables on the dependent variables were expressed using the 3D graphical presentation shown in Figure 1.

Physicochemical characterization of BUTE-SLN dispersion

PS and size distribution

The effects of various formulation components and process variables on the preparation of SLN can be observed from the particle size and PDI of BUTE-SLN gel, as depicted in Table 3. The particle size and PDI of BUTE-SLN increased with increasing lipid content. The range of particle size and PDI of the BUTE-SLN were observed to be 231 ± 2.79 nm to 278 ± 3.19 nm and 0.241 to 0.369, respectively. The particle size and PDI of optimized batch was found to be 261.25 ± 2.38 nm and 0.268 ± 0.01 respectively.

ZP

ZPs indicate the stability of the nanoparticles in SLN dispersion. The ZPs of all nine batches were found to be 11.7 ± 0.16 mV and 24.9 ± 0.41 mV, and for the optimized batch, it was observed to be 23.98 ± 0.27 mV.

EE and DL

Different concentrations of lipid and organic solvent showed significant effects on the EE and DL of butenafine loading in lipid nanospheres. The EE of BUTE was found to be higher in the selected formulation. The EE of eight formulations from run 1 to run 8 were found to be in between 87.45 ± 2.26% to 91.65 ± 1.69% with the DL in between 15.05 ± 0.58% to 22.86 ± 0.46%. The EE and DL of the optimized formulation were found to be 91.35 ± 2.35% and 19.692 ± 0.95%, respectively.[10,13]

TEM Imaging

With similar results to those obtained for PS, the TEM image of BUTE-SLN (Figure 2) revealed the presence of spherical nanometric-range particles.

Screening of the cryoprotectant

The cryoprotectant concentration was optimized on the basis of SLN aggregation, time of reconstitution, and particle size distribution upon reconstitution. From the different concentrations of cryoprotectant screened for lyophilization, mannitol at 5% *w/w* resulted in good lyophilized product upon reconstitution with quick reconstitution, no aggregation, and less difference in particle size distribution (PDI).[14,15]

Characterization of lyophilized BUTE-SLN

DSC

The thermal profiles of bulk lipid, pure butenafine, and lyophilized drug-loaded SLN formulation are given in Figure

Table 4. Observed and predicted responses for optimized formulation parameters

Reponses	Constraints	Set observed values*	Predicted values	Error %
Particle size (nm)	Minimize	261.250±2.38	264	1.041
PDI	Minimize	0.268±0.01	0.27	0.7407
ZP (mV)	Minimize	23.98±0.27	24.60	2.5203
EE %	Maximize	91.35±2.35	91.225	1.370
DL %	Maximize	19.692±0.95	18.705	5.2766

*Each value represents as mean ± standard deviation, PDI: Polydispersity index, ZP: Zeta potential, EE: Entrapment efficiency, DL: Drug loading

3[1]. The melting point of bulk lipid Olivem 1000 was determined using a DSC thermogram that showed a high endothermic peak at 63.20 °C (curve A). A sharp endothermic peak at 217.35 °C was observed on the second thermogram of pure butenafine. The endothermic peak wasobserved for BUTE-SLN at 46.91 °C, indicating the transformation of crystalline lipids and drugs into amorphous forms with numerous lattice defects in which the drug is encapsulated.[14]

X-ray Diffraction (XRD) analysis

The XRD patterns of the drug butenafine (a), lipid Olivem 1000 (b), and BUTE-SLN (c) areshown in Figure 3.[2] Pure butenafine showed a sharp peak, which is indicative of its crystalline nature. Lipid Olivem 1000 also showed a sharp peak revealing the crystalline nature of the lipid crystals. BUTE-SLN showed broad peaks instead of sharp ones, indicating an amorphous nature. All the major characteristicpeaks of butenafine and lipid

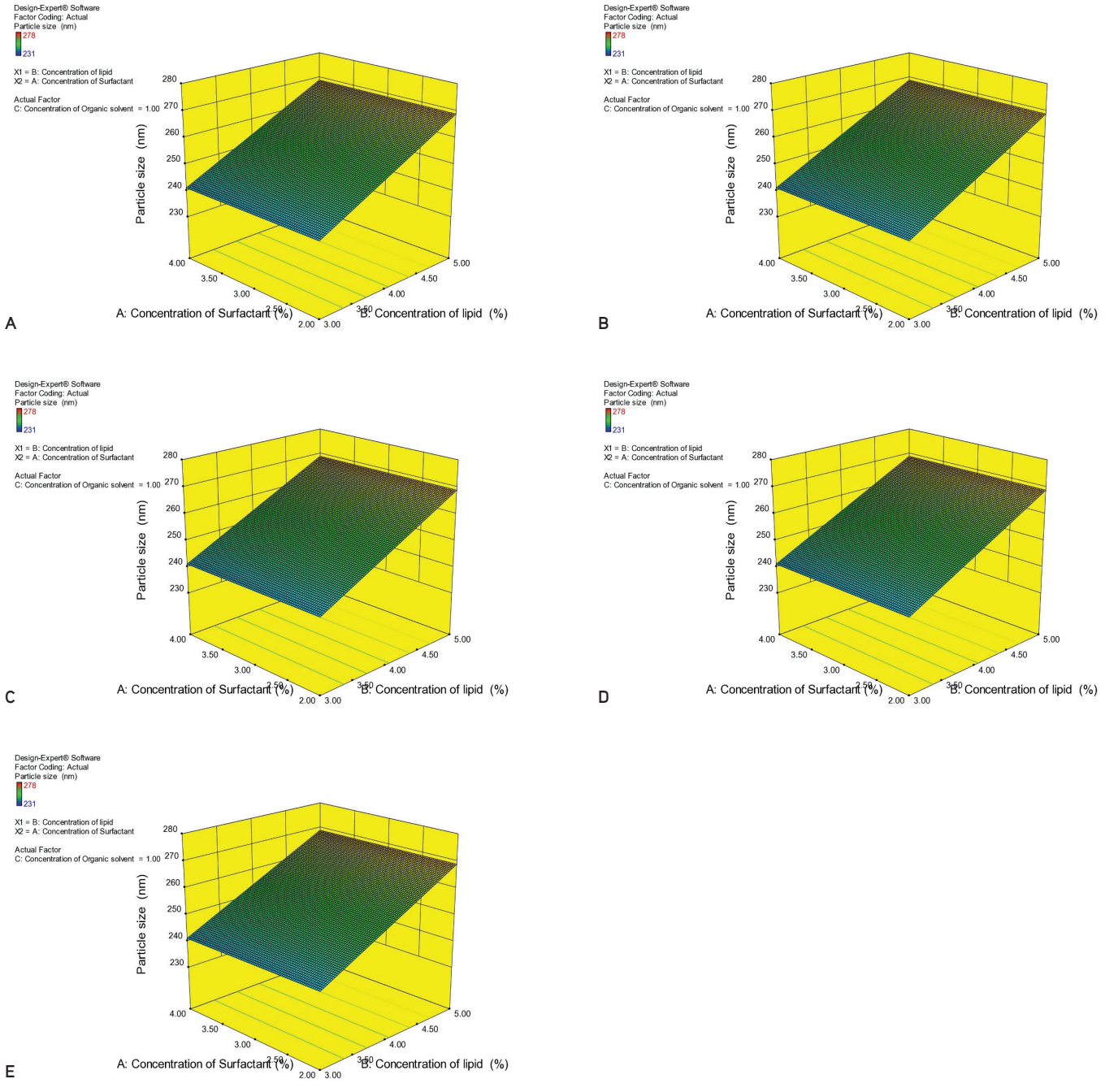


Figure 1. 3D response surface plot of BUTE-SLN showing. A) Effect of lipid Olivem 1000 and surfactant Olivem 300 on the particle size, B) Effect of lipid Olivem 1000 and organic solvent on the polydispersity index, C) Effect of lipid Olivem 1000 and surfactant Olivem 300 on the zeta potential, D) Effect of surfactant Olivem 300 and lipid Olivem 1000 on the percent entrapment efficiency and E) Effect of organic solvent and surfactant Olivem 300 on the percent drug loading

BUTE-SLN: Butenafine hydrochloride-solid lipid nanoparticles

Olivem 1000 were absent in BUTE-SLN. The presence of broad and small XRD peaks reveals the existence of butenafine and lipid Olivem 1000 in BUTE-SLN in the amorphous state.

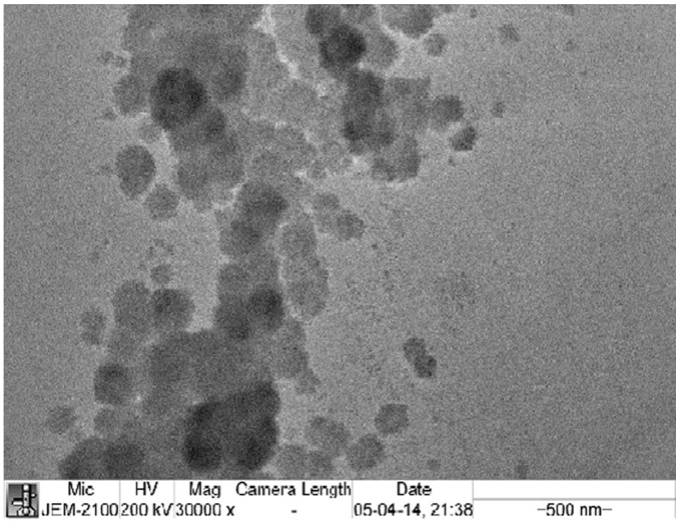


Figure 2. TEM image of BUTE-SLN

TEM: Transmission electron microscopy, BUTE-SLN: Butenafine hydrochloride-solid lipid nanoparticles

FTIR

The FTIR spectra of butenafine, pure lipid Olivem 1000, and BUTE-SLN are compared in Figure 4. The FTIR spectra indicated amorphization of the drug and lipid in the formulation.

BUTE-SLN showed no new peaks, revealing that there was no interaction between the formulation ingredients and no structural or functional changes in the formulation.

Preparation of BUTE-SLN into semisolid formulation

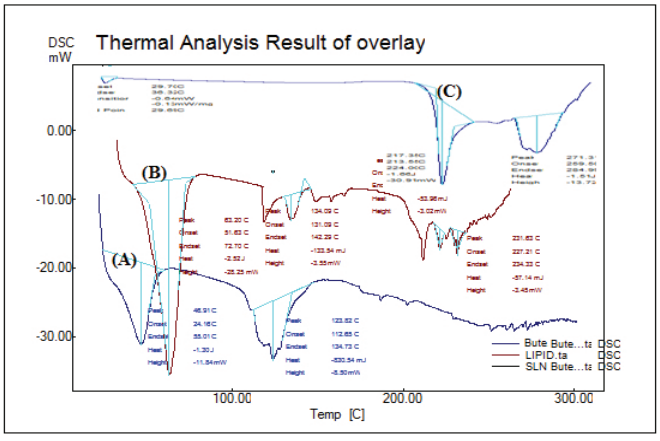
Instead of a conventional semisolid dosage form, we have formulated BUTE-SLN for incorporation into *aloe vera* gel. *Aloe vera* gel has been used for the external treatment of mild wounds as well as inflammatory skin diseases for a long time, with the aim of speeding up the healing process and reducing inflammation and tissue scarring. Mild skin irritation can be treated with this gel. Here, an attempt has been made to formulate a semisolid product that will have synergetic antifungal effects as well as anti-inflammatory, wound healing, and skin irritation effects by aloe *vera gel.*[24-26]

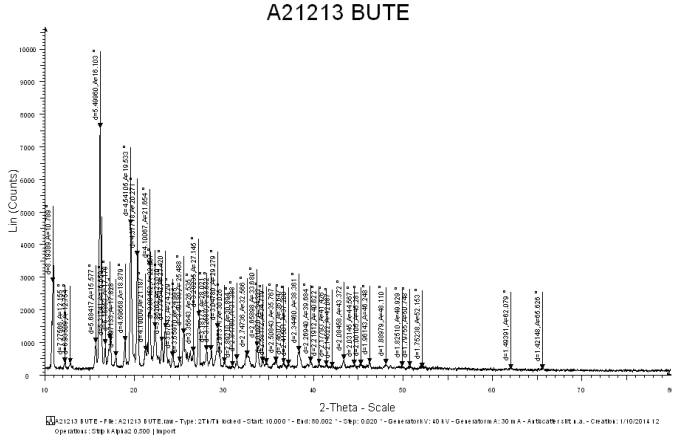
Evaluation of BUTE-SLN gel

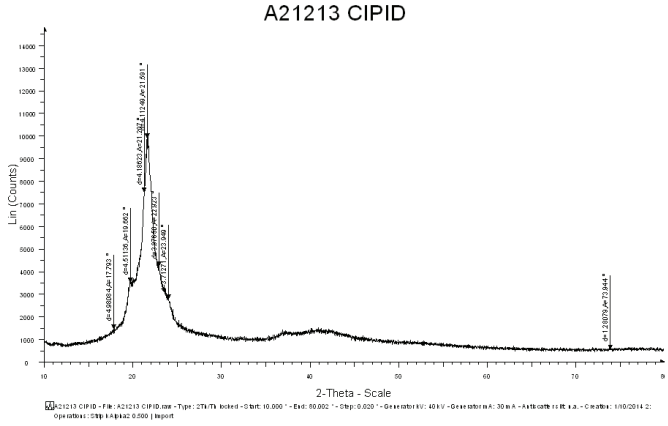
Rheological measurement

Viscosity

The viscosity of any semisolid formulation depends on its adhesion, ease of application, and spreadability. The results







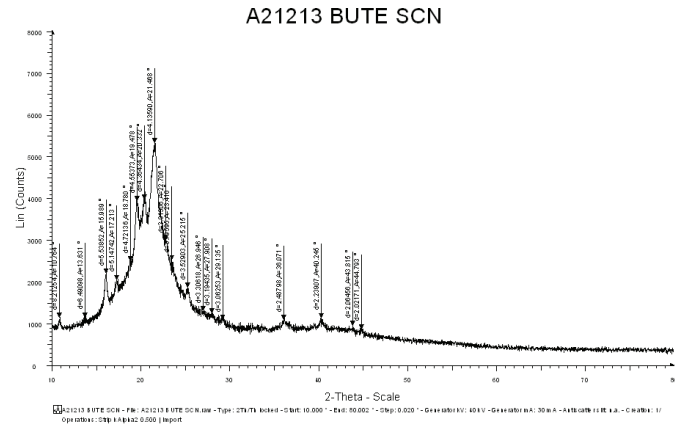


Figure 3. (1) DSC thermogram of sample, A) Butenafine, B) Pure lipid Olivem 1000, C) BUTE-SLN; (2) Comparison of XRD patterns: I. Butenafine, II. Lipid Olivem 1000; III. BUTE-SLN

DSC: Differential scanning calorimetry, BUTE-SLN: Butenafine hydrochloride-solid lipid nanoparticles, XRD: X-ray diffraction

were recorded for the BUTE-SLN *aloe vera* gel at 5 °C, 25 °C and at 40 °C after the storage of one week to check the consistency and compatibility of the gel with pharmaceutical excipients. A slight increase in the viscosity of the BUTE-SLN gel was observed compared to plain *aloe vera* gel due to existence of nanometric particle size of SLN and the polysaccharide crosslinking with the lipid component of SLN. The increase in total lipid content and particle size increases the viscosity of the gel. when a BUTE-SLN gel was placed between the slides. The spreadability of the BUTE-SLN gel was evaluated by placing it between two slides. The results showed that its spreadability was superior to that of pure aloe vera gel.

Spreadability

The potential application of semisolid topical dosage forms with desired consistency and adhesion onto skin was studied according to the spreadability values. Nanometric and uniform particle sizes lead to good spreadability, forming a uniform layer on skin. The presence of BUTE-SLN did not cause any changes in spreadability. The spreadability value of BUTE-SLN was found tobe 4.6 ± 0.37 s/g, and that for the plain gel base was 4.06 ± 0.41 s/g. A slight increase in the spreadability value indicates that SLN forms a 3D structure with a polysaccharide group, which leads to an increase in the viscosityand spreadability.

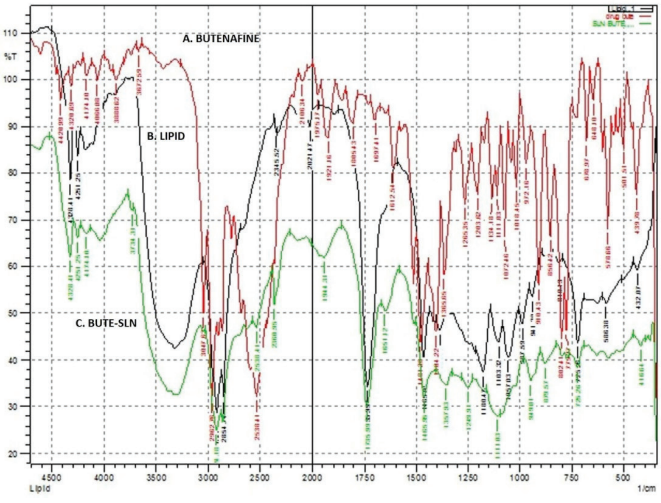


Figure 4. Comparative FTIR spectra of A) Butenafine, B) Lipid Olivem 1000 and C) BUTE-SLN

FTIR: Fourier transform infrared radiation, BUTE-SLN: Butenafine hydrochloride-solid lipid nanoparticles

Occlusive study

Successful topical drug delivery is based on the occlusive and skin hydration ability of semisolid formulations. Figure 6A shows a comparison of BUTE-SLN versus plain gel effects. After the 6 hours, 24 hours, 48 hours, and 72 hours study, occlusion factor F were found to be 31.362 ± 0.65%, 41.1372 ± 0.47%, 43.2659 ± 0.28%, and 45.7 ± 0.44%, respectively, as shown in Figure 6A. Whilst that of plain gel F were found to be 18.2795 ± 0.55%, 23.0020 ± 0.59%, 31.2094 ± 0.21% and 31.5209 ± 0.29% when compared to the control. BUTE-SLN showed higher occlusion factor *f* values in comparison to the plain gel owing to its high lipid content, which forms an impermeable layer on the surface of the skin.

Ex vivo skin hydration study

Using an optical microscope and image analyzer, the skin hydrating response of the SLN formulation was investigated in human cadaver skin and compared with that of the marketed cream Fintop® (Magnus MLX). This data analysis aimed to compare the effects of SLN gel and the marketed cream Fintop® on the stratum corneum, which is associated with skin moisturization and drug penetration. Figure 6B I shows a photomicrograph of untreated human cadaver skin. The photomicrograph's dark brown left side layer reflects the skin's top layer (*i.e.*, stratum corneum). The thickness of the stratum corneum changed significantly after the application of the marketed cream Fintop®, as seen in Figure 6B II. In contrast, the application of the gel enriched with SLN resulted in a substantial improvement in the thickness of the stratum corneum, nearly 3-fold when compared with the marketed

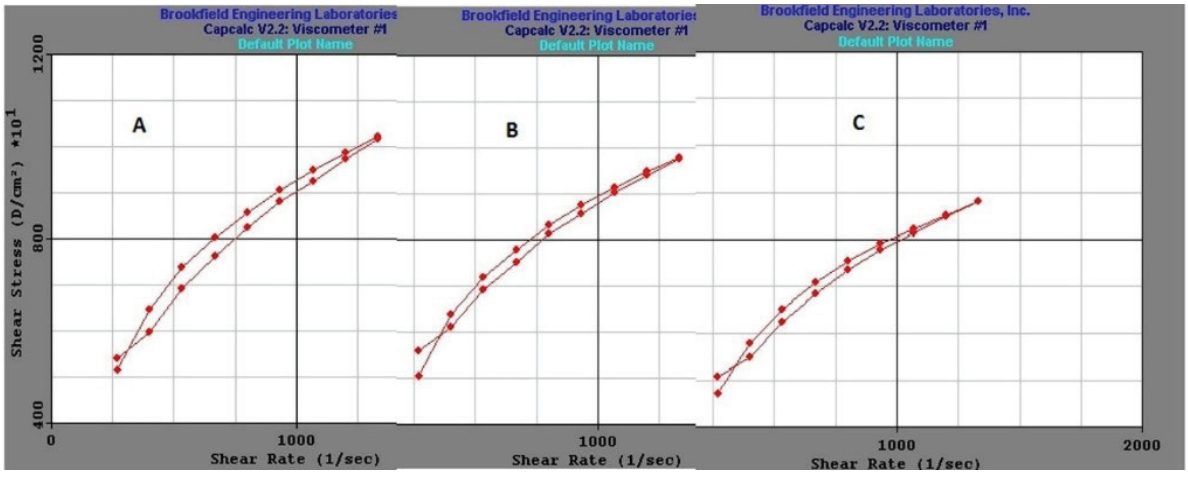


Figure 5. Effect of temperature on the viscosity of BUTE-SLN-enriched gel at A) 5°C, B) 25°C, and C) 40°C

BUTE-SLN: Butenafine hydrochloride-solid lipid nanoparticles

cream and 3.5-fold when compared with the untreated skin (Figure 6B).

In vitro drug diffusion study

BUTE-SLN was evaluated against Fintop® cream in an *in vitro drug release* study.The release pattern of butenafine from SLN was initially faster and was sustained after 2 hours. Sustained drug release from gels is further useful for maintaining drug release depots[27]. The marketed cream exhibited a more controlled release than the SLN gel (Figure 7A).

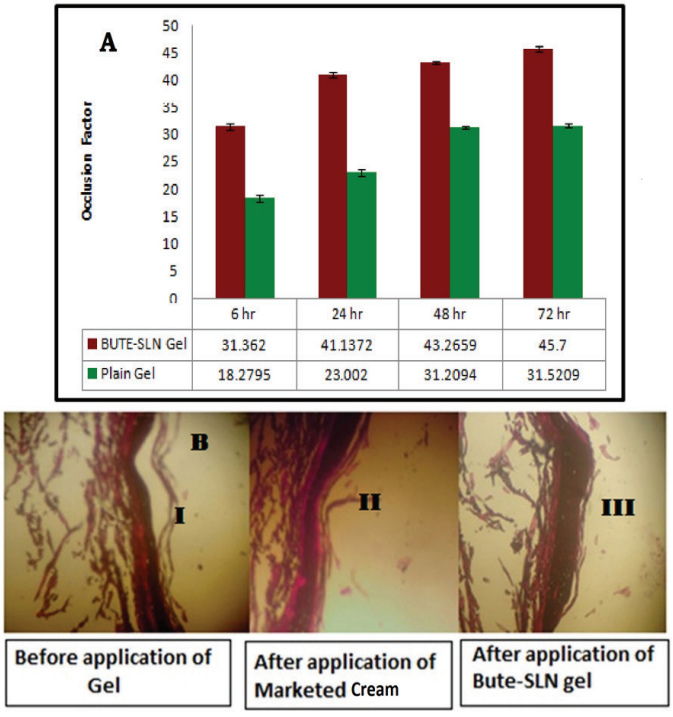


Figure 6. Comparison of the occlusion factor of BUTE-SLN-enriched gel and plain gel, B) Photomicrograph showing the skin hydrating potential of BUTE-SLN gel compared with a marketed cream and untreated human cadaver skin

BUTE-SLN: Butenafine hydrochloride-solid lipid nanoparticles

Drug permeation

The ability of BUTE-SLN to permeate pig ear skin was studied. The plot of the cumulative amount of drug permeated showed a 5.42-fold higher drug release for the BUTE-SLN gel compared with the reference cream. The flux value calculated from the linear portion of graph Q/A vs. time (Figure 7B) from SLN gel was found to be 1666.7 ± 0.198 ng $cm^{-2}h^{-1}$ and 818.181 ± 0.392 ng $cm^{-2}h^{-1}$ for reference cream.[27] Results of quantification of butenafine after 24 hours were expressed in percentage of the undiffused, deposited, and permeated drug through the skin, as depicted in Figure 8.

In vivo skin retention studies

The drug content in various epidermal layers was quantitatively estimated according to procedures or methods described in the literature. Butenafine was extracted into methanol from the stratum corneum by the tape stripping method. The epidermis was separated using the heat-separation technique for drug content. Using the UV spectrophotometer as an analytical method, the amount of butenafine present in different skin strata was depicted in the percent of the skin, as shown in Figure 8.

Skin irritation study

In a rabbit skin irritation analysis, the formulation did not display any signs of skin irritation, such as redness or inflammation, at the application site (erythema). As a result, it can be assumed that all the tested formulations are safe for topical use.

In vitro antifungal activity

The antifungal activity of butenafine was measured *in vitro* against *C. albicans* NCIM 3471 with the goal of evaluating the effect of process parameters on the intensity (potency) of butenafine and studying its antifungal activity in terms of the inhibition zone. In this study, the extract of BUTE-SLN equivalent to 500 µg/mL was compared with a marketed cream dilution equivalent to 500 µg/mL and different standard drug dilutions. From the present investigation, the test sample with a strength equivalent to 500 µg/mL showed a greater zone of inhibition (32 mm) comparedwith the standard 500 µg/mL butenafine solution (30 mm), as shown in Figure 9.

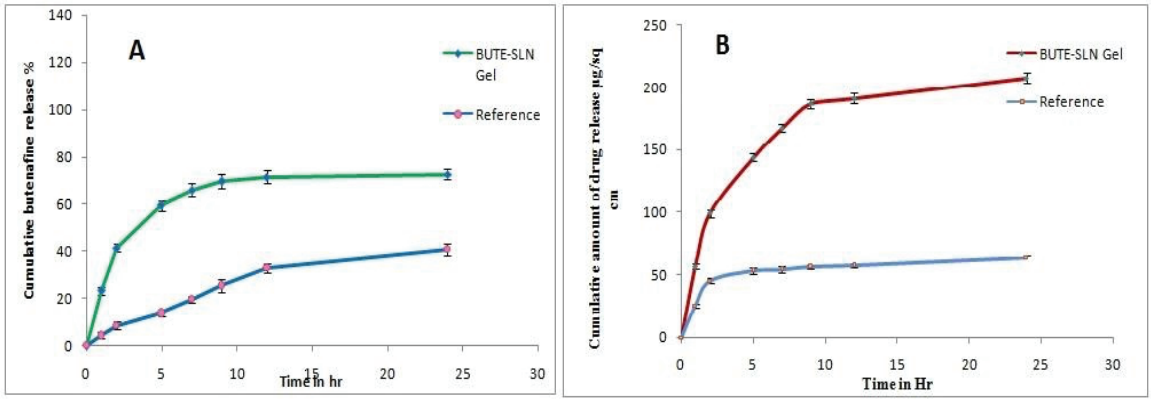


Figure 7. A) *In vitro* drug release profile of BUTE-SLN gel and its reference, B) *In vitro* skin permeation profile of BUTE from SLN gel and reference

BUTE-SLN: Butenafine hydrochloride-solid lipid nanoparticles

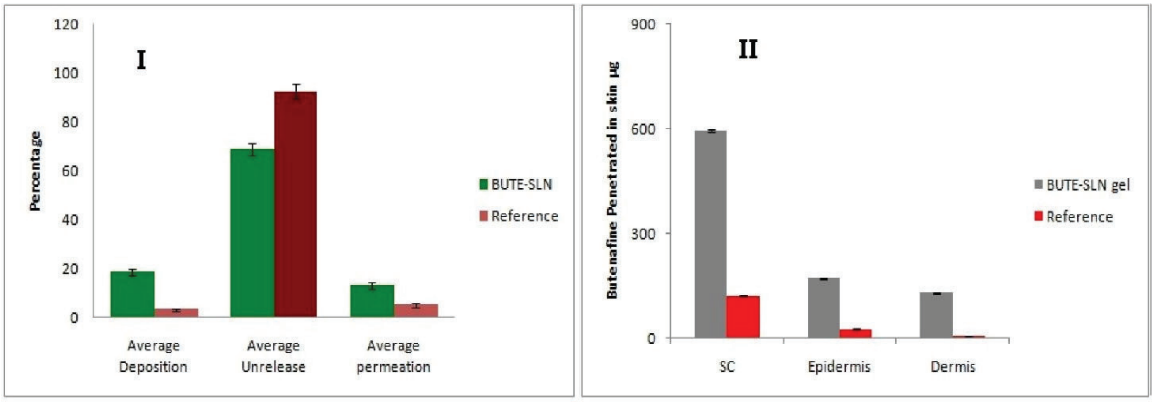


Figure 8. I) Percentage of drug permeated, deposited, and remaining unabsorbed in the skin, II) *In vivo* drug retention in different skin strata
BUTE-SLN: Butenafine hydrochloride-solid lipid nanoparticles

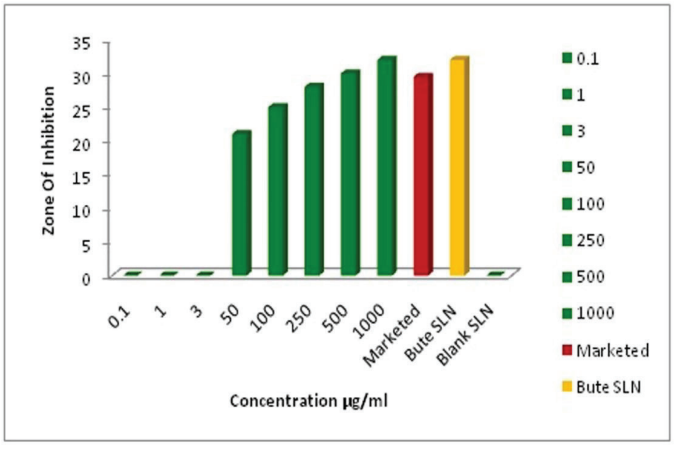


Figure 9. Comparative antifungal activity of different butenafine dilutions with marketed and BUTE-SLN formulation
BUTE-SLN: Butenafine hydrochloride-solid lipid nanoparticles

DISCUSSION

The objective of this study was to develop and evaluate antifungal drug BUTE-SLNs for treating topical fungal infections. The novelty of this work is the use of novel excipients such as natural surfactants and lipids, the use of organic solvents in the fabrication of SLN, and the very simple and novel modified solvent emulsification method of preparation.

The particle size model revealed that the concentrations of lipids, surfactants, and organic solvents and their interactions significantly affect the formation of nanometric particles. The response surface plot for the effect of factor X_1 and factor X_2 on particle size (Y_1) of BUTE-SLN is shown in Figure 1A. Changes in lipid concentration, in particular, resulted in increased particle size, whereas increases in surfactant concentration had negligible effects on particle size. The increase in particle size is attributed to an increase in lipid concentration, which allows the primary emulsion's globule size to increase. The smaller droplets enter the emulsion and redeposit onto the surface of nanoparticles to reach a more thermodynamically stable state. An insufficient surfactant concentration could not cover the formed new interfaces, resulting in an increase in particle size. The involvement of organic solvents in the BUTE-SLN formulation was a possible explanation for the increase in surfactant concentration, which contributed to a small increase in particle size. The surfactant present in the SLN formulation is in equilibrium with the organic solvent; hence, the surfactant covers the SLN at the interface and reduces the interfacial tension so that nano-lipid particles are formed, but the additional rise in surfactant concentration goes into organic solvent; hence, the rise in surfactant slightly increases the particle size.[28]

The regression equation no.4, generated for the PDI was significant, with an *f* value of 240.33 ($p < 0.0001$). The PDI model revealed that the percent concentration of lipids and organic solvents and their interactions have a significant impact on the PDI. The response surface plots (Figure 1B) illustrate that the PDI increases as organic solvent and lipid concentration (%) increase. An increase in organic solvent concentration causes non-uniform precipitation of SLN (heterogeneous SLN dispersion), leading to the deposition of smaller particles on larger particles as per the phenomenon of Ostwald ripening. This resulted in variable particles with bi-or tri-modal size distributions; hence, PDI increased.

The regression equation no.5, generated for the ZP was significant, with an *f* value of 87.80 ($p < 0.0001$). The model generated for ZP revealed that only the lipid concentration had a significant impact on ZP. The response surface plot in Figure 1C shows that increasing the lipid concentration lowers the ZP while increasing the surfactant concentration has less of an impact on particle charge. The charge on the nanolipid dispersion is the most important factor for the stability of the formulation of any colloidal system. Generally, particles with a ZP of ± 30 mV could be considered stable due to the electric repulsion between the particles.[11] The individual ZPs of the BUTE-SLN ingredients were -16.9 mV for butenafine, -4.81 mV for surfactant, and 20.5 for lipid Olivem 1000. From the given data, we can predict that lipids have a greater influence on ZP. The surfactant reduces the negative charge on the ZP of

particles. The surface-covered butenafine, surfactant, TPGS, and organic solvent collectively induce negative charges.

The regression equations no. 6 and 7 generated for EE and DL were significant with *f* values of 848.75 and 12.22, respectively ($p < 0.0001$). Figure 1D and Figure 1E demonstrate the response surface plots of the effect of surfactant and lipid concentrations on EE and the effect of organic solvent and surfactant concentrations on DL, respectively. The increase in the concentration of lipid showeda remarkable increase in EE as the solubility profile of the drug showed higher solubility in the selected lipid. Due to drug distribution into aqueous and lipid phases, an increase in surfactant concentration resulted in a slight increase in EE. An increase in the surfactant concentration causes the formation of micelles, which have the property to dissolve lipophilic drugs, hence reducing the solubility in the lipid phase.[29] Cosurfactant also helps to dissolve drugs into SLN as it tends to support the emulsifying property of surfactant and additionally improves drug solubilization.

Organic solvent and surfactant concentrations positively influenced DL. The organic solvent used here dissolves more drugs, and at the time of cooling of the SLN dispersion, the lipid core encapsulates DMSO between the imperfect crystal lattice structures of the lipid. Solidified SLN exhibited a nanostructured lipid carrier-like structure composed of solid lipids inamorphous form with various lattice defects in which the drug dissolved in organic solvent is presented as pockets. Along with independent variables, some practical experimentation work has been done forthe fabrication of modified SLN, such as sudden cooling of hot SLN dispersion leading to sudden precipitation of lipid and drug, which forms lipid in α form containing maximum amount of drug andorganic solvent.

The nanometric size of BUTE-SLN was determined by TEM analysis of the particle structure and surface morphology. Figure 2 illustrates that the BUTE-SLN formulated appeared to be spherical in shape with a narrow particle size distribution, which is in goodagreement with PCS and PDI analysis.

DSC is a powerful investigational tool used to determine the physical properties of compounds, such as the crystalline or amorphous nature of samples, which can be drawn on the basis of the fact that different lipid modifications exhibit different melting points and physicochemical properties. Figure 3A shows the thermal analysis results of pure butenafine, Lipid Olivem 1000 and BUTE-SLN. A sharp endothermic peak at the melting point was observed on a DSC thermogram of pure drug at 217.35 °C. The thermogram of pure lipid Olivem 1000 showed a sharp endothermic peak at 63.20 °C indicating lipid is present in pure crystalline form. Further small peaks are attributed to the decomposition of the formed liquid droplets. The curve for BUTE-SLN shows a small but broad peak at 46.91 °C. The result shows that crystalline lipids are converted into amorphous states as heat flow through larger and perfectly ordered crystals requires greater energy, resulting in large and sharp peaks, while heat flow through small and less ordered particles requires less energy to melt the particle. Our solid lipid nanoparticle formulation showed small and broad peaks with a difference of 16.29 °C with pure lipid, indicating the presence of nanostructure in amorphous form along with surfactant and pockets of organic solvent inside it.[30,31]

From the XRD data, we can clearly observe sharp diffraction peaks of butenafine at a 2 θ value of 16.0768 with a *d* value of 5.4996 and pure lipid 2 θ at 21.664 with a *d* value of 4.1124, which indicates a high crystalline nature of the compound, as shown in Figure 3B. Whereas BUTE-SLN formulation showed a broader and shorter peak with no specific diffraction peak for butenafine, revealing encapsulation of the drug within the lipid matrix, which existed in amorphous form.[32] The comparative FT-IR spectra of butenafine, pure lipid Olivem 1000 and BUTE-SLN shown in Figure 4. The FTIR spectra for butenafine gave a typical specific absorption character at 3047.63, 1365.65 and 1072.46 cm^{-1} corresponding to -C-H stretch, -C-C and -C-N vibrations, respectively, but the BUTE-SLN spectra showed the absence of these peaks. The FTIR results revealed that there was no strong interaction and no incompatibility, and butenafine was successfully encapsulated into the lipid structure.

For effective topical delivery, some constraints should be considered, including the physicochemical parameters of the drug, such as log *p* and *pKa* values, rheological study of gel-enriched SLN, occlusive and hydrating potential of formulation, and disease state. Natural *aloe vera* gel with many additive effects, such as excellent anti-inflammatory activity, natural healing ability, and moisturizing effect, is beneficial for skin conditions. *Aloe vera* gel contains the building blocks of glucomannan, C-glucosyl chromone, lupeol and 8 enzymes, *Aloe vera* gel is useful and comfortable for treating exaggerated skin conditions.[33] In the evaluation of semisolid formulation viscosity and spreadability parameters, we gave more attention because the ultimate formulation was going to be used on inflamed skin. BUTE-SLN *aloe vera* gel showed pseudoplastic rheological flow, which is expected for skin conditions. Small nanosized particles showed excellent slip properties required for gel spreading onto inflamed skin. The entrapment of SLN into the gel network slightly increased the rheology of the gel due to an increase in amphiphilic surfaces upon which water could bind and become immobile.

The process of SLN permeation starts with occlusion and skin hydration. Because SLN is composed of solid lipids and the stratum corneum also contains intercellular lipids such as cholesterol, phospholipids, and ceramide, SLN has a strong affinity toward the stratum corneum. Hence, they form an impermeable occlusive layer on the skin surface, preventing transepidermal water loss. Due to water pore dynamics, pores that exist in uniform spreading of the SLN gel attract permeated vapor, which condenses at the pores of the SLN. Hence, due to the negative pressure, they attract water rather than lose it. This particular mechanism is very useful in the treatment of superficial fungal infections, which cause scaling, dry skin, and irritation.[34,35] The stratum corneum comprises compactly packed corneocyte and intercellular lipids. Occlusive film formation and reduction in trans epidermal water loss result in hydration of the stratum corneum, which reduces the compact packing between

corneocyte and increases the pore size. This is the possible mechanism for the transport of SLN through the skin.

Drug release and permeation showed a biphasic release pattern of drugs from the SLN matrix. This can be due to the use of novel excipients such as surfactants such as lipid Olivem 1000, DMSO, and TPGS, which entrap the drug, forming nanosized mini pockets in SLNs. The process of fabrication provokedthe α state lipid transformation; hence, it could include more excipients. The formation of α state lipid structure slowly convert into β' form and then most stable β form then slowly expel the drug from the lipid matrix, which accounts for sustained release.

The possible mechanism for the diffusion of drug from the lipid matrix can start with possible events like distribution of drug between lipid and DMSO, lipid Olivem 1000, and TPGS, diffusion of drug through lipid, transformation of lipid from α state to most stable β form, distribution of drug between lipid and dissolution medium, and release of drug to dissolution medium. The incorporation of TPGS facilitates burst release from SLN matrix. This can occur at the time of sudden cooling of the dispersion. The saturated emulsion when cooled suddenly leads to solidification of the drug and lipids and forms the drug lipid matrix, whereas the presence of TPGS and DMSO partially solubilizes the drug in the aqueous phase from which it precipitates at the time of cooling. This forms a uniform coat around the SLN particles when applied to skin, resulting in initial burst release. The efficacy of the formulation also depends on the skin condition, such as the diseased state, type of infection, inflammation, edema, and dry skin. All these factors pose the problem of permeation of drugs intothe superficial layer of skin. In the disease state, the superficial layer of the skin is disturbed, exposing the epidermal cell along with the existence of various metabolites of fungal cells, enzymes secreted by fungal cells and the body's immune system (cytokines) degenerate the drug into inactive fragments. Marketed cream showed reduced diffusion of the drug into the skin layer compared to SLN, which forms a depotby concentrating in the SC and releasing the drug slowly. Skin retention studies revealed that BUTE-SLN dominantly concentrates in the stratum corneum, which forms a reservoir for further drug release,and natural surfactant Olivem 300 aids in the partitioning of drugs into epidermal cells.[35]

The skin irritation study revealed the non-irritant nature of BUTE-SLN gel, which explains its usefulness in treating fungal infections. An in vitro antifungal analysis was conducted to examine the comparative efficacy of butenafine with marketed cream and to establish the effects of different method parameters on the formulation of BUTE-SLN. The zone of inhibition of the marketed preparation was found to be 30 mm and that of BUTE-SLN was found to be 32 mm, which is greater than that of the marketed formulation proving the therapeutic efficacy of the SLN formulation.

CONCLUSION

Various formulation ingredients like lipid, surfactant and DMSO have an influence on the fabrication of SLN dispersions, which directly affects the particle size, EE, and ZP of SLN. The DSC and XRD studies indicated that the optimized formulation was amorphous in nature and contained the drug, DMSO, and surfactant entrapped inside the SLN. Rheological and spreadability study of BUTE-SLN *aloe vera* gel revealed excellent slip properties beneficial for inflamed skin application. Along with its excellent rheological and spreadability, the BUTE-SLN gel possesses good occlusive properties and hydrating potential. For long-term drug delivery through the stratum corneum, BUTE-SLN showed accumulation of the SLN in the stratum corneum with controlled drug release. BUTE -SLN demonstrated increased skin deposition, improved *in vitro* antifungal action, and a topical formulation that was less irritating. As a result, the BUTE-SLN *aloe vera* gel formulation is a cost-effective, novel, and reproducible alternative to the currently available traditional dosage method.

Acknowledgments

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Ethics

Ethics Committee Approval: The protocol for the study was approved by the Pravara Rural College of Pharmacy (approval number: 2013714-543, date: 07.01.2014).

Informed Consent: Not required.

Authorship Contributions

Concept: A.B., V.K., S.A., Design: A.B., S.A., Data Collection or Processing: A.B., S.A., D.B., M.A., Analysis or Interpretation: A.B., V.K., S.A., D.B., M.A., Literature Search: A.B., S.A., D.B., M.A., Writing: S.A., D.B., M.A.

Conflict of Interest: No conflict of interest was declared by the authors.

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