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4. EXPERIMENTAL INVESTIGATIONS

4.1. Preformulation Studies

4.1.1. Physico-chemical characterization of API

Preformulation study is the fundamental step in the development of new formulations which involves characterization of different physico-chemical parameters which directly influence on the development of formulations. In this study various physico-chemical and mechanical properties of drug and excipients to be characterized so as to develop an effective dosage form. A thorough understanding of the properties may ultimately provide a rationale for formulation design.

4.1.2. Identification of drug sample

The Nystatin drug, received as gift sample, was characterized and confirmed by studying its physicochemical parameters like appearance, solubility, absorption maximum and FTIR study. During the identification process the drug sample was used without further purification.

4.1.3. Description of drug sample

The drug sample was studied for organoleptic characters such as appearance, colour, odour and solubility. The drug sample appeared yellow in colour with cereal like odor.

The solubility study and determination of absorption maximum ($\lambda_{\text{max}}$) of the drug sample was studied in phosphate buffer saline of pH 7.4. The study was carried out as follows.
4.1.4. Preparation of phosphate buffer saline pH 7.4

Accurately, 1.564gm of sodium hydroxide and 6.804gm potassium dihydrogen phosphate were weighed and solubilized in small volume of distilled water. After complete solubilization of both the ingredients, the volume was adjusted to 1000ml with distilled water. The resulting solution pH was measured and adjusted to pH 7.4, if necessary.

4.1.5. Determination of absorption maximum ($\lambda_{\text{max}}$) of drug sample

A stock solution of drug sample was prepared by dissolving 20mg of drug sample in 100ml of phosphate buffer saline of pH 7.4. From the above stock solution, 2µg/ml solution was prepared by serial dilution. The prepared solution absorbance was recorded between the wavelengths 200-400nm by UV-Visible spectrophotometer. From the absorbance data, wavelength at which maximum absorbance was exhibited by the drug sample was noted. The observed wavelength was considered as absorption maximum and was found at 306nm. The absorption spectrum of given drug sample shown in figure 5.1.

4.1.6. Determination of Solubility of drug sample

100ml of supersaturated solution of nystatin was prepared by dispersing the nystatin in deaerated phosphate buffer saline of pH 7.4 and kept aside for 12 hrs to achieve equilibrium with occasional shaking at room temperature. From the above super saturated solution, 10ml of supernatant liquid was collected and centrifuged at
3000RPM for 10min. After centrifugation, 1ml of supernatant was transferred into the 100ml volumetric flask and volume adjusted to 100ml with PBS of pH7.4. The flask was shaken well and the resulting solution absorbance was measured using UV-Visible Spectrophotometer at 306nm. Based on the absorbance, the solubility of the drug sample in PBS of pH7.4 was calculated and tabulated in 5.1.

4.1.7. **FTIR study of the drug sample**

FTIR spectrum of drug sample was determined to find out the characteristic peaks for lactone carbonyl, N-H, C-OH, O-H bond stretching and Carboxylate ion. The FTIR spectrum of drug sample has shown in figure 5.2.

Based on the results of the solubility, absorption maximum and FTIR studies, the drug sample was confirmed as Nystatin.

4.2. **Standard curve of nystatin in phosphate buffer saline pH 7.4**

A stock solution of nystatin was prepared by dissolving 20 mg of drug in 100ml of phosphate buffer saline of pH 7.4. From the above stock solution, solutions having concentrations of 2, 4, 6, 8 & 10µg/ml were prepared by serial dilutions. The resultant solutions absorbance was recorded by UV–Visible spectrophotometer at 306 nm. The absorbance of the solutions tabulated in 5.2 and the standard curve is in figure 5.3.
4.3. **Formulation of nystatin loaded niosomes**

Different niosomal suspensions of nystatin were prepared by changing the proportions of the drug, surfactants and surfactant type using thin-film hydration technique as described by Ketul K Patel et al\(^{169}\) with slight modification. Accurately weighed quantities of drug, surfactant (Span – 60 & Span - 80) transferred into a round bottom flask and are dissolved in chloroform. The chloroform was evaporated at 60°C under reduced pressure using a rotary flash evaporator (Buchi type). After chloroform evaporation, the flask was kept under vacuum overnight to remove residual solvent. The thin film was hydrated with 10 ml of PBS pH 7.4 and the flask was kept rotating at 60°C. Formulations were sonicated three times in a bath-sonicator for 15 min with 5-min interval between successive times. The composition of all niosomal suspensions are tabulated in 5.3.

4.4. **Formulation of nystatin loaded ethosomes**

Ethosomal suspensions were developed according to the method reported by Touitou\(^{170}\). Lecithin and drug were dissolved in ethanol and the mixture was heated to 30±1°C. Double distilled water (consisting of dissolved cholesterol) was heated to 30°C±1°C and was added slowly as a fine stream to the lipid mixture with constant stirring (Mechanical stirrer-Remi equipments, Mumbai) at 700rpm. The entire process was carried out in a closed vessel to prevent the evaporation of ethanol at a temperature of 30°C±1°C. The stirring was continued for 5 more minutes after addition of aqueous solution to
lipid mixture which results in the formation of ethosomal suspension. Composition of all ethosomal suspensions are tabulated in 5.4.

4.5. Preparation of nystatin loaded transferosomes.

All transferosomal suspensions containing nystatin were prepared by the thin-film hydration method as described by Alpana Ram et al\textsuperscript{171} with slight modification. Accurately weighed quantities of drug, surfactant (Span – 60 & Span - 80) and lecithin were dissolved in chloroform in a round-bottom flask. The chloroform was evaporated at 40°C under reduced pressure using a rotary flash evaporator (Buchi type). After chloroform evaporation, the flask was kept under vacuum overnight to remove residual solvent. The thin film was hydrated with 10 ml of phosphate buffer saline (PBS) of pH 7.4 and the flask was kept rotating at 40°C. All the formulations were sonicated three times in a bath-sonicator for 15 min with 5-min interval between successive times. Composition of all transferosomal suspensions are tabulated in 5.5.

4.6. CHARACTERIZATION

4.6.1. Vesicle Morphology

Morphology (size, shape and surface) of the vesicles present in all vesicular suspensions were characterized. The shape and surface texture of the vesicles was observed by means of Scanning Electron Microscopy (SEM) and the size of the vesicles was measured using optical microscope. The SEM photographs of niosomes, ethosomes and transferosomes of optimized formulations are shown in figures 5.4, 5.15 and 5.27 respectively. The size of the vesicles presents in all
niosomal, ethosomal and transferosomal formulations were tabulated in 5.6, 5.7 and 5.8 respectively.

**4.6.2. Entrapment Efficiency**

Entrapment efficiency of all vesicular suspensions was determined by separating the unentrapped drug. Un-entrapped drug was separated by ultracentrifugation methods as reported by Labiba K. El-Khordagui et al.\textsuperscript{172} with slight modification.

Vesicular suspension was suitably diluted with Phosphate buffer saline solution and centrifuged at 12000rpm for 20min at 8°C. The supernatant liquid was collected and analyzed for concentration of an un-entrapped drug by UV spectrophotometer (PG instruments) at 306nm. Entrapment efficiency of all vesicular suspensions was determined by using formulae given below. The results are also confirmed by dialysis method. Entrapment efficiency of all vesicular suspensions was tabulated in 5.6, 5.7 and 5.8.

\[
\text{Amount of drug entrapped, } Q_e = \text{Total amount of drug, } Q - \text{Amount of unentrapped drug, } Q_u
\]

\[
\% \text{ entrapment efficiency} = \frac{\text{Amount of drug entrapped, } Q_e}{\text{Total amount of drug, } Q} \times 100
\]

**4.6.3. In vitro drug Release Studies**

In vitro drug release from vesicular suspensions was studied by exhaustive dialysis method and was carried out in an artificial diffusion cell\textsuperscript{173}.

Two side open ended glass tube was taken and one side has been closed with Dialysis membrane (HiMedia Laboratories Pvt. Ltd.,
M.Wt cut off: 12000). The fabricated tube was used as donor compartment, in which 1ml of vesicular suspension was taken and the entire set up placed in receptor compartment (Beaker, 250ml capacity) containing 100 ml of phosphate buffer saline. The dialysis was carried out at 50 rpm at 37±0.5°C for 12hrs. Every hour, 2ml of Phosphate buffer saline from receptor compartment was withdrawn and the same volume of fresh Phosphate buffer saline was replaced to maintain sink conditions. The withdrawn receptor fluids were suitably diluted and analyzed using UV spectrophotometer at 306nm. The *In vitro* drug release from all niosomal, ethosomal and transferosomal formulations was tabulated in 5.9-5.17, 5.19-5.27 and 5.29-5.37 respectively and the same was graphically presented in figures 5.5-5.6, 5.16-5.18 and 5.28-5.29 respectively.

**Specifications of the *In vitro* drug release study**

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Artificial diffusion cell</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Dialysis membrane (M.Wt cut off: 12000daltons)</td>
</tr>
<tr>
<td>Diffusion medium</td>
<td>Phosphate buffer saline pH 7.4</td>
</tr>
<tr>
<td>Volume of sampling fluid</td>
<td>2ml</td>
</tr>
<tr>
<td>Temperature</td>
<td>37±0.5°C</td>
</tr>
<tr>
<td>RPM</td>
<td>50</td>
</tr>
<tr>
<td>Absorption maximum</td>
<td>306nm</td>
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**4.6.4. Kinetic modelling of drug release:**

The order and mechanism of Nystatin release from all vesicular suspensions was determined by fitting the *In vitro* drug release data in
the following mathematical models. The release kinetics of drug from all formulations was evaluated by using the linear regression method.

1. Zero-order kinetics (cumulative % release vs time),
2. First-order kinetics (log % drug remaining vs time),
3. Higuchi kinetics (cumulative % drug release vs. square root of time),
4. Korsmeyer - Peppas (log cumulative % drug release vs log time) and
5. Hixson-Crowel models (cubic root of drug remaining vs time).

The \( r^2 \) and \( n \) values are calculated for the linear curves obtained by regression analysis of the above plots. The release pattern from all vesicular suspensions was concluded based on the \( r^2 \) and \( n \) – values. The \( r^2 \) and \( n \) – values of all niosomal, ethosomal and transferosomal suspensions was tabulated in 5.18, 5.28 and 5.38 respectively. Release kinetics of niosomal, ethosomal and transferosomal suspensions are graphically presented in figures 5.7-5.14, 5.19-5.26 and 5.30-5.37 respectively.

Based on the vesicle size, entrapment efficiency and \textit{In vitro} drug release profile of all vesicular suspensions, one vesicular suspension from each type of vesicular formulations, was selected as an optimized formulation. Elasticity of the membranes and surface charge of the vesicles present in the three optimized suspensions (each from one type vesicular suspension) was characterized as they are important factors which influence on the drug permeability across
the semipermeable membrane and physical stability of the vesicles respectively.

4.6.5. Elasticity measurement

Elasticity of the vesicles was studied by adopting extrusion method\textsuperscript{175}. Three optimized vesicular suspensions were allowed to pass through the filter paper having pore size of 50nm under vacuum conditions for 5min. After 5min, the volume of suspension permeated and the size of the vesicles present in the permeated suspension was measured. Then elasticity of the vesicles was calculated using the following formula. Elasticity study results of all three optimized suspensions are tabulated in 5.39.

\[ E = J \left( \frac{r_v}{r_p} \right)^2 \]

Where, \( E \) – Elasticity of vesicle membrane; \( J \) – Volume of suspension extruded in 5 min; \( r_v \) - Vesicle size after extrusion and \( r_p \) - Pore size of the barrier.

4.6.6. Zeta potential

Zeta potential\textsuperscript{176} of the optimized formulations was measured by instrument zetasizer nano ZS using DTS software (Malvern Instrument Limited, UK) using M3-PALS technology. Zeta potential study results of three optimized vesicular suspensions were tabulated in 5.40.

4.6.7. Preparation of conventional and vesicular gels

Using raw Nystatin and optimized vesicular suspensions, four gel was prepared having concentration of 0.01%w/w. for preparation
of gels, carbopol-971 and HPMC was used as gelling agents and are used at a ratio of 2:1. (Total concentration of gelling agents was maintained as 1.5%w/w) 177. Propylene glycol was used as co-solvent and as dispersion medium for nystatin. The performance of the gel, viscosity and physical properties was enhanced by adding HPMC to carbopol. Neutralization and pH of the gel was adjusted by adding triethanolamine (quantity sufficient).

Half quantity of the double distilled water was heated to 90°C and HPMC was added gradually to the above water with continuous stirring. The remaining half quantity of water was cooled to 5°C and this water added to the HPMC dispersion with continuous stirring. Required quantity of Carbopol which was passed through sieve no. 40 was weighed and added to the HPMC dispersion while stirring. The resulting gel pH was adjusted by adding triethanolamine. Required quantity of Nystatin was dispersed in propylene glycol and transferred in to the mixture of carbopol and HPMC with continuous stirring. Composition of all three formulations is tabulated in 5.41.

4.6.8. *In vitro* drug permeation from niosomal, ethosomal, transferosomal and conventional gels

*In vitro* drug release from vesicular and conventional gels was studied by exhaustive dialysis method and was carried out in an artificial diffusion cell.

Two side open ended glass tube was taken and one side has been closed with Dialysis membrane (HiMedia Laboratories Pvt. Ltd., M.Wt cut off: 12000). The fabricated tube was used as donor
compartment, in which 1gm of gel was taken and the entire set up placed in receptor compartment (Beaker, 250ml capacity) containing 100 ml of phosphate buffer saline. The dialysis was carried out at 37±0.5°C for 12hrs. Every hour 2ml of PBS of pH7.4 from receptor compartment was withdrawn and same volume of fresh diffusion fluid was replaced to maintain sink conditions. The withdrawn receptor fluids were suitably diluted and analyzed using UV spectrophotometer at 306nm. The *In vitro* drug release from all conventional, niosomal, ethosomal and transferosomal gels was tabulated in 5.42, 5.43, 5.44 and 5.45 respectively and the same were graphically presented in figures 5.41.

**Specifications of the *In vitro* drug release study**

<table>
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<tr>
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<td>RPM</td>
<td>50</td>
</tr>
<tr>
<td>Absorption maximum</td>
<td>306nm</td>
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### 4.6.9. *Ex vivo* drug permeation from the conventional, niosomal, ethosomal and transferosomal gels

*Ex vivo* drug permeation studies of all gels were carried out using an artificial diffusion cell consisting of effective diffusional area of 1.32 Sq.cm and 100 ml of PBS pH 7.4 was placed in receiver
compartment as diffusion medium. Between donor and receiver compartments rat abdominal skin was mounted and is used as semipermeable membrane\textsuperscript{178}.

Dehaired skin of anaesthetized male albino rats weighing between 200-250gms was separated from the adhering tissues. Circular section of suitable diameter was cut to place in the diffusion cell. The subcutaneous tissue from the skin was removed surgically and the fat content adhered to the skin was removed by washing with isopropyl alcohol followed by distilled water.

The prepared abdominal rat skin was arranged between donor and receptor compartments in such way that the stratum corneum, barrier of the skin, was faced towards the donor compartment and the dermal side faced towards the receiver compartment side.

In donor compartment, 1gm of gel was taken and the receiver compartment was filled with 100ml of diffusion medium, Phosphate buffer saline pH 7.4 and a magnetic bead was placed in the PBS to bring the agitation. The diffusion was carried out at 50 rpm at 37°C for 10hrs. Every hour 2ml of PBS, pH 7.4 from receptor compartment was withdrawn and same volume of fresh diffusion fluid was replaced to maintain sinc conditions. The withdrawn receptor fluids (PBS, pH 7.4) were suitably diluted and analyzed using UV spectrophotometer at 306nm. The \textit{ex vivo} drug release from all conventional, niosomal, ethosomal and transferosomal gels was tabulated in 5.46. 5.47, 5.48 and 5.49 respectively and the same were graphically presented in figures 5.42.
Specifications of *ex vivo* drug release study

Equipment : Artificial diffusion cell
Semipermeable membrane : Rat Skin
Diffusion medium : Phosphate buffer saline pH 7.4
Volume of sampling fluid : 2ml
Temperature : 37±0.5°C
RPM : 50
Absorption maximum : 306nm

4.6.10. Transdermal flux

Amount of material flowing through a unit cross-sectional barrier in unit time is said to be flux.

Mathematically it is expressed as,

\[ J = \frac{Q}{At} \]

Where,

J is the flux

Q is the quantity of compound traversing the membrane in time t

A is the area of exposed membrane in cm\(^2\)

Flux of drug from all gels was calculated and compared with each other.

4.6.11. Data analysis of the *ex vivo* drug release

Cumulative amount of nystatin permeated (µg/cm\(^2\)) through the membrane vs time (min) plot was constructed and parameters like transdermal flux (Jss, µg/cm\(^2\)/min) at steady state, permeation
coefficient \((K_p, \text{cm}^2/\text{min})\), etc was calculated. Slope of the linear portion of the cumulative amount of nystatin permeated vs time was considered as transdermal flux. Permeation coefficient \((K_p)\) calculated by dividing the flux with the initial concentration of the drug taken in the donor compartment and enhancement ratio \((E_r)\), extent of increase in permeation of drug by vesicular gels than the conventional gel, was calculated by dividing the nystatin permeation from vesicular gels by concentration of nystatin permeated from conventional gel in a fixed time.

\[
Permeation\ coefficients\ (K_p) = \frac{\text{Transdermal flux at steady state, } J_{rs}}{\text{Concentration of drug in donor compartment}}
\]

\[
Enhancement\ ratio\ (E_r) = \frac{\text{Permeation coefficient of vesicular gel}}{\text{Permeation coefficient of conventional gel}}
\]

4.6.12. Drug retention study:

This is an important parameter for characterization of transdermal drug delivery products which states the amount of drug reaches to the systemic circulation and amount of drug retained in and on the skin. This study will be carried out after completion of the \textit{ex vivo} drug release study. The study was carried out by adopting the method reported by Subheet jain et al\(^{179}\).

After completion of 12hrs \textit{ex vivo} diffusion studies, the skin placed between donor and receptor compartment was collected carefully and the gel present on the skin was scrapped off precisely and preserved for further studies.
The skin was sliced as very small thin pieces using surgical stainless steel scalpel. The pieces were transferred into a 250ml beaker containing 100ml of PBS pH7.4. The beaker was kept under a mechanical stirrer and stirring was initiated at a speed of 1000rpm and the same was continued for 24hrs. The entire process was carried out at a temperature of 37±1°C. After 24hrs, 10ml of solution collected from the beaker and filtered. The filtered solution was centrifuged at 3000rpm for 30min and supernatant fluid was collected and nystatin concentration was quantified using UV-Visible spectrophotometer at 306nm.

The resultant nystatin concentration indicates the amount of the drug retained in the skin. From the above data, amount of nystatin retained on the skin was calculated by subtracting the amount of nystatin permeated (receptor fluid concentration during ex vivo study) and retained within the skin. This was confirmed by analyzing the collected overlying gel. i.e. gel collected from skin surface. The data of skin retention study was tabulated in 5.50.

4.6.13. Stability Studies

In the present study, the stability of the vesicles was characterized as per ICH guidelines. Three optimized formulations preserved at refrigerated temperature (4-8±1°C) and room temperature (25±2°C) for 180days. After 90 and 180days, shape, size and % entrapment efficiency of vesicles were measured. The results were compared with the initial size, shape and % entrapment efficiency of all three samples and are tabulated in 5.51-5.53.

An approved reliable reversed-phase high-performance liquid chromatographic method was used for in vivo study. Four groups, consisting of two rabbits in each group, were taken. Each group was treated with 1gm of gel and is labelled properly.

At different time points, blood sample was collected from ear vein of the rabbits and are analysed by HPLC for concentration of nystatin in each sample. Nystatin is extracted by 1:2 (v/v) liquid-liquid extractions with methanol. Separation is achieved by HPLC after direct injection on a muBondapak C18 analytical column with a mobile phase composed of 10 mM sodium phosphate, 1 mM EDTA, 30% methanol and 30% acetonitrile adjusted to pH 6. Analysis was carried at UV detector at 305 nm. Quantitation is based on the sum of the peak area concentration of the two major isomers of nystatin, which elute at 7.5-8.5 and 9.5-10.5 min. The assay was linear over the concentration range of 0.05 to 50µg/ml. In vivo study results of conventional, niosomal, ethosomal and transferosomal gels are tabulated in 5.56, 5.57, 5.58 and 5.59 respectively. Summary of the nystatin concentrations in blood plasma at different time intervals was tabulated in 5.60 and pharmacokinetic parameters are tabulated in 5.61. HPLC chromatograms of conventional, niosomal, ethosomal and transferosomal gels in rabbits 1&2 are given in figures 5.53-5.68, 5.69-5.84, 5.85-5.100 and 5.101-5.116 respectively.