RESEARCH PAPER

Protransferosome-based gel for transungual delivery of luliconazole

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ABSTRACT

This study focused on the development of innovative luliconazole-loaded proTransfersomes (ProT) designed to optimize transungual delivery through a synergistic combination of a lipid blend and a chemical permeation enhancer. Luliconazole, a potent imidazole antifungal typically used to treat skin infections such as tinea pedis and tinea corporis, was encapsulated within these vesicles to overcome the physiological barriers of the nail plate. The resulting formulation exhibited a vesicle diameter of 198.29 ± 6.3 nm, a narrow polydispersity index of 0.109 ± 0.009, and a high encapsulation efficiency of 96.08 ± 2.7%. Notably, the proTransfersomes demonstrated superior nail permeation and retention capabilities compared to conventional forms, while stability testing confirmed optimal shelf-life under refrigerated conditions. Overall, these findings suggest that proTransfersome-based topical gels represent a highly viable and effective alternative for the treatment of localized fungal infections.

Keywords: Antifungal, Luliconazole, Drug delivery, Nail, Transfersomes, Onychomycosis, Transungual

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1. Introduction

The nail is a hard protective layer for the distal phalanges of the fingers and toes, shielding them from potential injury. The nail plate is a structurally coarse layer that overlays a nail bed. The basal cells of the nail unit at the nail root undergo continuous mitotic division, resulting in the perpetual growth of the nail plate throughout its lifespan. This growth is accompanied by the differentiation and keratinization of newly formed cells, displacing older cells near the dorsal surface [1,2]. The nail serves as a resilient protective barrier for the distal phalanges of the fingers and toes, safeguarding them against physical trauma. Structurally, the coarse nail plate overlays the underlying nail bed, originating from the nail root where basal cells undergo continuous mitotic division. This constant cellular proliferation drives the perpetual growth of the nail plate, as newly formed cells differentiate and keratinize, subsequently displacing older cells toward the dorsal surface [3].

Onychomycosis remains one of the most formidable challenges in pharmaceutical development, primarily due to the complexities of achieving a complete clinical cure and the high frequency of relapse. As the most prevalent nail disorder, it accounts for approximately 50% of all nail-related ailments. While dermatophytes—particularly Trichophyton species—are the primary causative agents, recent clinical evidence highlights an increasing involvement of non-dermatophyte yeasts, such as Candida albicans, and molds like Fusarium species. The condition is clinically characterized by distinct physical changes, including the progressive thickening and discoloration of the infected nail plate. [4,5].

Historically, fungal nail infections were managed through surgical avulsion, a painful and traumatic procedure for the patient. While systemic antifungal treatments—including agents such as fluconazole, Itraconazole, Terbinafine, and Griseofulvin—offer a non-invasive alternative, they are often hindered by the necessity for long-term administration, potential drug interactions, and significant systemic side effects such as gastrointestinal distress and hepatotoxicity. Consequently, topical therapy has emerged as a compelling alternative that avoids these systemic risks; however, developing a successful commercial

product remains a challenge due to the formidable barriers to effective drug penetration through the nail plate. [6,7].

Transfersomes represent a specialized class of liposomes composed of phosphatidylcholine and an edge activator, resulting in a highly flexible and malleable membrane designed to optimize drug transport. Etymologically derived from the Latin transfere ("to carry across") and the Greek soma ("body"), these engineered vesicles function as artificial carriers that mimic the exocytotic behavior of natural cellular vesicles. This unique structural adaptability allows them to act as sophisticated, stress-responsive aggregates capable of navigating restrictive barriers for controlled and potentially targeted drug delivery. [8,9]. Protransfersomes represent a stable precursor to transfersomes, typically formulated as a liquid crystalline or granular state that hydrates in situ to form highly deformable vesicles. By incorporating a lipid matrix and an edge activator, these carriers can effectively bypass the rigid barriers of the skin and nail plate, significantly enhancing the penetration of both lipophilic and hydrophilic drugs. This "pro-vesicular" approach addresses the stability issues often associated with traditional liposomes, such as aggregation or leakage during storage. Ultimately, protransfersomes offer a sophisticated platform for sustained and targeted drug delivery, improving therapeutic efficacy while minimizing systemic side effects [10,11].

Hence, to develop an alternate dosage form for increasing the skin transportation of the drug, the LFN-loaded trans- fersomes were developed systematically employing Quality by Design (QbD) tool and characterized for its drug release and drug permeation study.

Hence, to develop an alternate dosage form for increasing the nail transportation of the drug, the LCZ-loaded protransfersomes were developed and characterized for its ex vivo permeation and retention study.

2. Materials and Methods

2.1 Materials

Luliconazole was provided as a complementary gift by Glenmark, India. Phospholipon 90G was generously supplied by Lipoid GmbH, Germany. Absolute ethanol and was purchased from Research Laboratory—Fine Chem Industries, Mumbai, India. Tween-80 was purchased from Merck, India. Triple-distilled water was obtained from Siddhi Labs, Nashik, India. Triethanolamine was sourced from Loba Chemie, Mumbai, India. Complete Fruend's adjuvant (CFA) was ordered from Sigma Aldrich Corporation (USA). All other reagents used were of analytical grade.

2.2 Method

2.2.1 Fabrication of luliconazole loaded protransferosome gel (ProT-LCZ-gel) as Nanostructured lipid carriers:

Protransfersomal gel (**ProT-LCZ-gel**) was prepared with slight modifications as reported by Perrett *et al.* [12]. To be precise, Phospholipon 90G, Luliconazole (2% w/w), Tween 80, and alcohol were accurately weighed and transferred into an amber-colored vial. The components were blended using a magnetic stirrer at 60-80 ºC within a sealed environment to prevent solvent evaporation. Subsequently, small quantity of phosphate buffer was introduced at the same temperature under continuous agitation, resulting in a low-viscosity translucent liquid. Upon cooling at room temperature overnight, this liquid transitioned into the final ProT-LCZ-Gel formulation.

ProT-LCZ-Gel containing Rhodamine 123 dye (Rh^{123}) was prepared by using the same procedure as mentioned above except adding Rh^{123} in place of LCZ.

2.2.2 Characterization of ProT-LCZ-gel

2.2.2.1. *Vesicle size analysis*

Size (Z-Average diameter) and the polydispersity index (PDI) of transfersomes derived from ProT-LCZ-Gel was analyzed by PCS using Zetasizer Nano ZS (Malvern Instruments Ltd., USA) at 25ºC at an angle of 173º. Each experiment was executed in triplicate. The PDI relates with the width of vesicle size distribution, as its small value (< 0.1) points towards homogeneity, while a high value (> 0.3) indicates high heterogeneity.

2.2.2.2. *Preparation of Sephadex G-50 mini-column*

Sephadex G-50 (1 g) was swelled in 10 mL of 0.9% NaCl solution for 5 h at RT with occasional shaking. The gel so formed was stored at RT.

Preparation of Minicolumn: Whatman filter paper pads were retained at the bottom of 1 mL syringe barrels, which were then packed with the gel. Excess saline solution was removed with centrifugation at 2000 rpm for 4 min.

2.2.2.3. ***Determination of encapsulation efficiency (%)***

The encapsulation efficiency (EE) was determined by separating the unencapsulated drug using a Sephadex G-50 mini-centrifuge column. Specifically, 100 mg of ProT-LCZ-Gel was hydrated with 10 mL of 0.9% w/v NaCl solution under manual agitation. A 200 µL aliquot of the resulting vesicular suspension was added dropwise to the Sephadex G-50 column and centrifuged at 4000 rpm for 4 minutes at 4±1°C. This elution process was repeated with additional saline until all transfersomes were recovered, with clearance confirmed via digital microscopy. To quantify the entrapped drug, 1.2 mL of the collected eluate was treated 50% n-propanol to rupture the vesicles, diluted with methanol, and analyzed using HPLC. The EE (%) was then calculated using the following Eq.1.

$$\%EE = \frac{Amount\ of\ drug\ encapsulated}{Amount\ of\ drug\ used}\ x\ 100 \quad (1)$$

2.2.2.4. ***Spontaneity (Rate of hydration)***

Number of transfersomes formed without sonication per cubic mm were counted after 15 min of hydration, using a haemocytometer with Digital microscope [13]. Vesicles in 5 large squares were counted and rate of hydration was calculated by using Eq. (2):

$$No\ of\ Transferosomes\ formed = \frac{\text{Number of TransfersomesCounted in 5 Squares}}{\text{No of square counted (5 squares)}} \text{ x Dilution factor x } 10^4 \ldots\ldots\ldots\ldots(2)$$

2.2.2.5. ***Degree of deformability (D)***

Transfersomes possess the unique property of deformability [14], which was measured by extruding the vesicle through a polycarbonate membrane of 0.45 µm pore diameter (rp), at a constant pressure (0.5 Mpa). After 5 min extrudate was collected and weighed (J).

The average vesicle size after extrusion (rv) was obtained by using photon correlation spectroscopy. Elasticity of vesicular membrane (D) was calculated using Eq. (3):

$$D = J \times (rv/rp)^2 \ldots\ldots\ldots\ldots (3)$$

2.2.2.6. ***Evaluation of shape, size and surface morphology***

Microscopical examination

Photomicrographs were taken during progression of thin layer of liquid crystalline ProT-LCZ-Gel into ultra-deformable vesicular carrier while hydrating the formulation in a cavity slide with 0.9% saline solution under digital microscope (BA 210 Motic, China) at 100X.

2.2.2.7. Ex vivo studies

2.2.2.7.1 Porcine hoof samples preparation

For the permeation assays, full-thickness porcine hoofs were utilized as a model within a Franz diffusion cell setup. The hoofs were sourced from a slaughterhouse and preserved at -20°C until needed. Prior to use, the samples underwent a preliminary cleaning with double-distilled water, and any outer surface debris was carefully removed via manual rubbing. Finally, the hoof samples were polished with sandpaper to ensure uniform dimensions and thickness for consistent experimental results [15]. Hoof samples were made pre-equilibrated in phosphate buffer pH 7.4 at 25ºC for 2 h before experiments.

2.2.2.7.2. Ex vivo hoof permeation studies

A circular section of the prepared hoof was clamped between the donor and receptor compartments of a Franz diffusion cell, which provided an effective diffusion area of 2.5 cm² and a receptor volume of 25 mL (Figure 1). The hoof was oriented such that the dorsal surface faced the donor compartment while the ventral surface was in direct contact with the receptor medium. To maintain sink conditions, the receptor compartment was filled with Phosphate Buffered Saline (pH 7.4) supplemented with 2% Oleth-20. [16]. Fluid in diffusion cells was maintained at 32±1ºC under continuous stirring using a Franz-diffusion cell assembly (Orchid Scientific, India). Control (2% LCZ solution) and optimized LCZ (2% w/w) loaded ProT-LCZ-Gel were gently placed in the donor section. Solution containing LCZ was prepared by adding LCZ and tert-Butanol to PBS, pH 7.4 with sonication for 5 min. Samples were withdrawn periodically for 48 h and replaced with prepared stock. Samples were then analyzed by HPLC assay at 240 nm. Each preparation was studied in triplicate and the results represent the average value.

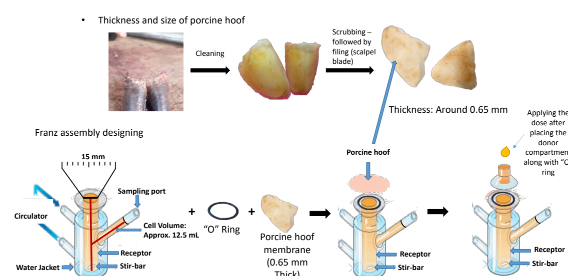

Figure 1: Arrangement for execution of ex vivo permeation and nail retention study

The permeation rate of drugs at steady state (JSS, ng/cm^2/h) through skin was obtained from the slope of linear portion of the cumulative amount permeated per unit area versus time plot. Permeation enhancement ratio (PEr) achieved (Eq. 4) is the ratio of transdermal flux from ProT-LCZ-Gel to LCZ solution.

$$PEr = \frac{\text{Flux achieved with ProT-LCZ-Gel}}{\text{Flux achieved with LCZ-Solution}} \quad \ldots\ldots\ldots.(4)$$

2.2.2.8. In vivo evaluations

Confocal laser scanning (CLS) microscopy

Confocal Laser Scanning (CLS) microscopy was utilized to visualize the permeation pathways and tissue localization of the fluorescent probe across various optical cross-sections, eliminating the need for cryofixation. For these studies, Nile Red-loaded protransfersomal gels (ProT-NR-Gel) were prepared. Freshly excised and cleaned porcine hoofs were mounted onto the Franz diffusion assembly, and 100 mg of the fluorescent-labeled gel was applied to the surface. After 6 hours, the samples were removed, cleaned of excess formulation, and fixed with 10% v/v formalin in PBS (pH 7.4).The hoof samples were then mounted on slides, and vertical cross-sections (1 mm²) were scanned optically at precise increments along the z-axis. Fluorescent dye penetration was monitored using a CLSM (Olympus FluoView™ FV1000, Japan) equipped with a 10× objective lens, utilizing excitation and emission wavelengths of 570 nm and 590 nm, respectively.

2.2.2.9. Stability studies

Optimized ProT-LCZ-Gel formulation was divided into 2 groups and stored at 5 ± 3ºC and room temperature, respectively for 3 months in screw capped amber colored glass bottles. Formulations were evaluated for drug crystals under digital microscope, drug leakage/drug remaining and vesicle size after every 30 days. Stability studies of each formulation were carried out in triplicate.

2.2.2.10. Statistical analysis

Data analysis was implemented with the software, GraphPad Prism (5.0). The data were reported as mean ± S.D. (n = 3 or 6) and multiple comparisons of means (one way ANOVA). Student's t-test was performed for comparing two samples. Results were said to be significant at ≥ 95% confidence interval, $p < 0.05$ and are shown as the mean ± SD.

3. Results and Discussion

3.1 **Development of Protransferosome Gel System**

ProT-LCZ-Gel is characterized by a lamellar liquid crystalline structure that spontaneously transforms into a stable transferosomal dispersion upon contact with an aqueous phase, driven by the hydration of Phospholipon 90G. This formulation was selected for transungual delivery due to its superior encapsulation efficiency (EE%), enhanced nail penetration, and efficient bioactive delivery. In this study, the phospholipid and Tween-80 were specifically screened for their ability to optimize skin permeation, vesicle deformability, and drug entrapment.

Upon initial hydration with a limited aqueous phase, the interaction between water and the polar head groups of the phospholipids facilitates the formation of elongated vesicular structures. Once hydration is complete, these transition into spherical multilamellar and multivesicular transfersomes. The high EE% observed in this study is attributed to the lipophilic nature of luliconazole (LCZ); however, exceeding a specific concentration threshold resulted in drug crystal precipitation. This suggests a saturation point where a fixed quantity of vesicular components can no longer accommodate additional drug within the formed transfersomes.

3. 2 **Characterization of ProT-LCZ-Gel System**

3.2.1 Size, uniformity and surface morphology

Information on globule size is particularly important for understanding the behavior of the nanovesicles. The optimized ProT-LCZ-gel formulation was found to have a globule size of 198.29 ± 6.3 nm (Table 1). The polydispersity index of the prepared formulation is having a value of 0.109 ± 0.009. Since the optimized ProT-LCZ-gel formulation vesicles size was much smaller than 200 nm, such particles are considered suitable for transungual drug delivery [17].

The surface morphology and three-dimensional structure of the hydrated vesicles was further

confirmed by digital microscope. (Figure 2). Microscopic evaluation confirms the formation of spherical transfersomes due to the hydration of ProT-LCZ-Gel. In this study transfersomes derived from the protransferosome gel was passed through the 50 nm filter at a constant pressure of 10 Kg/cm^2 at 37°C using the extruder and deformability was calculated to be 21.87 ± 1.1. Protransfersome gel (10 mg) was hydrated using PBS 7.4 (without sonication, 1.0 ml) and number of transfersomes formed was calculated after 10 mins using haemocytometer and optical microscope and the observed value was 1.44×10^6.

The % EE of the optimized LFN-transfersomes was found
to be ~ 86%. This high percentage efficiency suggests that
the method used to prepare LFN-transfersomes is effective

The % EE of the optimized LFN-transfersomes were found to be ~ 96%. This high percentage efficiency suggests that the method used to prepare ProT-LCZ-gel is effective (Table 1).

Table 1: Vesicles size, PDI and %entrapment of ProT-LCZ-gel

S.No	Parameter	Observed Value
1	Vesicles Size	198.29 ± 6.3
2	Polydispersity Index	0.109 ± 0.009
3	% Entrapment Efficiency	96.08 ± 2.7
4	Deformability	21.87 ± 1.1

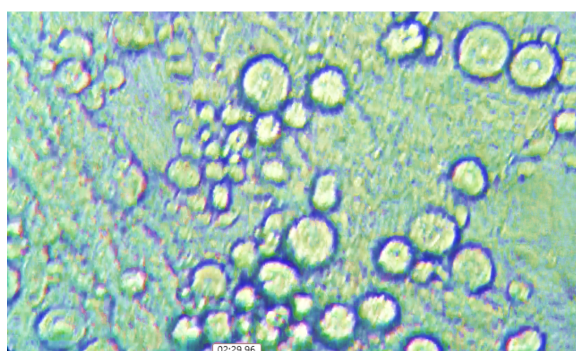

Figure 2 Photomicrograph of transfersomes reconstituted from ProT-LCZ-Gel formulation

3. 3 *Ex vivo* study

3.3.1 Nail retention and permeation study

Nail retention studies were carried out on with porcine hoof on the optimized formulation and was compared with the free drug solution. Significant augmentation in the nail retention of LCZ has been observed with vesicular formulation as the percent cumulative drug retention through the nail was 19.2 ± 1.8%, 1.8 ± 0.17% and 2.6 ± 0.29% in top, middle and bottom layer of nail respectively for drug solution whereas the amount of drug retained for ProT-LCZ-Gel formulation was 77.57 ± 7.8%, 6.2 ± 0.87% and 19.7 ± 1.02% in top, middle and bottom layer of nail respectively.

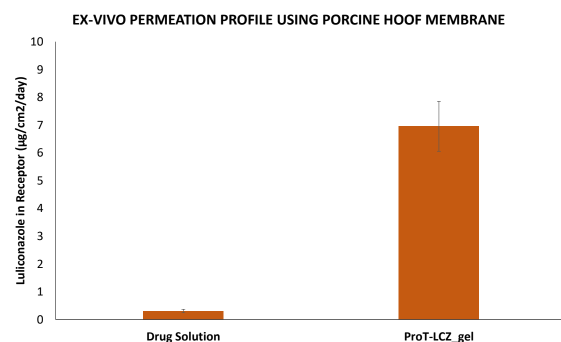

Figure: 3. Luliconazole permeated across nail (µg/cm^2/day)

he retention profile of ProT-LCZ-Gel demonstrated a superior sustained-release pattern for luliconazole (LCZ) compared to the plain drug solution, a behavior likely driven by the lipophilic nature of the drug within the protransfersomal matrix. The protransfersomal system yielded a significantly higher flux, resulting in elevated concentrations of LCZ within the receptor compartment and confirming that ProT-LCZ-Gel facilitates more efficient transungual drug transfer than the plain solution (Figure 3). This enhanced performance is primarily attributed to the spontaneous transformation of the gel into highly deformable transferosomal vesicles upon hydration. Under non-occlusive conditions, the penetration-enhancing effect and intact vesicular permeation operate simultaneously to drive drug transport, aided by high encapsulation efficiency, increased thermodynamic activity, and the complete intercalation of LCZ within the vesicular bilayers. Furthermore, the presence of alcohol as a penetration enhancer and the inherent membrane elasticity provided by the edge activators allow the vesicles to navigate the dense structure of the nail plate, resulting in the rapid initial permeation and superior retention observed. [18]. This mechanism certifies the more efficient permeation of transfersomes over the plain gel. Hence, the penetration improving effect and the intact vesicle permeation into the nails, plays a noteworthy role in the enhanced nail delivery under non-occlusive

conditions. This denouement is in accordance with Elsayed et al., [19] who suggested that either of the 2 mechanisms could overshadow as a result of the vesicle composition and characteristics, along with the physicochemical properties of the drug.

3.3.2 Confocal microscopy

In order to support the above hypothesis, optimized ProT-LCZ-Gel formulation was subjected to the CLS microscopic studies and the extent of retention was measured. The resulting images of very high fluorescence up to 180.00 µm from ProT-LCZ-Gel containing Rh123 pointed out that the retention of the ProT-LCZ-Gel encapsulated dye was enhanced to the middle portion of the hoof from where it can directly available for its therapeutic effect (Figure 4). Hence the carrier is successful enough to deliver enormous amount of drug to the nail and it clearly demarcates the potential of ProT-LCZ-Gel carrier for the transungual delivery [20].

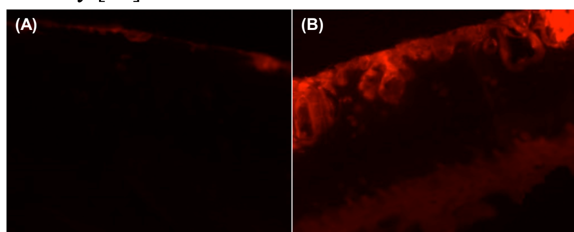

Figure 4. : CLSM images of (A) plain dye (Rh123) solution (B) Rh123 encapsulated ProT-LCZ-gel

3. 4 Stability study

The stability of a pharmaceutical product is defined by its ability to maintain its physical, chemical, therapeutic, and toxicological specifications throughout its intended lifespan. Stability studies are essential for determining the re-test period of the active pharmaceutical ingredient—identifying the duration it remains viable for use without immediate re-analysis—and establishing the shelf life of the final dosage form. Because active ingredients may undergo degradation or other acceptable changes during storage, the specifications for a product at the time of release may differ from those at the end of its shelf life. Consequently, evaluating storage stability remains a critical and ongoing priority in the development of a pharmaceutically acceptable product. In present study, the formulations were stored in refrigerator (5 ± 3ºC) and at room temperature for 3 months (Table 2). After 3 months the vesicular formulations were evaluated for any change in the vesicle size and residual drug content. The initial drug content was taken as 100%.

Table 2 Effect of storage on vesicle size and encapsulation efficiency of optimized final ProT-LCZ Gel at 5 ± 3°C and room temperature

Parameter	Time period	At 5 ± 3ºC	At Room temperature
Vesicle Size (nm)	0 days	198.29 ± 6.3	
	1 month	198.29 ± 6.3	222.87 ± 8.1
	2 months	201.68 ± 7.8	223.61 ± 11.6
	3 months	195.42 ± 9.2	237.64 ± 15.2
Encapsulation Efficiency (%) of LCZ	0 days	96.08 ± 2.7	
	1 month	95.21 ± 2.7	91.91 ± 1.2
	2 months	96.66 ± 3.1	90.22 ± 2.8
	3 months	95.01 ± 1.9	89.41 ± 3.7

The liquid crystalline nature of ProT-LCZ-Gel was not affected after 3 months of storage. Drug crystals were not observed even after 3 months of storage both at RT and refrigerated temperature (5 ± 3ºC). The storage stability of the colloidal carriers is of great concern as it is the major restraint in the development of clinically acceptable marketed formulations. The storage stability was evaluated by measuring the changes in vesicle size and encapsulation efficiency after 3 month storage in the refrigerator (5 ± 3ºC) and room temperature. The encapsulation efficiency (%) of the formulation was found to decrease significantly up to 89.41 ± 3.7% at room temperature. Vesicle size also increased significantly to 237.64 ± 15.2 nm after 3 month storage at room temperature (Table 2). Through ANOVA, it was concluded that no significant change ($p > 0.05$) in vesicles size of transfersomes was found when these are stored at 5 ± 3ºC compared to initial vesicle size. Size growth in case of colloidal carrier system is an indicator of instability. Vesicle size increased whereas polydispersity index decreased indicating complete swelling of bilayer and hence formation of more uniform vesicles upon storage [21]. The higher consistency of ProT-LCZ-Gel was maybe a result of the molecular interaction of polar head groups of surfactants with the solvent and permeation of solvent into bilayer. However, the solvent diffused into the bilayers did not disturb the liquid crystalline

structure. Instead it seemed to have resulted in complete bi-layer formation due to saturation of lipid polar head groups. This is imagined to increase the bilayer distance eventually resulting in overall increase in consistency. The effect of ageing on encapsulation efficiency was not significant at 5 ± 3ºC. However, the noted decrease in encapsulation efficiency after storage may be a result of leaching of drug from the formulation. Hence, formulation will be stable enough for a long period of time if stored at 5 ± 3ºC.

4. Conclusion

The ProT-LCZ-gel formulation was successfully engineered to enhance both the biopharmaceutical properties and therapeutic efficacy of luliconazole. Characterized by its nanometric dimensions and uniform particle size distribution, the formulation demonstrated significant potential in improving drug penetration and retention within the nail plate. By increasing drug availability at the localized site of infection, this system contributes to the more effective inhibition of disease progression. These results were further validated by CLSM analysis, which confirmed superior nail retention of the protransfersomal gel compared to the plain drug solution. Ultimately, this developed transfersomal gel represents an innovative approach to transungual delivery, offering a robust platform for the management of onychomycosis and other recalcitrant nail infections.

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