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A Novel System for The Treatment of Onychomycosis Through Transungal Route

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Abstract

Onychomycosis is a persistent fungal infection that affects both the nail plate and the nail bed. This condition is prevalent in older patients. Itraconazole is a widely used drug for the treatment of onchomycosis. In patients receiving various medication therapy regimens, oral antifungal therapy is typically linked to serious adverse effects that can exacerbate the illness. The study's current goal was to create a new itraconazole formulation that could be administered transungally. The optimized formulation was tested for a number of evaluation parameters, including drug release, entrapment efficiency, polydispersity index, morphology study, and particle size, and the results showed that the particle size, polydispersity index, and zeta potential were 277 nm, 0.265, and -30.6 mv, respectively. Itraconazole liposomes were made using the thin film hydration method to aid in drug penetration across the nail. The formulation's entrapment efficiency and drug release were determined to be 96% ±1.2 and 78% ±0.2, respectively. Physical characteristics, drug content, *in-vitro* drug release, antifungal activity, and in-vitro transungal permeation using goat hooves were all investigated for lyophilized liposomes of itraconazole integrated into a nail lacquer base. The drug content and drying time did not significantly change during the three months that the formulations were stable at 25°C and 60% relative humidity. It was determined that a promising delivery method for the treatment of onychomycosis is itraconazole-loaded liposomes in nail lacquer base.

Keywords

Onychomycosis, Transungal route, Itraconazole, Lyophilized liposomes, Nail lacquer, thin film hydration method

INTRODUCTION

Nail infections are not only problematic in terms of cosmetic application but can also cause pain affecting the quality of life. A persistent fungal disease that is frequently observed in older persons is onychomycosis. Although oral antifungal medications are strongly advised for onychomycosis, they can have major adverse effects in people with weakened immune systems. Other therapy approaches for onychomycosis include topical formulations, surgical or mechanical nail removal, and a combination of the two. [1,2]

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An antifungal agent has to permeate various nail plate layers in a defined effective concentration in order to achieve therapeutic effect. Medicated nail lacquer formulations are used for antifungal drug delivery via transungal route in order to maximize its therapeutic efficacy. The film applied on nail surface forms a depot layer of the drug thus providing sustained release for the entire duration of therapy. Itraconazole is a synthetic lipophilic antifungal drug that belongs to BCS class II which is used in treatment of fungal infections of skin as well as nails. Chronic oral intake of this drug results in severe

hepatotoxicity, cardiac arrhythmia and gastric side effects. Thus, the basic aim of the present study was to develop an itraconazole loaded liposomes in a nail lacquer base. Itraconazole was selected as a model drug as it has broad spectrum of activity against various fungal species and tends to accumulate in skin, nails and fatty tissues.[3]

The objective of the study was to develop liposomal formulation of itraconazole for topical application in order to avoid oral side effects associated with drug and to prepare liposomes vesicles with highest drug entrapment efficiency required for achieving high concentration of drug at site of action. The study aimed to enhance permeability of the drug in liposomes vesicle across the restrictive barrier of nail plate by incorporating drug loaded liposomes in a nail lacquer base for longer retention time and reduced frequency of administration. This study demonstrated enhanced ease of application and patient compliance.

MATERIALS AND METHODS

Itraconazole was obtained as a gift sample from Srijan medi solution private limited, Indore, Madhya Pradesh. Phosphatidylcholine was obtained from Lipoid GmBh, Santacruz, Mumbai. Cholesterol, Eudragit RL100 and Hydroxypropyl methyl cellulose E15 was obtained from Molychem, Evonik, Colorcon Asia, Goa, respectively. Polyethylene glycol, chloroform, methanol and acetone was obtained from Loba Chemie, Molychem, S.D. Fine Chemicals and Molychem, respectively. All the excipients and solvents used were of analytical grade.

1. **Determination of Melting Point of the drug:**
   Melting point of Itraconazole was determined using melting point apparatus.
2. **Determination of Solubility:**
   The solubility of the drug itraconazole was determined in various solvents. In this excess amount of drug was dissolved in 10ml of various solvents such as methanol and buffer pH 7.4 in a flask. These flasks were then kept on a mechanical bath shaker for 72 hours at 37±0.5^{o}C. The supernatant was separated, filtered, and after appropriate dilution with methanol, solubility was determined by UV-visible spectrophotometer.
3. **Compatibility studies:**
   Compatibility studies are conducted in order to guarantee that there are no unintended interactions between drug and excipients. Any kind of incompatibilities will affect the functional performance of pharmaceutical dosage form hence it forms an important study in formulation development.

3.1. FTIR Spectroscopy Analysis: Infrared spectrums of pure drug itraconazole, physical mixture with other excipients along with the final liposomal preparations were recorded on Attenuated Total Reflectance Shimadzu Fourier Transform Infrared (FTIR). Samples were directly placed on sample holder and scanned in the range of 4,000–600 cm^{-1}.

3.2. Differential Scanning Calorimetry (DSC) analysis: DSC studies were conducted to record the DSC thermograms of pure drug itraconazole and its physical mixtures with phospholipon 90G: Cholesterol in 1:1 ratio. The thermogram of final lyophilized product was also recorded. Samples (2.5–8.0 mg) were placed in an aluminum pan, firmly crimped with the lid to provide an adequate seal. Samples were scanned at 10°C/min over the temperature range 30°C–250°C.

4. **Formulation of liposomal suspension by thin film hydration technique using central composite design:**

4.1 ***Preparation of liposomal suspension***: Itraconazole liposomal formulations with different drug to lipid and phospholipon 90G to cholesterol molar ratios were prepared using thin film hydration technique. The phospholipid 90G to cholesterol with different ratios was dissolved in chloroform in a round bottom flask. To this 0.1M drug solution and 10gm of glass beads were then added. The organic solvent was then slowly evaporated under reduced pressure using rotary evaporator operated at 60^{o}C and 125 RPM until a thin film of dry lipid was formed on the inner surface of flask. The dried film was slowly hydrated with 10ml of phosphate buffer pH 7.4 solution for 2hr. The liposomal suspension formed was then subjected to probe sonication operated at 40 amplitudes, pulse of 180 sec for 10 min and left to mature overnight in refrigerator at 4^{o}C for complete hydration. [3,4,5]

4.2 ***Central Composite Design (CCD)***: The Design of Experiment (DoE) is the key element used in deriving a specific experimental setup to acquire the required information before the process optimization. Response Surface Methodology (RSM) is a technique used in predicting the effect of several independent variables influencing the dependent responses by varying independent variables simultaneously and conducting a series of experimental trial generated from the software. The CCD approach was used for the studying the influence of two factors such as drug

to lipid ratio and phospholipid to cholesterol ratio at three different level on the dependent variables i.e. entrapment efficiency and drug release using design experiment trial 11 version software. Both dependent and independent variables were selected based on literature survey. Using this design, the influence of two dependent factors was evaluated, and the experimental trials were carried out at all ten possible combinations.

5. Evaluation of liposomal dispersions: [6,7,8,9]

5.1 ***Appearance:*** The liposomal dispersions were visually inspected for colour and appearance.

5.2 ***Determination of particle size and polydispersity index (PDI):*** Particle size and polydispersity index of the liposomal formulation was measured using Zetasizer Nano (Malvern). The dynamic range is 0.3mm-8um, depending upon physical properties of sample. Helium laser was used as a light source and scattering was monitored at 25 °C temperature and 90° detector angle. 2ml of liposomal suspensions was diluted to 10 folds with purified water and particle size as well as PDI was determined.

5.3 ***Determination of Zeta Potential:*** Every particle in colloidal or nano form carries a surface charge in a suspension. In application of electric field, this charged particles tends to move towards the oppositely charged electrode. The direction and velocity of the motion is the function of its own charge, the surrounding medium and the electric field strength. The particle velocity is measured by observing Doppler shift in scattered light. The instrument used in the measurement is Zetasizer Nano (Malvern). Few milliliters of liposomal formulation were transferred by micropipette specialized cuvettes which was then place in to the instrument.

5.4 ***Determination of Entrapment Efficiency:*** This was done using indirect centrifugation technique. The resulting solution was analyzed for presence of free drug by using UV-visible spectrophotometer at 225nm.The percent drug entrapment was calculated using formula:

Percent drug entrapment =

$$\frac{drug_{total} - drug_{supernatant}}{drug_{total}} \times 100$$

5.5 ***Drug content:*** Aliquots of 1ml liposomal suspension was diluted with 10ml of methanol and the subjected to sonication for 15min. The resulting solution was filtered, diluted and analyzed by UV-visible spectrophotometer at 225nm.

5.6 ***in-vitro drug release:*** This was conducted using dialysis bag method. In this a volume of 2ml of liposomal suspension was introduced into a dialysis bag of MWCO 10000 14000Da Himedia. Both the ends were tied in such a manner that there was no formulation leakage from the bag. The dialysis bag was suspended in 50ml of receiver compartment composed of phosphate buffer pH 7.4 with 20% methanol maintained at 37^{o}C. The dispersion was rotated at 150rpm. At predetermined time intervals of 0.5, 1, 2, 4, 6, 8, 10 ,12 hours, 5ml of aliquots were sampled and replaced with 5ml of fresh buffer containing 20% methanol. The concentration of drug in the filtered sample was then analyzed using UV spectrophotometer at 225nm. The data was analyzed to determine percent drug release and mechanism of drug release.

6. Optimization of liposomal formulation: [10,11]
Response surface methodology was employed to determine the influence of independent variables i.e. drug to lipid ratio and lipid to cholesterol ratio on entrapment efficiency and percent drug release. The response plot was generated using design software 11 version to enable modelling of the responses over an entire range of varying factors with limited number experimental runs. Analysis of Variance (regression ANOVA) was employed to determine the model and significance of various factors.

7. Characterization of optimized batch: [12,13,14,15]

7.1. ***Particle size, zeta potential and polydispersity index determination:*** The determination of the physical properties was carried out as per standard procedures.

7.2. ***Morphology study of particle:*** This study was conducted by Cryosem analysis.

7.3 ***Determination of entrapment efficiency:*** This test was conducted as per given procedure mentioned in evaluation of liposomal dispersion.

7.4. ***In vitro drug release:*** This test was conducted as per given procedure mentioned in evaluation of liposomal dispersion.

8. **Lyophilization of optimised formulation:** Five percent of sucrose was added as a cryoprotectant to itraconazole-loaded liposomal suspension, filled into vials. The sample was then placed in freezer at a temperature of -28°C for 24hr. Primary drying was achieved by maintaining the sample chamber to temperature of -50°C and pressure to 0.2 mill bar for 24h. After primary drying, the sample was then subjected to secondary drying.

9. Characterization of freeze-dried liposomes: [16,17]

9.1 ***Determination of Entrapment Efficiency:*** This test was conducted as per given procedure mentioned in evaluation of liposomal dispersion.

9.2 ***Particle size, polydispersity index and zeta potential:*** This test was conducted as per the given procedure mentioned in evaluation of liposomal dispersion.

9.3 ***Morphology study:*** This test was conducted using scanning electronic microscopy

9.4 ***Drug content:*** This test was conducted as per the given procedure mentioned in evaluation of liposomal dispersion

9.5 ***in-vitro drug release:*** This test was performed as per the given procedure mentioned in the evaluation study of liposomal dispersion.

10. Formulation of Nail Lacquer Base:

10.1. Formulation of Itraconazole loaded nail lacquer base:

The nail lacquer of itraconazole loaded liposome was prepared by using both hydrophilic as well as hydrophobic polymer. Accurately weighed amount of Eudragit RL 100 was dissolved in half quantity of the acetone using sonication. To this HPMC E15 dissolved in to other half of solvent containing PEG 400 was added under controlled temperature condition. The solution was then kept on magnetic stirrer for stirring until homogenous nail lacquer was formed. Finally, the drug loaded lyophilized liposomes equivalent to 1%w/v were dispersed in the prepared nail lacquer with gentle stirring.

10.2. Formulation of Itraconazole lacquer base:

Drug equivalent to 1%w/v dissolved initially in 1ml of chloroform used as cosolvent was then added in to Eudragit RL 100 polymer solution. The drug containing polymer mixture was then added in to HPMC EI5 containing PEG 400 solution. The solution was then subjected to stirring under temperature-controlled condition until homogenous preparation formed.

Table 1: Composition of Itraconazole Loaded Liposome in Nail Lacquer

Formulation code	Eudragit RL 100	HPMC E15	PEG 400	Acetone
ERL 1	1.5%	1.5%	12%	qs to 100 ml
ERL 2	2%	2%	12%	qs to 100 ml
ERL 3	2.5%	2.5%	12%	qs to 100 ml

11. Evaluation of Nail Lacquer Formulation:

11.1 ***Non-Volatile Content:*** 1±0.2g of sample was taken in a glass petri dish of about 8cm in diameter. Samples were spread evenly with the help of tared wire. The dish was placed in the oven at 105^{o}C ± 2^{o}C for 1hr. After 1hr the petri dish was removed, cooled, and weighed. The difference in weight of sample after drying was determined.

$$\% \text{ non-volatile matter} = \frac{\text{mass of the dried film residue}}{\text{mass of the wet film residue}} \times 100$$

11.2. ***Drying Time:*** A thin film of lacquer formulation was cast on the petri dish with the help of brush. The time to form dry-to-touch film was recorded.

11.3. ***Smoothness of flow:*** The sample was poured to approximately 1.5 inches and spread on a glass plate and made to rise vertically.

11.4. ***Drug Content:*** Nail lacquer equivalent to 10 mg of the drug was accurately weighed, dissolved and made up to the mark in 100ml volumetric flask with methanol and sonication for period of 15min. The resultant solution was then filtered through a 0.22-µm membrane filter and quantified for drug content through double-beam UV spectrophotometer.

11.5. ***Film thickness:*** Single film layer of the nail lacquer formulation was cast on a petri plate with 12.5 cm^2 area, using applicator brush. The film was allowed to dry at room temperature. The dried film was peeled off from the petri plate, and the thickness of the formed film was determined using a digital Vernier caliper

11.5. ***Viscosity determination:*** Viscosity analysis of the formulated nail lacquer was carried out using Brookfield viscometer (DVE)

11.6. ***Adhesive strength:*** In-house modified equipment designed in pharmaceutical laboratory was used to determine the adhesive property of nail lacquer. The instrument is modification of balance used in laboratory. One pan of the balance was replaced with two glass slides. In between the glass slides a film of 8cm^2

was applied on it. The equilibrium of the balance was adjusted by adding a weight to the right pan of balance. The force required to pull away the plates was recorded as force of adhesion.

Force of adhesion = Mass x Acceleration

Adhesive strength = Force of Adhesion (N) / Surface area (m^2)

12. ***in-vitro* studies:**

Culture preparation: Test species- Candida Albicans and Aspergillus Niger organisms was used for determination of *in-vitro* antifungal inhibitory activity of the itraconazole loaded liposome in nail lacquer formulations.

Stock culture suspension for Aspergillus Niger was prepared from pure culture on the sabouraud dextrose agar medium. For Candida Albicans cultures containing 10 5-10-7 cfu/ml of microorganism were used and diluted with distilled water to obtain turbidity.

12.1 ***Antifungal activity test:*** This was determined by diffusion test employing cup plate technique. 20ml of sterilized sabouraud dextrose agar was poured on to sterilized petri plate (basal layer) and was allowed to solidify. 100µl of culture suspension prepared in 0.85 percent saline was added aseptically to sabouraud dextrose agar and cyclomixed. This was then poured over the basal layer and allowed to set. Using a sterilized cork borer, a well was made in center of the plate. Nail lacquer formulation equivalent to 1mg of Itraconazole was added in to each well. Standard Itraconazole nail lacquer equivalent to 1mg of the drug and lacquer without drug were used to compare the zone of inhibition. The plates were kept in refrigerator for 30min. After 30min the plates were incubated at 27±^oC for 3days. The results were recorded by measuring the zones of inhibition surrounding using Vernier calliper. All determinations were made in triplicate for each test organism. For positive control, dimethyl sulfoxide (DMSO) solution of itraconazole (1%) was used.

12.2 in-vitro drug release from nail lacquer formulation:

Activation of dialysis membrane: The dialysis membrane was soaked in phosphate buffer pH 7.4 containing 20% methanol.

***in-vitro* drug release** was performed using in-house modified Franz Diffusion cell. Sample equivalent to 5mg itraconazole was applied on the surface of the cellophane membrane and it was tied to the end of donor compartment. The diffusion tube was placed such that the entire surface of the membrane was in contact with receptor compartment containing phosphate buffer pH 7.4 with 20% methanol. The whole assembly was maintained at 37^oC and the speed of stirring was kept constant (120) for 12hrs. A 5ml aliquot was withdrawn at specified time interval and was then replaced with fresh media. The withdrawn sample was further diluted and analysed UV spectrophotometrically at 225nm.

12.3. *in-vitro transungal permeation studies:*

Preparation of Hooves as membrane: Hooves from freshly slaughtered goat, free of adhering connective and cartilaginous tissue were used membranes of about 1cm thickness were cut from distal part of hooves. Two 4x4 cm sheet of cellophane membranes were used as support, a rectangular portion of area 1cm^2 was cut in the middle of the cellophane membrane. The square cut portion of the hooves, were heat laminated between previously cut cellophane membranes. The hooves were kept for soaking in distilled water for 24hrs.

***in-vitro* transungal permeation studies** were carried out by in house modified Franz Diffusion cell. The hoof membrane was placed carefully on the cell, and the surface area available for permeation was 1cm^2. Then the formulation equivalent to 5mg was applied evenly on the surface of the membrane. The receptor compartment was filled with phosphate buffer pH 7.4 containing 20% methanol. The whole assembly was maintained at 37^oC with constant stirring at 15rpm for 12hr. The 5ml aliquot of drug sample was withdrawn at specified time interval and was then replaced with fresh media. The drug analysis was done using UV spectrophotometer at 225nm.

12.4 ***Stability studies:*** For any formulation of dosage form, the stability of the active drug plays an important role in its acceptance and rejection.

RESULTS AND DISCUSSION

1. **Standardization of Itraconazole**

1.1 ***Determination of λ_{max} of Itraconazole:*** A solution of 10µg/ml of Itraconazole was prepared in Phosphate buffer pH 7.4 containing 20% methanol. This solution was scanned in the UV range of 200-400nm (Figure 1).

2. **Preformulation studies of drug**

2.1. ***Determination of Melting point:*** The observed melting point of Itraconazole was 167^oC.

2.2. ***Determination of Solubility:*** The solubility of the drug in methanol was found to be around 1.73mg/ml. The solubility of the drug in phosphate buffer was about 0.023mg/ml.

3. Compatibility studies

3.1. ***FTIR spectroscopy analysis:*** FTIR spectra of the pure drug and physical mixture with drug were recorded on Attenuated Total Reflectance Shimadzu Fourier Transform Infrared (FTIR).

3.2 ***Differential Scanning Calorimetry (DSC) analysis:*** Differential scanning calorimetry was performed to confirm the purity as well as the compatibility of drug with the excipients. The DSC thermogram of pure Itraconazole exhibited an endothermic peak at 167.56^{o}C. The physical mixture of Itraconazole with phospholipon 90G showed an endothermic peak at 163.47^{o}C respectively, nearly the same temperature as that of pure drug, indicating no interaction between drug and excipients. The DSC thermogram of final product indicated that the drug maintained its crystallinity form during the lyophilization process.

4. Formulation of liposomal suspension by thin film hydration method using central composite design

4.1 Formulation study:

a. **Selection of solvent:** Chloroform: methanol in ratio of (2:1) was tried for dissolving the lipid component. With chloroform: methanol solvent there was slight precipitation of lipid fragment before drying to avoid this, chloroform alone was selected as solvent for lipid dissolution.

b. **Selection of lipid: cholesterol ratio**: Formulation of varying lipid: cholesterol in range of 6:4-8:2 was prepared. When the ratio of 5:5 was used, it resulted in lipid precipitation in hydration medium. c. **Selection of glass bead:** The time of hydration of the lipid film was observed to be higher in absence of beads. The glass beads allowed formation of uniform thin film, and proper mixing of lipid film with hydration media. This prevented any chance of lipid fragment precipitation.

4.2. Central Composite design: Response Surface Methodology (RSM) is a combination of statistical and mathematical methods used to select the best experimental parameters requiring the lowest number of trials in order to get appropriate results. A Central Composite Design (CCD) with two independent variables at three levels was used. A total of 10 experimental runs comprising center points and edge midpoints lying on the cube was developed using central composite design. Centre points were replicated for two times to design a total of 10 experimental runs. The independent variables were selected based on literature survey. The observed responses were used to derive a response surface plot and second order polynomial equation was generated employing multiple regression analysis. The observed experimental responses value was fitted through various model such as linear, cubic, quadratic and 2FI using design software. Based on the multiple correlation coefficient values, model was selected.

5. Characterization of liposomal dispersion

5.1. Appearance: Liposomal dispersion appeared to be milky white in colour.

5.2. Determination of particle size, polydispersity index and zeta potential of the formulation: Particle size analyses of the liposomal dispersion were done using dynamic light scattering technique. Results of the particle size and PDI are displayed in Table No 2.

Table 2: Particle size, PDI and Zeta potential values of Itraconazole Loaded Liposome in Nail Lacquer

Formulation	Particle size(nm)	PDI	Zeta potential(mV)
F1	379	0.398	-25.3
F2	165	0.402	-31.7
F3	1065	0.827	-23
F4	209.4	0.410	-37.5
F5	197.2	0.374	-29.5
F6	584	0.712	-26
F7	1073	0.700	-62
F8	620	0.459	-23
F9	277	0.265	-30.6

5.3. Determination of zeta potential: The zeta potential of the formulations was measured using Malvern zeta sizer analyzer. The negative potential value is due to neutral charge of the lipid since phosphatidylcholine is a zwitterion compound with isoelectric point 6 and 7. Results of the zeta potential of the formulation are mentioned in Table no 2.

5.4. Determination of Entrapment Efficiency: Entrapment efficiency of the formulation batches was conducted. The influence of the independent variables on the response was studied using 3D response surface plot. With increase in the amount of lipid: cholesterol ratio the entrapment efficiency increases. For every lipid: cholesterol concentration

for the selected 9 formulations, it was observed there was an increase in entrapment efficiency with decrease in cholesterol concentration. In case of 8:2 lipid: cholesterol ratio, a higher entrapment was observed. This kind of behaviour was due to individual molecular interaction between phospholipids, cholesterol and drug. Cholesterol is responsible for increase in hydrophobicity in the central region of the membrane bilayer and inclusion of hydrophobic molecules. Both drug and cholesterol prefer to align themselves in the hydrophobic region of the membrane and there is limited space available for both, cholesterol and drug that will end up in competition for the space between acyl chains in phospholipid thereby resulting in low entrapment. The drug: lipid ratio showed minimal effect on the entrapment efficiency.

5.5. Drug content: For various liposomal dispersion, the drug content was found to be in range 83 ±1.59% to 97.4 ±2.05%. The results are tabulated in table 3. The data was analyzed for mean and standard deviation.

5.6. in-vitro Drug release: The drug release from liposome was found to increase with increase in drug: lipid ratio. In case of lipid: cholesterol the drug release was increasing with increase in lipid: cholesterol. The concentration of cholesterol plays an important role in mediating the membrane permeability and fluidity. For all drug: lipid ratio, at its lowest cholesterol concentration, the drug release was observed to be highest.

6. Optimization of the liposomal dispersion: Design space was generated from the overlay plot. The optimized formula was predicted within the design space. The level of independent variables that would give the optimum response was predicted as A (1), B (1). The responses predicted at the mentioned levels of independent variables were entrapment efficiency 92 ±1% and drug release 78.07 ± 0.32%. The observed response for the batch was 96 ±1.2% and drug release 78 ±0.28. Since the data mean falls within prediction interval range the model becomes suitable for the experiment.

Table 3: Drug Content of the Liposomal Formulations

Formulation	Drug Content (%)
F1	93.9 ± 1.69
F2	94.8 ± 1.59
F3	97.8 ± 1.57
F4	95.2 ±1.99
F5	90.5 ±1.84
F6	97.4 ±1.55
F7	96.5 ±1.85
F8	83.0 ±1.59
F9	97.4 ±2.05

7. Characterization of optimized formulation

7.1 Particle size determination: This test was conducted using Malvern zeta sizer

7.2. Determination of zeta potential: This test was conducted using Malvern zeta sizer

7.3. Morphology study of particle: This test was conducted by Cryosem analysis.

7.4. Determination of Entrapment Efficiency: The entrapment efficiency of the optimized formulation was found to be 96 ±1.2

7.5. in-vitro drug release: *in-vitro* release data of the optimized formulation F9 was fitted to various models that are Zero order, First order, Higuchi's plot and Peppa's plot. The data from *in-vitro* release study of formulation F9 was subjected to linear regression analysis and values for regression coefficient (R) and release rate constants (K) were determined.

8. Lyophilization of the optimized formulation: Lyophilization is an important process that helps in improving stability of liposomal formulation by overcoming most of the stability issues associated with liposomes.

9. Characterization of lyophilized liposomes:

9.1 Determination of Entrapment Efficiency: The entrapment efficiency of lyophilized formulation was found to be 96±1.2 which was in close to value before lyophilization. Thus, it was concluded that cryoprotectant has stabilizing effect on vesicles in freeze dried state there by protecting it against fusion and leakage.

9.2 Determination of particle size polydispersity index and zeta potential: The particle size of formulation was found to be 309.4nm with PDI of 0.474 and zeta potential -29.8mv. There was slight increase in particle size and PDI in an acceptable range.

9.3 Morphology study: This test was carried out using scanning electron microscopy.

9.4 Drug content: The drug content of freeze-dried formulation was found to be 96% ±0.25.

9.5 in-vitro drug release: The drug release of lyophilized formulation was found to be 77.8% ±0.2 which was similar to that observed before lyophilization indicating there was no change in drug release profile.

10. Formulation of nail lacquer base

10.1 Formulation study:

A. Selection of polymer: Eudragit RL 100, RS 100 and various grades of HPMC polymer were used for film forming agent. Solubility of the polymer in the given solvent base was ideal criteria of selection. Eudragit RS 100 was found to be insoluble resulting in turbid solution hence Eudragit RL 100 was selected. Amongst its various grades, HPMC E 15 grade was found to be soluble in acetone.

B. Selection of concentration range for polymer: Ideal concentration range for HPMC E 15 must be maintained in range of 1-1.5%w/v to avoid its precipitation.

C. Selection of plasticizer: Castor oil and PEG 400 was first choice of plasticizer for the formulation but there was turbidity observed upon addition of castor oil hence concluding that PEG 400 was an appropriate plasticizer for the formulation.

11. Evaluation of nail lacquer formulation

11.1 Non-Volatile Content determination: The results were given in following table no 5

11.2. Drying time: Results given in table no 6

11.3 Smoothness of flow: Results given in table no: 6

11.4. Drug content determination: Results given in table no 7

11.5. Film thickness determination: Results given in table no 7

11.6. Adhesive strength determination: Results given in table no 8

11.7. Viscosity determination: Results given in table no 8

Table 4: Values of Regression Coefficient and Kinetics for Optimized Formulation (F9)

Formulation Code	Zero Order plot		First Order plot		Higuchi plot		Peppa's plot	
	R^2	K	R^2	K	R^2	K	R^2	K
F9	**0.943**	**5.47**	**0.964**	**-0.046**	**0.9742**	**21.1**	**0.9534**	**0.43**

Table 5: Non-Volatile Content Determination of Lacquer Base

Formulation	Non-volatile content (%)
ERL I	43.04 ± 0.16
ERL II	44.03 ± 0.16
ERL III	44.13 ± 0.18
Standard Lacquer	42.4 ± 0.18

Table 6: Physical Appearance and Drying Time Determination of Nail Lacquer Base

Formulation	Smoothness of Flow	Non-volatile content (%)
ERL I	Smooth dry film without any coarse particles or pinholes	43.04 ± 0.16
ERL II		44.03 ± 0.16
ERL III		44.13 ± 0.18
Standard Lacquer		42.4 ± 0.18

Table 7: Drug Content and Film Thickness Determination of Nail Lacquer Formulation

Formulation	Drug content (%)	Film thickness (mm)
ERL I	98 ± 1.0	0.18
ERL II	96.4 ± 1.0	0.17
ERL III	94.2 ± 1.0	0.18
Standard Lacquer base	96 ± 1.0	0.18

Table 8: Adhesive Strength and Viscosity Determination of Nail Lacquer

Formulation	Adhesive strength(N)	Viscosity determination(cP)
ERL I	14.15	525
ERL II	15.5	580
ERL III	15.75	636
Standard Lacquer base	14.25	520

12. *in-vitro drug release*

12.1 Antifungal activity: The optimized formulation was subjected to antifungal studies. The zone of inhibitions obtained for candida species was found to be higher than aspergillus species. The zone of inhibition of itraconazole loaded liposomes in lacquer base for both candida and aspergillus species was found higher than pure drug. There was no significant difference in terms of antifungal activity exhibited by standard itraconazole nail lacquer and itraconazole loaded formulation thus concluding that itraconazole loaded liposomes in lacquer base has efficient antifungal activity.

The pure drug showed a release of 20.6% at end of 12 hr. The drug in standard lacquer base showed a release of 18.4% whereas the drug in liposome formulation showed a release of 68.7% at the end of 12hrs indicating that liposome has improved the solubility of drug. The optimization of the nail lacquer base was done depending upon the *in-vitro* release. The itraconazole loaded liposomes in ERL I (1.5%) as optimized base showed drug release of 68.7% at the end of 12hr. The phosphate buffer with 20% methanol was taken to maintain the sink condition required during release. The R^2 value obtained for optimized base was 0.904 which was close to 1.0, thus indicating that drug release from nail lacquer formulation follows zero order kinetics. The R^2 value of optimized formulation when subjected to Higuchi model was found to be 0.975 which is nearly approaching 1.0. The result indicated that itraconazole liposomes in nail lacquer base exhibited diffusion mechanism in drug release. The n value derived from Peppa's plot was greater than 0.5 thus concluding that drug release follows Fickian diffusion.

12.2. *in-vitro transungal permeation:* The *in-vitro* transungal permeation data was subjected to various kinetic models where the R^2 and slope values of various models were calculated.

13. Stability studies: Stability study of liposomal dispersion was conducted for three months at two different temperatures i.e. room temperature and refrigerated temperature. Particle size, poly dispersity index, zeta potential and entrapment efficiency were evaluated for optimized liposomal dispersion. The particle size was found to increase for stored samples at different temperature conditions. There was a decrease observed in entrapment efficiency which could be possibly due to fusion or aggregation of particles leading to the rupture of vesicles and increase in free drug concentration. In case of itraconazole loaded liposomes in nail lacquer, there was no significant change found in drug content and drying time. The drug content and drying time of itraconazole loaded liposome in lacquer base was found to be 97% and a drying time of 03 min.

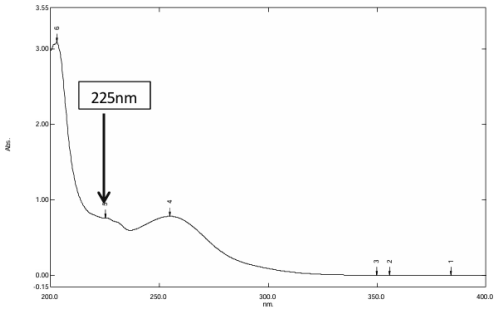

Figure 1: UV-Scan of Itraconazole (10µg/ml) in Phosphate buffer pH7.4 with 20% Methanol

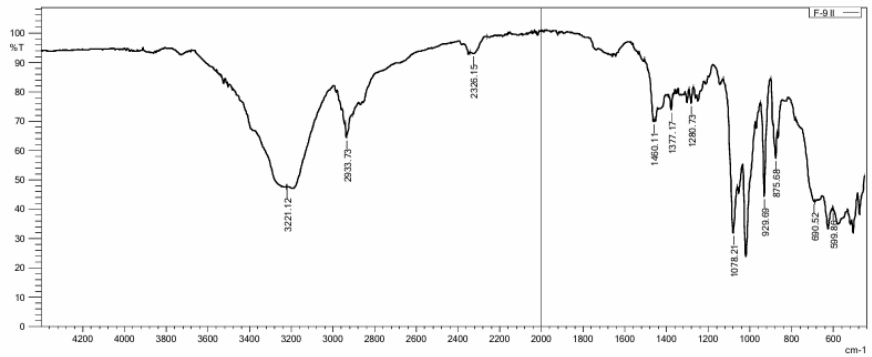

Figure 2: IR Spectra of Freeze-Dried Itraconazole Loaded Liposomes

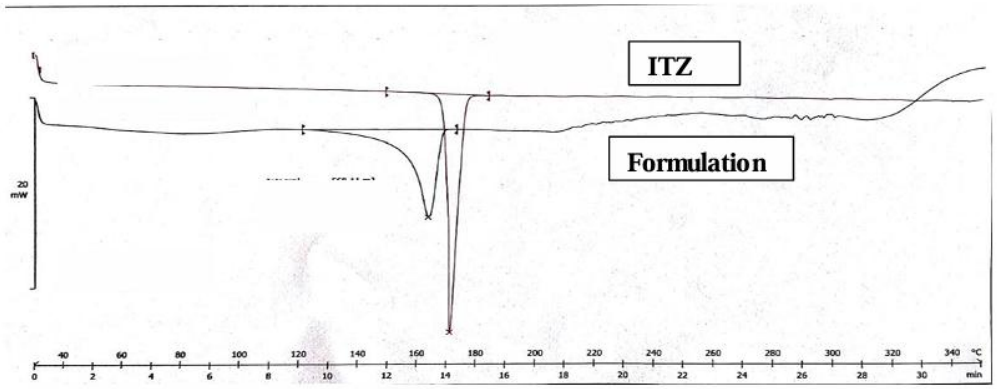

Figure 3: DSC thermogram of Freeze Dried Itraconazole Liposomes

Figure 4: Formulation Batches of Itraconazole Liposomes

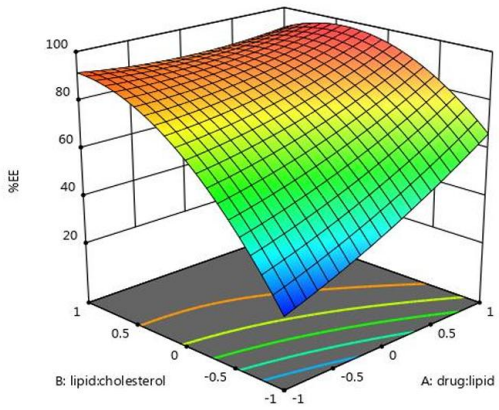

Figure 5: 3D Response Plot for Entrapment Efficiency

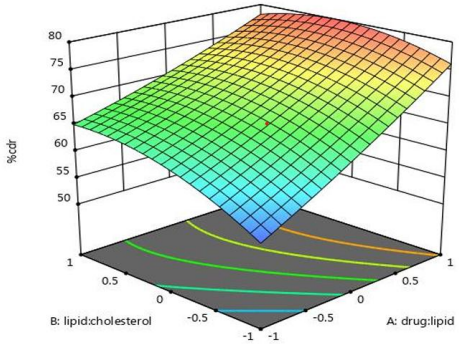

Figure 6: Response Surface Plot for % Cumulative Drug Release

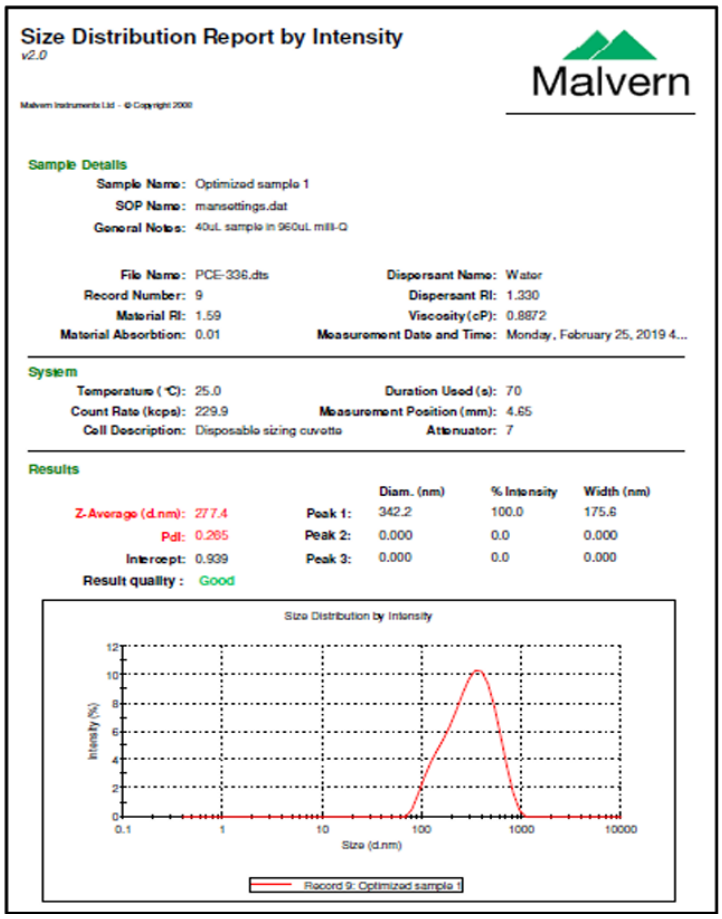

Figure 7: Result of Particle Size and Polydispersity Index of Optimized Formulation (F9)

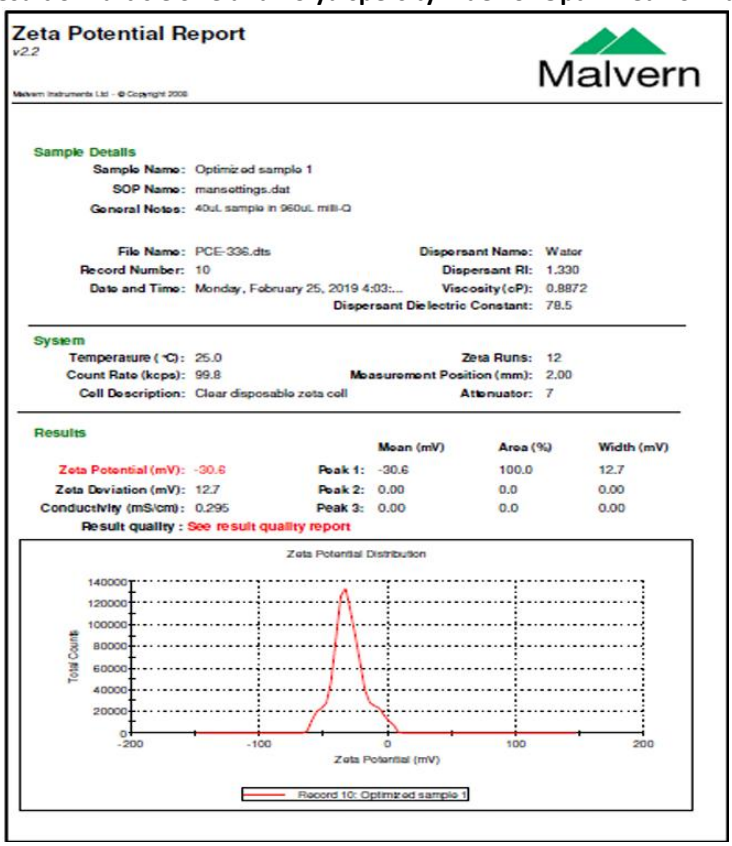

Figure 8: Result of Zeta Potential of Optimized Formulation (F9)

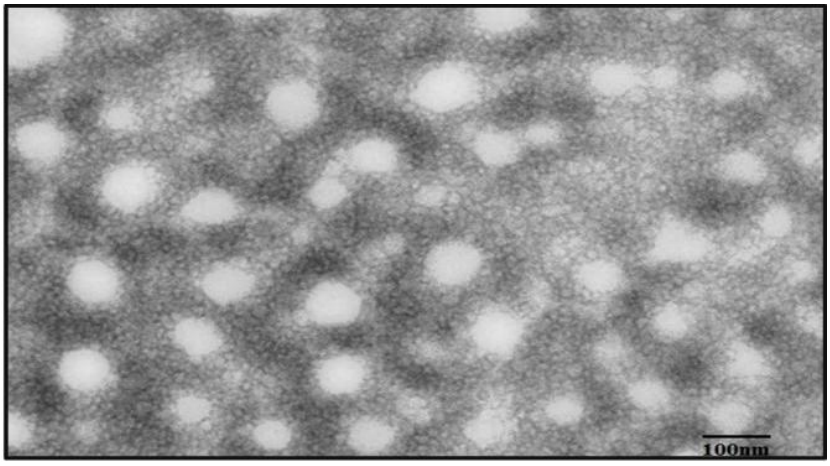

Figure 9: Cryo-Sem Images of Optimized Liposomal Formulation (F9)

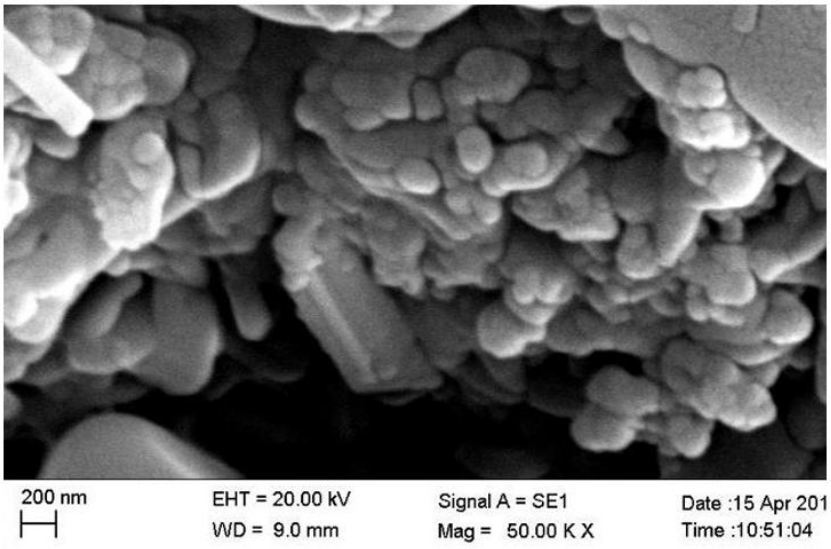

Figure 10: SEM Analysis of Lyophilized Formulation (F9)

Figure 11: Itraconazole Loaded Liposome in Nail Lacquer

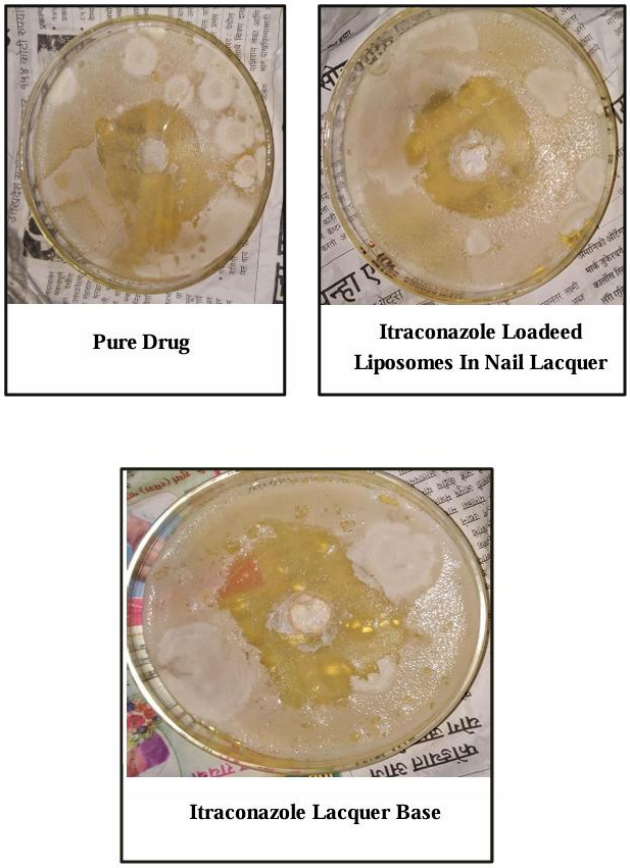

Figure 12: Antifungal Activity Test of Itraconazole Formulations Against Candida Species

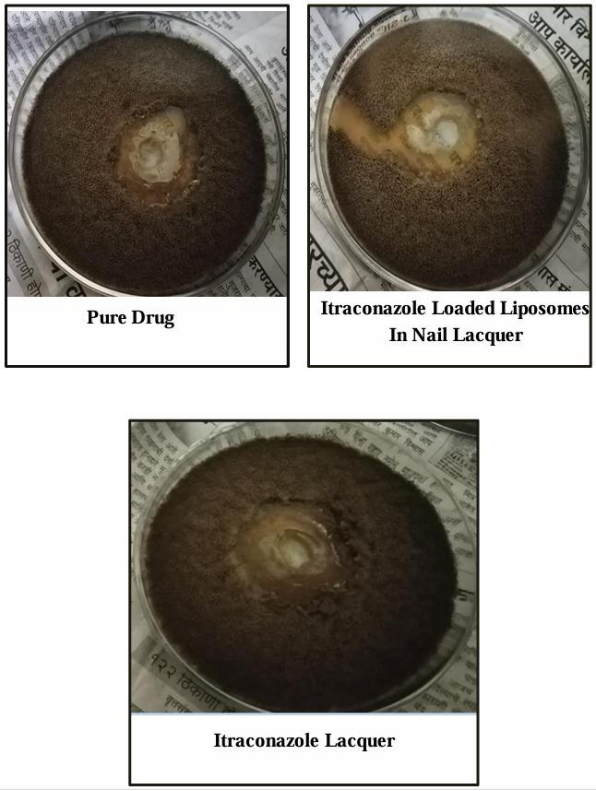

Figure 13: Antifungal Activity Test of Itraconazole Formulation Against Aspergillus Niger

Figure 14: *in-vitro* Transungal Permeation Assembly

SUMMARY AND CONCLUSION

Novel drug delivery systems are investigated and used in today's research field since they have proved to be a suitable system for delivery of drug in an effective way. Liposomes are generally known for their enhancing permeation property of drugs across the biological membrane thereby improving the therapeutic efficacy of many drugs. In the present work, a topical liposomal formulation of an antifungal agent of itraconazole in a nail lacquer base was developed with an aim of overcoming the oral side effects associated with the drug. Liposome helps in permeation of the drug across the nail. Itraconazole liposomal dispersion was formulated by thin film hydration method using rotary evaporator. Phosphotidylcholine and cholesterol were used as lipid bilayer forming agent. The influence of various ratios of drug: lipid, lipid: cholesterol on the entrapment efficiency and drug release of the formulations were studied by using the software "Design of Expert" which was used to optimize the liposomal formulation. The optimized formulation was than evaluated for various tests such as particle size, polydispersity index, zeta potential, entrapment efficiency and morphology studies. The particle size, PDI and zeta potential of the formulation was found to be 227nm, 0.265and -30.5mv respectively. Entrapment efficiency and in vitro drug release of the optimized formulation was found to be 96 %±1.2 and 78%±0.28 respectively. The stability related issues of the liposomal dispersion were overcome by lyophilization using 5%w/v sucrose. Lyophilized liposomes were subjected to evaluation tests such as particle size, PDI, zeta potential, entrapment efficiency and drug release. Morphology determination was done using SEM analysis. The results obtained were in acceptable ranges. The finally optimized itraconazole liposome was incorporated in nail lacquer base. Standard drug lacquer base was prepared for determination of efficiency of liposome loaded in nail lacquer preparation over plain nail lacquer.

The formulations were subjected to various evaluation tests such as drying time, non-volatile content, viscosity determination, drug content, adhesive strength and smoothness of flow. In vitro studies such as determination of antifungal, drug release and transungal permeation studies were conducted. Optimization of itraconazole loaded liposomes nail lacquer was done based on its release parameter. The formulation showed acceptable physiochemical property. % CDR of optimized formulation was found to be 68.9% at end of 12hr. The itraconazole liposome lacquer base has effective antifungal activity against the fungal species.

Transungal permeation of drug across the goat hoof was found to be 67.4% at end of 12hr.

The calculated flux for both standard lacquer and itraconazole liposome nail lacquer was about to be 8.68µg/cm^2/h. and 33.51µg/ cm^2/h. A stability study of liposomal dispersion was conducted for 3 months at two different temperatures i.e. refrigerated and normal temperature. There was an increase observed in particle size and PDI along with a decrease in entrapment efficiency. Hence, lyophilization of the formulation was found to be a solution to overcome the stability related problem. In case of itraconazole loaded liposomes in nail lacquer base, it was observed that there was no significant change in the drug content and drying time. Hence, it can be concluded that itraconazole loaded liposome in nail lacquer base can be a promising delivery system in the treatment of onychomycosis through transungal route.

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