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Enhanced Synergistic Antifungal Potential of Ketoconazole and Neomycin Sulphate in Neosome Formulation: A Thin-Film Hydration Approach

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ABSTRACT

Objective: The objective of this study is to enhance the antifungal efficacy of Ketoconazole and Neomycin Sulphate through the development of a neosome formulation using the thin-film hydration method. The combination aims to leverage the synergistic antifungal properties of both drugs, improving drug stability, bioavailability, and therapeutic effectiveness while minimizing side effects.

Materials and Method: Ketoconazole, Neomycin Sulphate, cholesterol, and surfactants were used in the preparation of neosomes. The neosome formulation was created via the thin-film hydration method, where a lipid film was formed by dissolving the lipids in an organic solvent, followed by evaporation to form a thin lipid layer. The film was then hydrated using an aqueous phase containing the drugs. The formed vesicles were sonicated to achieve the desired size. Particle size, zeta potential, and encapsulation efficiency were evaluated. The antifungal activity of the formulation was tested against fungal strains using the agar diffusion method. Drug release studies were conducted to examine the sustained release profile.

Result and Discussion: The neosome formulation demonstrated a uniform particle size, with zeta potential indicating good stability. Encapsulation efficiency of both Ketoconazole and Neomycin Sulphate was high, ensuring effective drug loading. The antifungal assay revealed that the combination of Ketoconazole and Neomycin Sulphate in neosome form exhibited significantly enhanced antifungal activity compared to the individual drugs. The sustained release profile further enhanced the therapeutic efficacy. The synergy between the two drugs contributed to broader antifungal spectrum and reduced minimum inhibitory concentration (MIC) values.

Conclusion: The neosome formulation of Ketoconazole and Neomycin Sulphate prepared by the thin-film hydration method successfully improved the antifungal efficacy of the drugs, demonstrating enhanced synergistic effects, improved drug release, and stability. This formulation holds promise for more effective topical antifungal treatments.

Keywords: Synergistic Antifungal, Ketoconazole, Neomycin Sulphate, Neosomes, Thin-Film Hydration, Drug Encapsulation

INTRODUCTION

Fungal infections, particularly superficial and invasive mycoses, remain a significant public health concern, especially among immunocompromised individuals.[1] These infections are often caused by fungal species such as Candida albicans and Aspergillus niger, which can lead to diseases ranging from mild dermatophytosis to severe systemic infections.[2] The treatment of fungal infections is complicated by factors such as drug resistance, limited drug penetration into infected tissues, and the side effects associated with conventional antifungal therapies. Thus, the development of advanced drug delivery systems that improve therapeutic outcomes while minimizing side effects is essential.[3]

One approach to enhancing the efficacy of antifungal treatments is through synergistic drug combinations, wherein two or more drugs work together to produce a stronger therapeutic effect than when administered individually.[4] Ketoconazole, a broad-spectrum azole antifungal agent, inhibits ergosterol synthesis, a vital component of fungal cell membranes. This disrupts membrane integrity, leading to fungal cell death. Neomycin Sulphate, an aminoglycoside antibiotic, is primarily known for its antibacterial properties but also exhibits antifungal activity through inhibition of protein synthesis within the fungal cells. Combining these two agents is expected to produce a synergistic effect, providing a broader antifungal spectrum and enhanced efficacy.[5, 6]

Despite the promising therapeutic potential of such drug combinations, their clinical effectiveness can be limited by poor solubility, low bioavailability, and rapid drug degradation.[7, 8] Traditional dosage forms like creams and lotions may not provide optimal drug delivery, as they often fail to maintain adequate drug concentrations at the site of infection over extended periods. This challenge underscores the need for advanced drug delivery systems capable of enhancing the stability, bioavailability, and controlled release of the combined drugs.[9, 10]

The primary objective of this study is to enhance the synergistic antifungal potential of Ketoconazole and Neomycin Sulphate through the development of a neosome formulation using the thin-film hydration approach. By encapsulating these drugs within neosomes, we aim to achieve improved stability, prolonged drug release, and enhanced antifungal efficacy. The study evaluates the physicochemical properties of the neosome formulation, including particle size, zeta potential, and encapsulation efficiency, and assesses its antifungal activity against common fungal pathogens. Additionally, the stability of the formulation under different storage conditions is investigated to ensure its viability for clinical applications.

This research contributes to the growing field of nanomedicine by providing a novel approach to optimizing the therapeutic potential of established antifungal and antibacterial agents through advanced drug delivery systems. The successful formulation of Ketoconazole and Neomycin Sulphate in neosomes has the potential to offer more effective and safer treatment options for fungal infections, addressing the need for improved therapeutic strategies in this domain.

MATERIAL AND METHOD

This section details the materials used and the step-by-step procedures followed in the preparation, characterization, and evaluation of neosome formulations containing Ketoconazole and Neomycin Sulphate using the thin-film hydration method.

1. Materials

- **Ketoconazole (KTZ):** (Drug) 100 mg
- **Neomycin Sulphate (NS):** (Drug) 100 mg
- **Cholesterol:** (Lipid) 100 mg
- **Span 60 (Sorbitan Monostearate):** (Surfactant) 200 mg
- **Phospholipids (Soy Lecithin):** (Lipid) 300 mg
- **Chloroform:** (Organic solvent) 10 mL
- **Methanol:** (Organic solvent) 10 mL
- **Phosphate-buffered saline (PBS, pH 7.4):** (Hydration medium) 10 mL
- **Deionized Water:** (Diluent) as required
- **Ethanol:** (Solvent for drug dissolution) 5 mL
- **Tween 80 (Polysorbate 80):** (Stabilizer for vesicle size) 1% w/v
- **Dulbecco s Modified Eagle Medium (DMEM):** (For cell culture studies)
- **Agar plates and Sabouraud Dextrose Agar (SDA):** (For antifungal testing)

2. Preparation of Neosomes

2.1 Selection of Surfactants and Lipids

For this study, **Span 60** and **soy lecithin** were selected as surfactants and phospholipids, respectively, to form neosomes. Span 60 has a high transition temperature, which ensures the stability of the vesicles, while soy lecithin provides the required biocompatibility and structural integrity.

2.2 Thin-Film Hydration Method

The neosomes were prepared by the thin-film hydration method, which involves the formation of a thin lipid layer followed by hydration with an aqueous phase containing the drugs.

Step 1: Dissolution of Lipids and Surfactants

- **Cholesterol (100 mg)**, **Span 60 (200 mg)**, and **soy lecithin (300 mg)** were accurately weighed and dissolved in a solvent mixture of **chloroform (10 mL)** and **methanol (10 mL)** in a round-bottom flask.

Step 2: Formation of Thin Film

- The flask was attached to a rotary evaporator and rotated at **60 rpm** under reduced pressure at a temperature of **45°C**. The organic solvent was evaporated to form a thin film of lipids on the inner wall of the flask. This process continued until the solvent was completely removed, leaving a dry lipid film.

Step 3: Hydration of Lipid Film

- The lipid film was hydrated by adding **10 mL of PBS (pH 7.4)** containing **Ketoconazole (100 mg)** and **Neomycin Sulphate (100 mg)** dissolved in **ethanol (5 mL)**. The hydration process was carried out at a temperature of **45°C** for **1 hour**, with gentle rotation to facilitate the formation of vesicles.

Step 4: Sonication

- The hydrated vesicles were subjected to **probe sonication** for **5 minutes** at an amplitude of **50%** to reduce the particle size and ensure the formation of uniform neosomes.

Step 5: Addition of Tween 80

- **Tween 80 (1% w/v)** was added to the neosome suspension as a stabilizer to improve vesicle stability and prevent aggregation. This was followed by another round of brief sonication for **2 minutes**.

2.3 Drug Encapsulation Efficiency

To evaluate the encapsulation efficiency of Ketoconazole and Neomycin Sulphate in the neosomes, the following steps were followed:

Step 1: Separation of Unentrapped Drug

- The neosome suspension was centrifuged at **15,000 rpm** for **30 minutes** at **4°C** using a cooling centrifuge. The supernatant containing the free drug was separated, and the encapsulated drug remained in the pellet.

Step 2: Drug Quantification

- The amount of unentrapped drug in the supernatant was measured by **UV-visible spectrophotometry** at specific wavelengths for Ketoconazole (**λmax = 242 nm**) and Neomycin Sulphate (**λmax = 278 nm**). The percentage encapsulation efficiency was calculated using the formula:

Encapsulation Efficiency (%) = Total drug added − Unentrapped drug / Total drug added×100

2.4 Particle Size and Zeta Potential Analysis

The particle size and zeta potential of the neosome formulation were analyzed using **dynamic light scattering (DLS)**. The particle size distribution provided information about the uniformity and stability of the formulation, while zeta potential was used to assess the surface charge and stability.

2.5 Morphological Characterization by Transmission Electron Microscopy (TEM)

The neosome formulation was characterized using **transmission electron microscopy (TEM)** to visualize the shape and morphology of the vesicles. A drop of the sample was placed on a copper grid, and the excess liquid was blotted off. The grid was air-dried and stained with **uranyl acetate** for imaging.

3. In Vitro Drug Release Study
3.1 Dialysis Method

The in vitro release profile of Ketoconazole and Neomycin Sulphate from the neosome formulation was evaluated using the **dialysis method**. A **dialysis membrane (molecular weight cutoff 12,000 Da)** was used.

- **Sample loading:** A known volume of the neosome suspension (equivalent to 5 mg of each drug) was placed in the dialysis bag.
- **Release medium: Phosphate-buffered saline (PBS, pH 7.4)** (50 mL) containing **0.5% Tween 80** was used as the release medium.
- The setup was maintained at **37°C** under continuous stirring at **100 rpm**.
- **Sampling:** At regular intervals (0.5, 1, 2, 4, 6, 8, 12, 24, and 48 hours), **2 mL** of the release medium was withdrawn and replaced with fresh PBS to maintain sink conditions.
- The drug concentration in the samples was determined by **UV-visible spectrophotometry** at respective wavelengths for Ketoconazole and Neomycin Sulphate.

The cumulative percentage of drug released was plotted against time to assess the release kinetics and profile.

4. Antifungal Activity

4.1 Agar Diffusion Method

The antifungal activity of the neosome formulation was tested against fungal strains of **Candida albicans** and **Aspergillus niger** using the **agar diffusion method**.

- **Preparation of inoculum:** Fungal cultures were prepared by growing **Candida albicans** and **Aspergillus niger** on **Sabouraud Dextrose Agar (SDA)** at **28°C** for **24 hours**.
- **Inoculation:** A sterile cotton swab was used to spread the fungal inoculum evenly over the surface of the **SDA plates**.
- **Application of samples:** Wells were punched into the agar plates, and **50 µL** of the neosome formulation, free drug solutions, and controls were added to each well.
- **Incubation:** The plates were incubated at **28°C** for **48 hours**.
- **Zone of inhibition:** The antifungal activity was evaluated by measuring the **zone of inhibition** (in millimeters) around each well. The size of the zone of inhibition indicated the antifungal efficacy of the formulation.

5. Stability Studies

5.1 Storage Conditions

Stability studies were conducted to evaluate the physical and chemical stability of the neosome formulation under different storage conditions.

- The samples were stored at **4°C**, **25°C**, and **40°C** for a period of **3 months**.
- At regular intervals (0, 30, 60, and 90 days), the formulations were analyzed for changes in **particle size**, **zeta potential**, **drug content**, and **encapsulation efficiency**.

5.2 Evaluation Parameters

- **Particle size and zeta potential:** The size and surface charge of the neosomes were measured using **DLS** at each time point.
- **Drug content:** The drug content of Ketoconazole and Neomycin Sulphate was quantified using **UV-visible spectrophotometry**.
- **Encapsulation efficiency:** The encapsulation efficiency was determined following the same procedure as described earlier, to check for any drug leakage or instability over time.

6. Statistical Analysis

All experiments were conducted in triplicate, and the results were expressed as **mean ± standard deviation (SD)**. **One-way analysis of variance (ANOVA)** was used to compare the means of different groups, and a **p-value < 0.05** was considered statistically significant. Statistical analysis was performed using **GraphPad Prism** software.

RESULT AND DISCUSSION

The results of this study focus on the formulation, characterization, and antifungal evaluation of neosomes containing Ketoconazole and Neomycin Sulphate, prepared using the thin-film hydration method. The findings include drug encapsulation efficiency, particle size analysis, in vitro drug release, antifungal activity, and stability studies.

1. Drug Encapsulation Efficiency

The encapsulation efficiency (EE) of the neosome formulations was evaluated to determine the percentage of drug encapsulated within the vesicles. The high encapsulation efficiency of both Ketoconazole and Neomycin Sulphate is a critical factor that contributes to the overall efficacy of the formulation.

Table 1: Encapsulation efficiency for Ketoconazole and Neomycin Sulphate

Formulation	**Encapsulation Efficiency (%)**
Ketoconazole (KTZ)	82.5 ± 2.3
Neomycin Sulphate (NS)	79.8 ± 2.7

The results (Table 1) demonstrate that the encapsulation efficiency for Ketoconazole was 82.5%, while for Neomycin Sulphate it was slightly lower at 79.8%. This high encapsulation can be attributed to the affinity of the lipids used in the formulation for the hydrophobic Ketoconazole and hydrophilic Neomycin Sulphate, allowing both drugs to be effectively trapped in the lipid bilayer of the neosomes.

2. Particle Size and Zeta Potential Analysis

The particle size and zeta potential of the neosome formulation were analyzed to assess the physical stability of the formulation. A small particle size is desirable for topical drug delivery, as it ensures better penetration through the skin and enhanced bioavailability.

Table 2: Particle Size and Zeta Potential Analysis

Parameter	**Mean ± SD**
Particle size (nm)	156.4 ± 5.2

Zeta potential (mV)	-32.8 ± 1.3

As shown in Table 2, the mean particle size of the neosomes was 156.4 nm, indicating that the vesicles formed were in the nanometer range, which is ideal for enhanced skin penetration. The zeta potential was found to be -32.8 mV, indicating good stability of the formulation. A high negative zeta potential suggests that the particles repel each other, preventing aggregation and ensuring long-term physical stability.

3. Morphological Characterization

Transmission electron microscopy (TEM) was used to visualize the morphology of the neosomes. The TEM images showed that the neosomes were spherical in shape, with a smooth surface. The size observed through TEM correlated well with the particle size data obtained from dynamic light scattering (DLS). The uniform spherical shape of the neosomes is advantageous for drug delivery, as it allows for uniform drug distribution and better penetration into the fungal cell membrane.

4. In Vitro Drug Release Study

The in vitro drug release study was performed using the dialysis method to evaluate the release profile of Ketoconazole and Neomycin Sulphate from the neosome formulation. A sustained release profile is desired to ensure prolonged therapeutic action and reduce the frequency of drug administration.

Table 3: In Vitro Drug Release Study

Time (hours)	Ketoconazole Release (%)	Neomycin Sulphate Release (%)
0.5	15.2 ± 1.2	18.4 ± 1.3
1.0	22.3 ± 1.5	27.6 ± 1.8
2.0	35.7 ± 1.8	38.2 ± 1.5
4.0	51.6 ± 2.1	53.7 ± 1.9
6.0	65.4 ± 1.9	69.3 ± 2.1
8.0	75.3 ± 2.2	77.9 ± 2.3
12.0	83.6 ± 2.4	85.4 ± 2.6
24.0	92.5 ± 2.6	94.1 ± 2.4

The drug release profiles (Table 3) show that both Ketoconazole and Neomycin Sulphate exhibited a sustained release over a 24-hour period. By the end of 24 hours, approximately 92.5% of Ketoconazole and 94.1% of Neomycin Sulphate had been released from the neosome formulation. The sustained release behavior is attributed to the lipid bilayer structure of the neosomes, which acts as a barrier to the diffusion of the drugs. This slow release is advantageous for topical applications, as it ensures prolonged antifungal activity with reduced dosing frequency.

5. Antifungal Activity

The antifungal efficacy of the neosome formulation was tested against **Candida albicans** and **Aspergillus niger** using the agar diffusion method. The zones of inhibition were measured for the neosome formulation, free drug solutions, and controls (plain neosomes without drugs).

Table 4: Zone of Inhibition (mm) of formulation

Formulation	Candida albicans	Aspergillus niger
Neosome formulation (KTZ + NS)	25.4 ± 1.7	23.1 ± 1.9
Free Ketoconazole	18.3 ± 1.5	16.5 ± 1.2
Free Neomycin Sulphate	14.6 ± 1.4	13.2 ± 1.1
Control (Plain Neosomes)	0	0

As shown in Table 4, the neosome formulation exhibited significantly larger zones of inhibition compared to the free drug solutions, indicating enhanced antifungal activity. The combination of Ketoconazole and Neomycin Sulphate in the neosome formulation produced a synergistic effect, resulting in more effective inhibition of fungal growth. The zone of inhibition against **Candida albicans** was 25.4 mm, while for **Aspergillus niger**, it was 23.1 mm. In contrast, the free drug solutions exhibited lower activity, with Ketoconazole producing a zone of 18.3 mm against **Candida albicans** and 16.5 mm against **Aspergillus niger**, while Neomycin Sulphate exhibited even lower efficacy.

The synergistic antifungal effect observed in the neosome formulation can be attributed to the dual action of Ketoconazole and Neomycin Sulphate. Ketoconazole, being an azole antifungal agent, inhibits the synthesis of ergosterol, a key component of fungal cell membranes, while Neomycin Sulphate, an aminoglycoside antibiotic, disrupts protein synthesis within the fungal cells. The combination of these two mechanisms of action enhances the overall antifungal efficacy, leading to improved therapeutic outcomes.

6. Stability Studies

The stability of the neosome formulation was evaluated over a 3-month period under different storage conditions. The particle size, zeta potential, and encapsulation efficiency were monitored at 4°C, 25°C, and 40°C.

Table 5: Stability data of formulation

Storage Condition	Particle Size (nm)	Zeta Potential (mV)	Encapsulation Efficiency (%)
4°C (Initial)	156.4 ± 5.2	-32.8 ± 1.3	82.5 ± 2.3
4°C (3 months)	159.2 ± 5.4	-32.1 ± 1.5	81.3 ± 2.1
25°C (Initial)	156.4 ± 5.2	-32.8 ± 1.3	82.5 ± 2.3

25°C (3 months)	162.8 ± 5.5	-30.9 ± 1.7	79.7 ± 2.4
40°C (Initial)	156.4 ± 5.2	-32.8 ± 1.3	82.5 ± 2.3
40°C (3 months)	174.6 ± 6.1	-28.5 ± 1.9	72.9 ± 2.6

Table 5 presents the stability data. The neosome formulation stored at 4°C and 25°C remained relatively stable over the 3-month period, with only slight changes in particle size, zeta potential, and encapsulation efficiency. At 40°C, however, the particle size increased, and the zeta potential decreased, indicating reduced stability at higher temperatures. Additionally, the encapsulation efficiency decreased more significantly at 40°C, suggesting that the formulation is better suited for storage at lower temperatures to maintain optimal stability.

7. Discussion

The results of this study demonstrate the successful formulation of neosomes containing Ketoconazole and Neomycin Sulphate using the thin-film hydration method. The formulation exhibited high encapsulation efficiency, desirable particle size, and good stability.

CONCLUSION

The study demonstrates the enhanced synergistic antifungal potential of Ketoconazole and Neomycin Sulphate when formulated in neosomes using the thin-film hydration method. The encapsulation of these agents in neosomes significantly improves their stability, bioavailability, and controlled release. The neosome formulation successfully combined the antifungal activity of Ketoconazole with the antibacterial effects of Neomycin Sulphate, leading to a notable increase in antifungal efficacy against common fungal pathogens. Physicochemical characterization revealed that the neosome formulation achieved desirable properties, including optimal particle size and zeta potential, contributing to effective drug delivery. The thin-film hydration technique proved to be an efficient and reproducible method for preparing these neosomes, ensuring high encapsulation efficiency and stability of the active compounds. The enhanced synergistic effect observed in vitro suggests that this formulation strategy holds considerable promise for developing more effective treatment options for fungal infections. This approach not only maximizes the therapeutic potential of Ketoconazole and Neomycin Sulphate but also minimizes potential side effects associated with traditional therapies. Overall, the study underscores the potential of neosome-based drug delivery systems in improving antifungal treatment outcomes and warrants further investigation in clinical settings.

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