RESEARCH ARTICLE

Utilization of Box Behnken Design for the Development and Evaluation of Luliconazole-loaded Nanoemulgel

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ABSTRACT

Pharmaceutical research is always looking for novel ways to distribute drugs and developing these methods to increase the effectiveness of existing medications. The physicochemical characteristics of the drug determine the dose form. Lipophilic API development is a result of recent discoveries in high-throughput screening. A topical broad-spectrum antifungal agent is luliconazole. Due to its lower water solubility, topical administration is limited, and topical absorption is confined. The stratum corneum's lipid phase's solubility of the medication also serves as a rate-limiting step for penetration. Because fungal infections impact the skin's epidermis, dermis, and deeper layers, medicine administration must be tailored to target high drug concentrations at the layers of the epidermis and dermis.

Keywords: Antifungal agent, Drug delivery systems, Lower water solubility, Luliconazole.

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INTRODUCTION

Lipophilic API development is a result of recent discoveries in high-throughput screening. The low bioavailability of several recently authorized medications is preventing them from moving forward in the development process. Despite developments in DDS and numerous techniques utilized to improve therapeutic results.[1-3] Given this complex scenario, a multidisciplinary approach is required to solve it. Therefore, it is crucial to enhance biopharmaceutical performance, particularly their aqueous solubility and permeability. To increase the solubility of poorly soluble pharmaceuticals, a variety of techniques including physical and chemical modification and innovative formulation approaches, have been used. A topical broad-spectrum antifungal agent is luliconazole (LLC).[4-6] Due to LLC's lower water solubility, topical administration is limited, and topical absorption is confined. The stratum corneum's lipid phase's solubility of the medication also serves as a rate-limiting step for penetration.[7] Fungal infections affect the deeper layers of the skin, necessitating the customization of medication administration to target high drug concentrations at the epidermis and dermis layers. However, the 1% w/v cream LUZU® used in commercial topical LLC formulations is linked to poorer skin permeability and reduced drug retention in the skin.[8] To make medications that are poorly soluble in water more soluble, numerous formulation strategies have been developed, including amorphous formulation, crystal engineering, nanocarrier systems, micronization, and different lipid formulations. One of the expanding technological fields that has seen increased use in a variety of fields, particularly in the food, biopharmaceutical, and cosmetic industries, is nanotechnology.[9,10] Better delivery of active substances because of their outstanding qualities. Because of their small droplet size, large interfacial area, and excellent solubility, products utilizing nanotechnology have potential for use in the market. A nanoemulsion is one of the more recently created nanocarriers that have attracted a lot of interest due to several benefits, most notably their small particle size, which would offer improved absorption and, as a result, improve the bioavailability of the poorly soluble drug. Additionally, it might increase solubility and allow regulated drug release with improved stability.[11] NE is a single-phase system composed of, on occasion, a co-surfactant, an aqueous phase and oily phase, and a surfactant. Thermodynamic stability is present. NE shows more stability as compared to conventional emulsions in terms of flocculation, phase separation, sedimentation, and creaming.[12,13] It is a potentially effective drug delivery method that might be used for parenteral, oral, and topical modes of administration. However, it is preferable to combine the created nanoemulsion with a hydrogel basis to create a unique dosage form known as NE for topical drug delivery.[14,11]

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MATERIALS AND METHODS

Materials

Luliconazole was supplied as a gift specimen by Glenmark Pharmaceuticals, Mumbai. Castor oil and triethylamine were obtained from Analab Fine Chemicals, Mumbai. Tween 80 was acquired from Fine Chemicals Analab, ethanol Analab Fine Chemicals, all reagents are of lab grade and distilled water was produced from the laboratory. The analysis employed chemicals and reagents of analytical grade.

Selection of Oil, Surfactants, and Co-surfactants

The drug-saturated solubility of different oils, including castor oil, oleic acid, olive oils, surfactant tween 20, Cremophor RH40, tween 80 and co-surfactant propylene glycol and ethanol, was confirmed. An adequate quantity of luliconazole was introduced into 5 mL of , co-surfactant, surfactant and oil. It agitated at room temperature for 48 hours using a magnetic stirrer at 500 rpm. After passing the solutions using the Whatman filter paper, the solvent content was measured in milligrams per ml utilizing a UV spectrophotometer.[15-17]

Building a Pseudo-ternary Phase Diagram

The development of a pseudo-ternary phase diagram using the water titration method was used to represent the nanoemulsion zone and provide component concentration ratios. S/Co-S and oil were combined in multiple glass tubes at weight ratios of 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, and 9:1. The aqueous phase was then injected dropwise into each tube (at 26 ± 2°C), mixed for 3 to 5 minutes in a vortex, and then left to equilibrate after 30 minutes of magnetic stirring. Following the formation of equilibrium, the mixtures were defined in terms of phase clarity. A nanoemulsion was observed with a clear emulsion and good flowability. Ternary phase.com was used to construct the ternary diagram.[18-21]

Preparation of Nanoemulsion

From the ternary phase diagram, suitable weight ratios (low, middle, and high) for the oil and S/Co-S were chosen, and formulas were proposed based on the NE region in the diagram. The lower oil ratio than water led to the formation of O/W nanoemulsion at the weight ratios. The clarity was observed in these NE systems.[22-24]

Preparation of Luliconazole Loaded Nanoemulsion and Nanoemugel

Luliconazole was added to the oil and stirred for 30 minutes. This mixture was further mixed for 30 minutes with a vortex mixer by adding S/Co-S. To obtain a homogeneous nanoemulsion, a specified weight of water was added drop by drop. Carbopol 940 was used to gel the luliconazole nanoemulsion formulations.[25,26] To make the gel base, Carbopol 940 was dissolved in purified water and continuously agitated to achieve a homogeneous base of 1, 2, and 3%. Triethanolamine was then used to balance the pH. The optimized NE preparations were mixed individually with 2% gel base concentration in a homogenizer while being stirred at 1000 rpm for up to 4 hours, resulting in a smooth and viscous nano emulgel.[27]

Box-Behnken Design of Luliconazole Loaded Nanoemulgel

Considering the data from the pseudo-ternary phase diagram and screening studies, three independent variables, including the concentration of S_{mix} (tween 80: ethanol) (X1), castor oil (X2) and water (X3) on particle size, were studied for preparing LLC-NE. Box-Behnken design as response surface method was used for the luliconazole loaded nanoemulgel optimization process (Design-Expert® V13, Stat-Ease Inc., Minneapolis).[28] Three independent variables include the effect of S_{mix} (tween 80: Ethanol) (X1), castor oil (X2), and water (X3) concentration with particle size as dependent variables.[29-31] All NEs were formulated and kept overnight. Developed NE was mixed with polymeric solution in the 1:1 parts. Gradually stirring the mixture produced homogenous LLC-NE4G. LLC-NEG formulations are presented in Table 1.

$$y = \beta0 + \beta1X1 + \beta2X2 + \beta3X3 + \beta12X1X2 + \beta13X1X3 + \beta23X2X3 + \beta11X1X1 + \beta22X2X2 + \beta33X3X3 \quad (1)$$

Characterization of Nanoemulgel Formulations

Particle size

A particle size analyser (HORIBA SZ-100 for Windows [Z] Ver2.40) (Montaseri et al., 2017) was used to determine the particLe size of developed LLC -NEG.[32,33]

Zeta potential

The zeta potential of LLC -NEG was measured using Zeta Check (HORIBA SZ-100 for Windows [Z] Ver2.40) (Montaseri *et al.*, 2017) at a temperature of 25°C.[34,35]

pH determination

A pH of 1% w/w LLC-NEG was measured using a pH meter (Lab India pH CON centre, PICO+).[35,36]

Table 1: Box-Behnken design in terms of actual factors

Formulation	S_{mix} *(X1)*	*Castor oil (X2)*	*Water (X3)*	*Partial size (Y)*
R1	40	55	18	750
R2	20	49	6	2014
R3	60	55	12	821
R4	60	43	12	863
R5	40	43	6	260
R6	20	49	18	2435
R7	20	55	12	3016
R8	60	49	6	260
R9	20	43	12	4201
R10	40	49	12	286
R11	60	49	18	240
R12	40	55	6	724
R13	40	43	18	445

The response surfaces of the obtained results were also plotted.

Drug content

A predetermined volume of LLC-NEG was diluted using deionized water, combined with acetonitrile, then centrifuged for fifteen minutes at 10,000 rpm. After that, the validated analysis method was used to examine the composition of the supernatant.[37] The final optimized batch was selected further to prepare a gel using Carbopol 940. Different concentrations of Carbopol 940 gels were prepared and finally 1% of the gel was selected further for the preparation of LLC loaded nanoemulsion gels (LLC - NEG).[38-40]

Viscosity

The viscosity of LLC -NEG formulae was determined by Brookfield viscometer RV using spindle 6 with a spindle speed of 20 rpm. Each test was conducted three times, and the mean ± SD of the results was given.[40]

Texture analysis

TecturePro CT V1.7 Build 29 was used by Brookfield Engineering Labs for texture analysis (11). The texture profile is based on the product's compression and/or stretching behavior. Monitoring the force during compression provides insight into the product's uniformity. The result will be more consistent if the force required to produce the prescribed movement is greater. Monitoring the force during stretching provides information on the product's adhesion quality, how it stretches (stringy effect or elastic return), and how much the product remains on the probe. All these variables indicate how easy it is to take up.[41,28]

In-vitro drug release

Drug release studies employed a modified Franz diffusion cell with a dialysis membrane separating donor and receptor compartments. The formulation was applied to the membrane, and tests were directed in the buffer of pH 7.4 at 37°C using a thermostatic jacket. These simulated *in-vitro* drug release conditions are crucial for pharmaceutical research. The assembly, placed on a magnetic stirrer, ensured continuous stirring of the drug release system by means of a magnetic blob. A corresponding control firm was concurrently run. Trials (5 mL) were reserved at intervals, substituted with new solution, and analyzed at 271 nm using spectrophotometry. The cumulative %drug release was calculated for evaluation.[41]

Skin irritation study

The acute cutaneous toxicity of a NEG was assessed on female Wistar rats weighing around 250 g following OECD 402 criteria. The rats had 10% of their body surface area removed from the dorsal region. Subsequently, three sequential doses of the test substance (200, 1000, and 2000 mg/kg) were topically administered to the shaved area using a micropipette. The treated region was subsequently covered with a gauze bandage, and the rats were then monitored daily for 14 days while also being periodically observed every 30 minutes, every 2 hours, and every 6 hours. On days 1, 7, and 14, the rats' body weights were noted.[44,45] To ascertain any potential topical toxicity, a section rat skin was subjected to several treatments, including formalin-treated (positive control), control group, and LLC-NE5G skin samples were obtained by excision after the animals had been executed using a ketamine/xylazine overdose. Utilising a microtome, samples were prepared and sectioned. Additionally, the sectioned samples were stained with eosin and hematoxylin. The samples' coloration made the cross-sectioned samples easier to see under a microscope. A Leica optical microscope was used to adequately see and photograph each slide at a 400X magnification.

RESULTS

Preparation of Pseudo-ternary Phase Diagram, LLC Nanoemulsion and Nanoemulgel

The LLC-NEG was created using a homogenizer, with castor oil as the oil phase, Tween 80 as the surfactant, and ethanol as the co-surfactant, in varying percentages, as displayed in Table 2. A pseudo-ternary phase diagram was used to pinpoint the nanoemulsion (NE) zone without luliconazole, selecting a S_{mix} ratio of 1:2, as shown by the shaded area in Figure 1. Various oil, Smix, and water concentrations were tested to maximize the number of NE formulations. Increasing the oil concentration significantly altered the particle size, which could rise with further oil increases. Optimal surfactant/co-surfactant mixtures and water amounts were determined for each NE formula based on the oil percentage. Luliconazole was incorporated at a 1% dosage into the selected oil for each formula.[21]

Luliconazole-loaded NEG Characterization

Zeta potential

LLC-NE7G formulations showed zeta potential of the batches as listed in Table 1. The observed range was between -0.1 to -73 mv. The selected LLC-NEG R 10 had a zeta potential of -68 mv.[7]

pH and drug content

The range obtained for the LLC-NEG formulations was 5.8 ± 0.08 to 6.0 ± 0.00. The selected LLC-NEG R 10 had a pH of -5.8 ± 0.09. Drug content ranged from 79.02 ± 0.38 to 95.01 ± 0.33%.

Table 2: Composition of castor oil, S_{mix} and water for preparation of pseudo ternary phase diagram

Formulation	*Castor oil (mL)*	*S_{mix} (mL)*	*Water (mL)*
A1	1	9	1
A2	2	8	1
A3	3	7	0.5
A4	4	6	0.4
A5	5	5	0.5
A6	6	4	0.5
A7	7	3	0.7
A8	8	2	1
A9	9	1	0.8

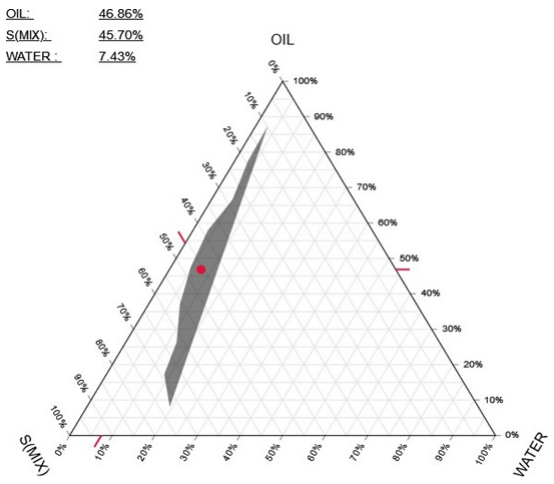

Figure 1: Pseudo ternary phase diagram depicting the nanoemulsion region

Optimization of LLC-loaded NEG

NEs optimization was done by Box-Behnken model with Design-Expert® software (Version 13.1, Stat-Ease Inc., Minneapolis, MN). Three independent variables including the effect of Smix (tween 80: Ethanol) (X1), castor oil (X2), and water (X3) concentration with particle size as dependent variables. Based on the outcomes of 13 experiments recommended by the software (Table 1), the model exhibited a quadratic nature, indicating a noteworthy correlation between variables. The absence of fit originated to be non-significant (0.0768 and 0.569), affirming the adequacy of the model (see Table 2) 1, 21, 28. The 3D-model graphs depicted in Figure 2 further illustrate the significant relationship between variables. These findings, coupled with the quadratic model, suggest the existence of an optimum surface for achieving the desired particle size in NE formulations.

The regression equation is as follows-

$$Y = 286 - 1185.25X1 - 57.25\ X2 + 76.50\ X3 + 76.506 + 285.75\ X1X2 - 118.25\ X1X3 + 1315.58\ X1X1 + 632.38\ X2X2 - 364.62X3X3;\ R2 = 0.9572 \quad (2)$$

As for each equation (2) and Figure 2, the standards of numbers specify an effective fit. Results in the equation with the predominant negative sign X1 showed that during the nanoemulsion preparation led to an increase in the particle dimensions, whereas the increase in the increase in the concentration of the Smix. X12 showed a positive impact on the preparation of NE. Charge on the particles. A similar negative impact was obtained for the X22 individual factor and when increased, the opposite mechanism was observed. Individual interaction X1X2 showed a positive sign. Both S_{mix} and castor oil might have impacted positively on the decrease in particle size and the same can be explained by the hydrophilicity property of the Smix. An increase in the Smix concentration might result in unwanted particle aggregation, leading to coarse particle size. The increased concentration of X1 might have helped in decreasing the particle size of the nanoemulsion. Surfactants have a vital character in promoting the development of nanoemulsions (NEs). Consequently, elevating surfactant concentration to an optimal level may reduce the particle size of NEs, preventing undesirable particle aggregation. Conversely, exceeding this optimum level could lead to the induction of micelle formation by surfactants, with the potential prediction of increased aggregation.[11,14,27,36]

Texture analysis

As shown in Figure 3, the formulation demonstrated a pseudoplastic flow, a shear thinning system. A non-linear graph was obtained, the viscosity decreases with increasing shear. In this system, at rest or low shear, the long-chain molecules are disarranged and matted together. At high shear stress, these structures align themselves and thus become less viscous and hence less resistance to the flow.[14,41]

Skin irritation study

The presence of irritation signs can limit the applicability of the preparation. Therefore, the preparation need to be devoid of any irritating effects. To assess skin irritation, the luliconazole-loaded nanoemulgel was evaluated following the OECD 402 criteria. The luliconazole-loaded nanoemulgel formulations, both fabricated and commercial, exhibited no indications of irritation (such as erythema and edema) even after being exposed for 48 hours. This implies that

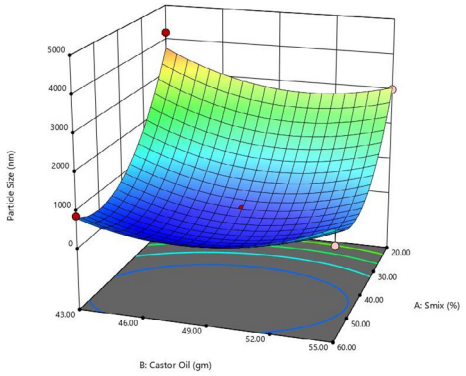

Figure 2: 3-D plot display the relative outcome of the independent variables on LLC-NE

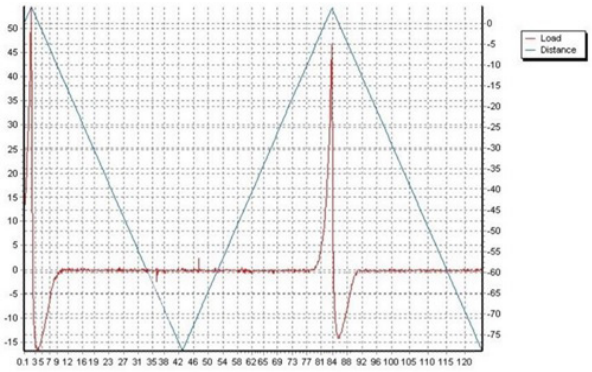

Figure 3: The association between the shear rate and the functional stress on LLC-NEG

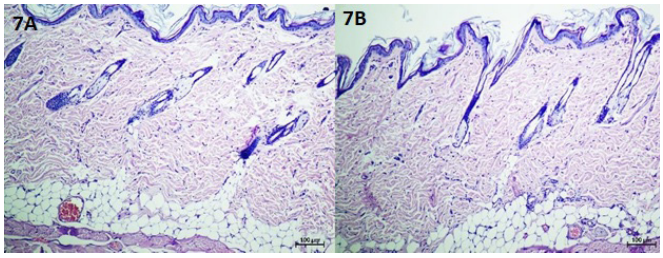

Figure 4: Histopathological studies of (A) normal dermis layer (B) Luliconazole-loaded nanoemulgel-treated group of the dermis layer

the experimental animals' skin responded to the formalin treatment by becoming red and itchy.[19,23] Conversely, groups I and III animals displayed no indications of erythema. Hence, it can be inferred that both the polymeric blank nanoemulgel (NEG) and the luliconazole-loaded nanoemulgel demonstrated an absence of inflammation or irritation, indicating a secure way to apply the product to treat fungal infections.[3,11] The Institutional Animal Ethics Committee approved the protocol with reference number SOPMITWPU/IAEC/2021-22/M2/04 for above mentioned studies.

Histopathological Study

To ensure the safety of the enhanced LLC-NEG formulation, histopathological tests were performed. Rat skin was exposed to a formalin solution as a positive control, and the effects of both luliconazole-infused and blank nanoemulgel were compared to this benchmark. The control rat skin sections, stained with H&E, showed a normal keratin layer consisting of three to four layers of keratinized stratified squamous cells. The epidermis displayed its typical four-layer structure: Stratum corneum, stratum granulosum, stratum spinosum, and stratum basale, arranged from the outermost to the innermost layer. The junction of the epidermis and dermis featured various epidermal ridges and dermal papillae.[39] The dermis was observed to contain the reticular layer, characterized by thick, dense, irregular connective tissue, and the papillary layer, a thin layer just beneath the epidermis. Within the dermis, sweat glands and sebaceous hair follicles were visible (referenced in Figure 4(a)). The formalin-treated group showed pathological changes such as a thickened and deteriorated epidermis, intercellular edema, and infiltration of inflammatory cells (as seen in Figure 4(b)). However, the rat skin tissue treated with the luliconazole-containing nanoemulgel showed no unusual alterations when compared to the control group (also in Figure 4(b)).[17]

SUMMARY AND CONCLUSION

The main goal of the research was to formulate a luliconazole-based topical emulgel for treating patients with *Candida albicans, Malassezia* spp., and *Aspergillus* infections. The study concentrated on luliconazole, which is classified under Biopharmaceutical Classification System (BCS) class II, indicating that it has low solubility but high permeability characteristics. The investigation aimed to explore the potential of luliconazole in formulations, given its classification, which often presents challenges in drug delivery due to its limited solubility in aqueous media despite its ability to permeate biological membranes efficiently. The study involved formulating luliconazole emulgel using varying concentrations of methanol, propylene glycol, liquid paraffin, span, and tween to achieve an optimal formulation. Among all the preparations, F1-F4 demonstrated suitability, with formulation F3 proving to be the most stable. Storage at room temperature (25–35°C) for one month revealed no phase separation or bacterial growth in any of the formulations. %Drug release studies indicated that formulation F3 exhibited the highest drug release within five hours, making it the optimal formulation. The findings suggest that luliconazole emulgel could be a more effective and efficient system for topical fungal treatment compared to commercially available traditional luliconazole systems.

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