CHAPTER 2

Materials & Methods
Materials

The drugs, excipients, chemicals/reagents, instruments and animal used for various experiments are enlisted in Table 2.1, 2.2 and Table 2.3 respectively.

**Table 2.1: Materials list**

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>List of Ingredients</th>
<th>Procurements From</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Nystatin (gift sample)</td>
<td>Cipla Pvt. Ltd., Mumbai</td>
</tr>
<tr>
<td>2.</td>
<td>Triamcinolone Acetonide (gift sample)</td>
<td>Galentic Pvt. Ltd., Mumbai</td>
</tr>
<tr>
<td>3.</td>
<td>Sorbitan Monostearate (Span 60)</td>
<td>Loba Chem. Pvt. Ltd., Mumbai</td>
</tr>
<tr>
<td>4.</td>
<td>Cholesterol</td>
<td>E. Merck (India) Ltd., Mumbai</td>
</tr>
<tr>
<td>5.</td>
<td>Stearic acid</td>
<td>Colorcon Asia Pvt. Ltd., Goa</td>
</tr>
<tr>
<td>6.</td>
<td>Ether A.R</td>
<td>E. Merck (India) Ltd., Mumbai</td>
</tr>
<tr>
<td>7.</td>
<td>Sodium hydroxide A.R</td>
<td>E. Merck (India) Ltd., Mumbai</td>
</tr>
<tr>
<td>8.</td>
<td>Potassium Di-hydrogen phosphate A.R</td>
<td>E. Merck (India) Ltd., Mumbai</td>
</tr>
<tr>
<td>9.</td>
<td>Carbopol 934</td>
<td>E. Merck (India) Ltd., Mumbai</td>
</tr>
<tr>
<td>11.</td>
<td>Methanol</td>
<td>E. Merck (India) Ltd., Mumbai</td>
</tr>
</tbody>
</table>

**Table 2.2: List of Equipments**

<table>
<thead>
<tr>
<th>Sr. no.</th>
<th>List of Equipments</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Single pan balance</td>
<td>B154, 1113371982, Mettler Toledo, Switzerland</td>
</tr>
<tr>
<td>2.</td>
<td>Magnetic Constant speed stirrer</td>
<td>Heidolph RZR 2051</td>
</tr>
<tr>
<td>3.</td>
<td>Inverted Microscope</td>
<td>Nikon</td>
</tr>
</tbody>
</table>
4. Centrifuge \hspace{2cm} Eppendorf
5. UV Visible Spectrophotometer \hspace{1cm} Shimadzu-1800, Japan
6. Fourier Transform InfraRed Spectroscopy (FTIR) \hspace{1cm} Agilent, Germany
7. Transmission electron Microscopy \hspace{1cm} CM12; Philips, Bothell, Washington, USA
8. Malvern Instruments \hspace{1cm} Mastersizer 1000, Malvern, UK
9. Stability chamber \hspace{1cm} Remi Motors Ltd., Mumbai
10. Ultra-sonicator \hspace{1cm} Spectra Lab, Mumbai
11. X-ray diffractometer (XRD) \hspace{1cm} PW 1729, Philips. The Netherlands
12. pH meter \hspace{1cm} Remi Motors Ltd., Mumbai
13. Differential scanning calorimetry (DSC) \hspace{1cm} NETZSCH DSC 200F, Diya lab, Mumbai
14. Rheometer \hspace{1cm} Stress-Tech, Rheological, Sweden

Table 2.3: Animal used in experiment

<table>
<thead>
<tr>
<th>Sr. no.</th>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Species / Common name</td>
<td>Rabbits (New Zealand, White)</td>
</tr>
<tr>
<td>2.</td>
<td>Weight</td>
<td>1.6-2 Kg</td>
</tr>
<tr>
<td>3.</td>
<td>Gender</td>
<td>Any Sex</td>
</tr>
<tr>
<td>4.</td>
<td>Source</td>
<td>Animal House of Tatyaasaheb Kore College of Pharmacy, Warananagar, MS, India</td>
</tr>
</tbody>
</table>
2.1.1 Drug Profile

- **Nystatin**

**CAS**: 5687-27-1

**Chemical Name**: 2-(4-isobutyl-phenyl) propionic acid

**Empirical Formula**: \( \text{C}_{47}\text{H}_{75}\text{NO}_{17} \)

**Molecular weight**: 926.13

**Structural Formula**:  

![Structure of Nystatin](image)

**Figure 2.1: Structure of Nystatin**

**Description**: Yellowish crystalline powder, with slight odour

**Physical properties**

- **Melting point (°C)**: 153-155
- **Dissociation constant pKa**: 5.3
- **Partition coefficient**: 4.49

**Solubility**: Practically water insoluble, easily soluble in dimethyl formamide, dimethyl sulfoxide, soluble in 1 in 1 of methanol.

**Mechanism of action**: Act through binding to candidal cell membrane sterol which causes an increase in fungal cell wall permeability leading to its death.
**Pharmacokinetics:** Oral bioavailability 0% orally used for oral candidiasis and superficial mucus membrane infections.

**Adverse effects:** Minor: In oral formulation the adverse effects are diarrhea, nausea or vomiting. In topical formulation itching is the problem but these adverse effects should disappear as your body adjusts to the drug.

**Preparations:** For oral use: Tablets (five lakh units / g), suspension (one lakh unit / ml with not more than 1% alcohol), lozenges (200,000 units per gm)

For Topical use: Cream, Ointment, and Powder (100,000 units per gm)

- **Triamcinolone Acetonide**

  **CAS** : 76-25-5
  **Chemical Name** : Pregna-1, 4-diene-3, 20-dione, 9-fluoro-11, 21-dihydroxy-16, 17-[(1-methylethylidene) bis (oxy)]-, (11β, 16α-)
  **Empirical Formula** : C_{24}H_{31}FO_{6}
  **Molecular weight** : 434.50

  **Structural Formula:**

  ![Figure 2.2: Structure of Triamcinolone Acetonide](image)

**Description:** Crystalline powder with white color and slight odor in nature.

**Solubility:** Soluble in ethanol and chloroform, slightly soluble in ether and insoluble in water.
Mechanism of action: TA act as an anti-pruritic, anti-inflammatory and vaso-constrictive action.

Pharmacokinetics: Corticosteroids are capable to absorb through skin layers. Increase in corticosteroid precutaneous absorption in inflammation and skin disease condition. Metabolism occurs via liver and excretion via kidneys.

Adverse effects: In dermal formulation itching is the problem but these adverse effects should disappear as your body adjusts to the drug.

Preparations: For Topical use like Cream, Ointment, and Powder (1 mg per gm)

2.1.2. Excipient Profile

- **Span 60**: [Konno K, 1974 & Hannuksela M, 1976]

  - Non-proprietary Name: Sorbitan stearate
  - Chemical Name: Sorbitan mono-octadecanoate
  - CAS number: [1338-41-6]
  - Molecular Formula: \( \text{C}_{24}\text{H}_{46}\text{O}_6 \)
  - Molecular Weight: 430.62

  **Structural formula:**

  ![Figure 2.3: Structure of Span 60](image)

  **Description**: Sorbitan mono-stearate occurs as a light off-white to tan-coloured, hard, waxy solid. It is manufactured via reaction between stearic acid and sorbitol.
Solubility data reveal that span 60 is insoluble in water and soluble in ethanol, dioxane, methanol, ether and toluene.

**Specifications:**

<table>
<thead>
<tr>
<th>Specification</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid value</td>
<td>5-10</td>
</tr>
<tr>
<td>Hydroxyl value</td>
<td>235-260</td>
</tr>
<tr>
<td>Saponification value</td>
<td>147-157</td>
</tr>
<tr>
<td>Water content (%)</td>
<td>≤ 1.0</td>
</tr>
<tr>
<td>Melting point (°C)</td>
<td>53-57</td>
</tr>
</tbody>
</table>

**Applications:** Span 60 is used as emulsifying agent (1-15% w/w), wetting agent (0.1-3% w/w) and solubilizing agent (1-10% w/w).

**Stability and storage:** Gradual soap formation occurs with strong acids or bases; sorbitan esters are stable in weak acids or bases. Sorbitan esters must be stored away from humidity.

- **Cholesterol**

**Nonproprietary Names**

- BP: Cholesterol
- JP: Cholesterol
- PhEur: Cholesterolum
- USPNF: Cholesterol
- Synonyms: Cholesterin; cholesterolum

**Chemical Name and CAS Registry Number:** Cholest-5-en-3β-ol [57-88-5]

**Empirical Formula and Molecular Weight:** \(C_{27}H_{46}O\); 386.6

**Structural Formula:**
**Functional Category:** Emollient, Emulsifying agent.

**Applications:** Cholesterol used as emulsifying agent at concentrations of 0.3–5.0% w/w.

**Description:** Cholesterol occurs as white or faintly yellow, almost odorless, pearly leaflets, needles, powder, or granules.

**Pharmacopeial Specifications determination**

<table>
<thead>
<tr>
<th>Test</th>
<th>JP 2001</th>
<th>PhEur 2005</th>
<th>USPNF 23</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOD</td>
<td>≤0.3%</td>
<td>≤0.3%</td>
<td>≤0.3%</td>
</tr>
<tr>
<td>Melting range</td>
<td>147–150°C</td>
<td>147–150°C</td>
<td>147–150°C</td>
</tr>
<tr>
<td>Residue on ignition</td>
<td>≤0.10%</td>
<td>—</td>
<td>≤0.1%</td>
</tr>
<tr>
<td>Solubility in alcohol</td>
<td>—</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sulfated ash</td>
<td>—</td>
<td>≤0.1%</td>
<td>—</td>
</tr>
<tr>
<td>Assay</td>
<td>—</td>
<td>95.0–97.0%</td>
<td>—</td>
</tr>
</tbody>
</table>

**Density:** 1.052 g/cm³ for anhydrous form

**Dielectric constant D:** 5.41
Table 2.5: Solubility of Cholesterol

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Solubility at 20°C unless otherwise stated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>Soluble</td>
</tr>
<tr>
<td>Ethanol (95%)</td>
<td>1 in 78 (slowly)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>1 in 147 at 0°C</td>
</tr>
<tr>
<td>Ethanol</td>
<td>1 in 78 at 20°C</td>
</tr>
<tr>
<td>Ethanol (95%)</td>
<td>1 in 3.6 at 80°C</td>
</tr>
<tr>
<td>Methanol</td>
<td>1 in 294 at 0°C</td>
</tr>
<tr>
<td>Methanol</td>
<td>1 in 153 at 20°C</td>
</tr>
<tr>
<td>Vegetable oils</td>
<td>Soluble</td>
</tr>
<tr>
<td>Water</td>
<td>Practically insoluble</td>
</tr>
</tbody>
</table>

Specific rotation \( [\alpha] \_D \): 39.5° (2% w/v solution in chloroform) and 31.5° (2% w/v solution in ether).

Incompatibilities: Cholesterol is incompatible with digitonin formulation because it precipitated by cardiac glycoside.

Safety: It was reported that cholesterol at 0.3 – 5% w/w concentration was safe and non irritant. At high concentration there may be mutational or teratogenicity occurs.

Storage: Stored in a dry place.

- **Stearic acid** [Yalkowsky S, 2003 & Mores L, 1980]
**Synonyms:** Acidum stearicum; Crodacid; Crosterene; Glycon S-90; Hystrene.

**Appearance:** It is crystalline in nature & yellowish white in color.

**Chemical name and CAS registry number:** Octadecanoic acid [57-11-4]

**Molecular formula:** $\text{C}_{18}\text{H}_{36}\text{O}_2$

**Molecular weight:** 284.47

**Structural Formula:**

![Structure of Stearic Acid]

**Solubility:** Stearic acid is highly soluble in chloroform, benzene and insoluble in water.

**Melting Point:** 57-60 °C

**Application in pharmaceutical formulations or technology:** Stearic acid utilized in oral formulation capsule and tablet as a lubricant (1-3%). In topical formulation it acts as emulsifying agent. When partially neutralized with alkalis or triethanolamine. Stearic acid is used in the preparation of creams concentration range (1-20%).

**Functional category:** Act as Lubricant in solid dosage form and emulsifying agent in semisolid dosage form.

**Stability and storage conditions:** Stearic acid is a stable compound. Stearic acid stored in dry place.

**Incompatibilities:** Incompatible with most metal hydroxides and oxidizing agent. There may be problem of lumpiness when compounded with zinc and calcium salts.

- **Carbopol**
  
  **Description:** Carbomer are white coloured, “fluppy”, and characteristics odour and acidic in nature.
Chemical name and CAS number: Carbomer 910, 934, 934P, 940, 941, 971P and 974P and CAS No. 9003-01-4

Structural Formula:

![Structural Formula](image)

Figure 2.6: Acrylic acid monomer unit in Carbomer polymer

Use: Bioadhesive, release modifier, viscosity modifier and binding agent. 
Application: Used as emulsifying agent (0.1-0.5% w/w), gelling agent (0.5-2% w/w), suspending agent (0.5-1% w/v) and tablet binder (5-10% w/w). [Carnali J, Naser M (1992]

Moisture content: Normal water content: 2 %w/w at 25°C/50% RH

Physiochemical properties: The Carbopol was evaluated for physiochemical properties and the results obtained were as follows


Nonproprietary Names

- BP: Triethanolamine
- PhEur: Trolaminum
- USPNF: Trolamine
Synonyms: TEA, triethylolamine, trihydroxytriethylamine, tris(hydroxyethyl) amine.

Chemical Names and CAS Registry Number: 2, 2', 2"-Nitrilotriethanol [102-71-6]

Empirical Formula and Molecular Weight: $\text{C}_6\text{H}_{15}\text{NO}_3$ 149.19

Functional Category: Alkalizing agent, emulsifying agent.

Applications: Triethanolamine acts as buffering agent mostly in topical dosage form. It utilized as solvents and polymer plasticizer.

2.2 Methods

2.2.1 Preformulation study

In order to develop effective and stable dosage form initial step of development chain was found to be preformulation testing. In that active pharmaceutical ingredient and excipients physical as well as chemical parameters were evaluated which give safe formulation.

Generally aim of preformulation study indicates green signal to formulation development and assurity to develop safe & effective formulation.

2.2.1.1 Characterization of Drug’s & Excipients

Organoleptic properties (Indian Pharmacopoeia, 2007 & British Pharmacopoeia, 2009)

Nystatin & Triamcinolone Acetonide were tested for organoleptic properties such as appearance, colour, taste, etc.

M.P.
Nystatin & Triamcinolone Acetonide melting point was done by open capillary method. Melting point determination gives idea regarding purity of the provided sample, if M.P. was found to be higher or lower than the reported value then there are chances of impurity in test sample. (Indian Pharmacopoeia, 2007 & British Pharmacopoeia, 2009)

**Solubility**
It was carried out in solvent system in which test sample is soluble. (Indian Pharmacopoeia, 2007)

**pH**
pH of Nystatin and Triamcinolone Acetonide was determined in a 5% w/v solution prepared with the aid of gentle heat. (Indian Pharmacopoeia, 2007)

**Loss on drying**
Loss on drying was determined by keeping 0.5 g of Nystatin and 0.5 g of Triamcinolone Acetonide in oven at 105°C for 2 hours. (Indian Pharmacopoeia, 2007 and British Pharmacopoeia, 2009)
Results were compared with specifications given in Pharmacopoeia and certificate of analysis provided by drug sample provider.

**Specific gravity**
It was found out using specific gravity bottle (g / mL). The results of Nystatin & Triamcinolone acetonide are shown in table no. 3.1 & 3.3 respectively.

**IR Spectrum determination**
To characterize sample IR apectroscopy was carried out. The definite concentration of drug is transferred on the stage of instrument and an IR spectrum was recorded using FT-IR Cary 630 model. IR spectrum of Nystatin & Triamcinolone acetonide is indicated in figure 3.1 & 3.2 respectively.

**2.2.1.2 Analytical method development and validation**

**2.2.1.2.1 Introduction**
Analytical branch gives the selectivity of the drug. It involves the more sensitive, simple and specific data for the bulk drug powders and its dosage form. It is easy for the detection of sample purity and standardization with accurate result for
pharmaceutical uses. [Sethi P, 1993] In this study Analytical method was developed and validated for Nystatin and Triamcinolone Acetonide.

A detailed survey literature was carried out and revealed that several methods based on varied techniques, viz. High Performance Liquid Chromatography, High Performance Thin Layer Chromatography and Spectrophotometry were available individually for both Nystatin and Triamcinolone Acetonide. [Kenyon A. 2002, Gupta V. 2006& Andreas H. 1999] No method is available for the simultaneous estimation of these drugs by UV spectrophotometric method.

Literature review suggests that selected API’s i.e. nystatin and triamcinolone acetonide having various analytical techniques like thin layer chromatography, high performance chromatography and spectrophotometry for individual quantitation of drug. But there is no any spectroscopic method which can analyse both the drug’s simultaneously.

Therefore objective was to develop and validate spectroscopic method able to analyse simulataneously Nystatin and Triamcinolone Acetonide in topical formulations.

2.2.1.2.2 Simultaneous equation method development

Stock preparation

The solution 1000 µg/mL of Nystatin and Triamcinolone Acetoinide done separately via adding 10 mg API in 10 mL Ethanol.

Preparation of Calibration Curves

10 ppm of Nystatin and Triamcinolone Acetoinide were prepared separately. Scanning studies was carried out in UV region. The maximum absorbance of Nystatin and Triamcinolone Acetonide was observed at 304 nm and 240 nm respectively. Nystatin and Triamcinolone Acetonide revealed linearity within 2 – 10 µg/mL and 5 – 30 µg/mL on maxima as given in 3.4 & 3.5 graphs. Correlation coefficient was 0.998 for Nystatin and 0.976 for Triamcinolone Acetonide. The obtained contant values ae given in Table 3.7.

Simultaneous Equation Method

Standard solution of 10µg/mL of Nystatin and Triamcinolone Acetonide undergo scanning in UV region as shown in figure 3.3. Wavelengths of Nystatin 304nm,
wavelength of Triamcinolone Acetonide 240 nm were taken for simultaneous estimation of drug. A1%, 1 cm was established for both API’s. Lastly obtained values were added in following equation for quantitative evaluation of respective drug. [Jefferey G, 1989 & Beckett A, 1997]

Concentrations in the sample were obtained by using following equations.

\[
C_x = \frac{A_1 a y_2 - A_2 a y_1}{a x_1 a y_2 - a x_2 a y_1} \quad \text{.........Eq. (i)}
\]

\[
C_y = \frac{A_1 a x_2 - A_2 a x_1}{a y_1 a x_2 - a y_2 a x_1} \quad \text{.........Eq. (ii)}
\]

Where,
A1 (304 nm) and A2 (240 nm) = Absorbances of mixture
ax1 and ax2 nystatin = A1%, 1 cm at 304 nm and 240 nm
ay1 and ay2 TA = A1%, 1 cm at 304 nm and 240 nm
Cx and Cy = Concentration of Nystatin and TA respectively

2.2.1.2.3 Validation

Developed analytical method was validated as per ICH Q2 B guidelines. In validation various parameters were determined like linearity, accuracy, precision inter-intra day. [ICH, 2005 & International Conference on Harmonisation, 1995]

Accuracy

Accuracy means test output match with true value. Recovery study was carried out to assure accuracy validation parameter at 100%, 80% and 50% levels. Accuracy in terms of % recovery Nystatin and Triamcinolone Acetonide was found within the range 97 % to 99 %.

Linearity
It was carried out by determining various concentrations in calibration curve of Nystatin and Triamcinolone Acetonide. Linearity study revealed that Beer-Lambert’s applicable in the range 2 – 10 $\mu$g/mL for Nystatin and 5 – 30 $\mu$g/mL for Triamcinolone Acetonide.

**Precision**

Intra day day and interday precision were carried out for Nystatin and Triamcinolone Acetonide. Intra day precision was carried out in same day otherwise interday precision was carried out on different days. The results are tabulated in Table 3.8.

### 2.2.2 Niosome dispersion

#### 2.2.2.1 Introduction

Non ionic surfactant vesicle is best alternative in topical and transdermal delivery system. Vesicle enhances penetration of API through the skin layer and also showed sustained release pattern. Advantages of surfactant vesicle are biodegradable in nature, low side effect; enhance skin penetration and deposition through skin. Driving force essential for permeation of hydrophobic moiety is high thermodynamic gradient of the active pharmaceutical ingredient at the interface. Vesicle acts as penetration enhancer by diminishing horny layer barrier properties. [Schreier H, 1985]

Surfactant and lipid play important role in the formation of stable niosomes. The niosomes were prepared by Ether injection method.

Formulation development was planned in the following manner

- Preparation of Blank niosomes
- Process optimization with respect to bilayer component and conditions such as rate of addition, stirring speed, stirring time, evaporation time etc.
- Selection of optimize formula
- Preparation of drug loaded niosomes
- Preparation of batches using factorial design
2.2.2.2 Ether injection method

Different concentration of span 60: cholesterol: stearic acid were weighed and dissolved in ether. Drug (10 mg) was dissolved in same solution. This oily solution was injected in 10 ml of preheated aqueous phase at 60-65°C with continuous stirring at 500 rpm on magnetic stirrer. [Agarwal S, 2001] Method is summarized in Figure 2.8.

![Flow chart for preparation of niosomal dispersion](chart)

**Figure 2.8: Flow chart for preparation of niosomal dispersion**

2.2.2.3 Preparation of Blank niosomal dispersions

The various concentration ranges of span 60, cholesterol and stearic acid were dissolved in ether in a small beaker. In another beaker double distilled water was taken. Then the dissolved surfactant / lipid were injected slowly at a rate of 0.25 mL/min, via a 24 gauge needle in double distilled water which was magnetically stirred continuously and maintained at 60-65°C for 30 minute and to ensure complete evaporation of the solvent and to get a uniform suspension of niosome. Batches were prepared wherein the content of span 60 % varied since 0.1 to 1% w/v, whereas cholesterol concentration varied from 0.1 to 0.5% w/v. All batches contain 0.05% w/v of Stearic acid. Various batches were prepared using various concentrations of span 60 and Cholesterol. The dispersions were filled in amber coloured glass vial and observed for appearance, colour, pH, odour and redispersion time.

2.2.2.4 Preliminary characterization of Blank niosomal dispersion
• **Organoleptic properties**
The blank dispersions were evaluated for colour, odour, appearance and texture.

• **Creaming volume**
The blank dispersion were kept undisturbed for 24 hrs in measuring cylinder and evaluated for separation, creaming and redispersibility.

• **pH**
The pH of blank formulation was checked with digital pH meter.

• **Microscopic evaluation**
Formulation of 1 ml was put on slide and evaluated at 400X magnification under microscope to determine any cluster preparation.

• **Changes after two weeks**
The dispersions were kept undisturbed for two weeks and were observed for any changes in the formulations. The results are given in table 3.10.

**2.2.2.5 Effect of processing parameters on formation of niosomes**
After optimizing the concentration of span 60 and cholesterol in the preparation of niosomal dispersions. The processing parameters such as rate of addition & stirring time were optimized.

**Effect of Rate of addition**: The ether solution was injected rapidly as well as dropwise into aqueous phase to study its effect on vesicle size.

**Effect of Stirring time**: The dispersion was stirred for varied time period of 5, 10 and 15 minutes. The results are given in table 3.11.

**2.2.2.6 Optimizing & Preparation of Drug loaded niosomal dispersions**
Optimization was found to be complex process because in that various chemical were used may be having interaction with each other. So to simply this complex process there is use of factorial design and statistics to carry out the less number of experiments in optimization phase. Also it clarifies the effect of factors on formulation development. [Kincl M and Turk S, 2005]
Optimization of niosomal dispersion process is critical one that requires consideration of lot of factors also their interaction among each other. Use of statistical technique like factorial design can help in better understanding of these interactions while requiring lesser experiments and consequently make the formulation development economical.

A $3^2$ factorial design approach clarify the effect concentrations of span 60 and concentrations of cholesterol on particle size of vesicle were studied. The factors and the levels selected for optimization are showed in table 2.6.

**Table 2.6: Factors and levels selected for optimization**

<table>
<thead>
<tr>
<th>Factors</th>
<th>Higher level</th>
<th>Middle level</th>
<th>Lower level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Span 60</td>
<td>60</td>
<td>50</td>
<td>40</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>40</td>
<td>30</td>
<td>20</td>
</tr>
</tbody>
</table>

**Table 2.7: $3^2$ Factorial batches**

<table>
<thead>
<tr>
<th>Batch no.</th>
<th>Span 60 (mg)</th>
<th>Cholesterol (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1(-1,-1)</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>B2(-1,0)</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>B3(-1+1)</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>B4(0,-1)</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>B5(0,0)</td>
<td>50</td>
<td>30</td>
</tr>
<tr>
<td>B6(0,+1)</td>
<td>50</td>
<td>40</td>
</tr>
<tr>
<td>B7(+1,-1)</td>
<td>60</td>
<td>20</td>
</tr>
<tr>
<td>B8(+1,0)</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>B9(+1,+1)</td>
<td>60</td>
<td>40</td>
</tr>
</tbody>
</table>

(All batches contain drug 10 mg & stearic acid 5 mg)
2.2.2.7 Characterization of drug loaded niosomal dispersions

- **Oraganoleptic properties**
The drug loaded dispersion was evaluated for colour, odour and appearance.

- **pH**
The pH of niosome dispersion was checked by digital pH meter.

- **Total drug content**
Assay of drug loaded niosomal dispersions were carried out by U.V. method. Two ml of niosomal dispersion was dissolved into 50 ml Phosphate buffer solution having pH 6.8. After addition of 1% isopropanol, sample was stirred at 100 rpm to break the niosomes. Drug content was determined using UV spectrophotometer at respective absorption maxima.

- **Zeta potential (\(\zeta\))**
Zeta potential of the dispersion was determined by Malvern zetameter. Time duration for zeta potential determination was 60 seconds and charge was find out. Typical graphs for zeta potential of Triamcinolone Acetonide loaded noisome and Nystatin loaded noisome were shown in figure 3.7 and 3.15 respectively.

- **Mean particle size and Polydispersibility index**
The particle size analyses of the formulation (T7 & N7) were determined using Beckman particle size determination technique. The results of Triamcinolone Acetonide and Nystatin loaded noisome are shown in figure 3.6 & 3.14 respectively.

- **Encapsulation efficiency**
Unentrapped drug from niosomal dispersion was separated by centrifugation method. Niosomes were centrifuged at 20,000 rpm at controlled temperature of 4°C for 60 min. By using UV spectroscopy unentrapped drug was quantified at respective absorption maxima. The entrapped drug in niosomes verified using following equation. The results of Triamcinolone Acetonide and Nystatin loaded noisome are shown in table 3.13 and 3.16 respectively.
Entrapment efficiency (%) = \[ \frac{(C_t - C_f)}{C_t} \times 100 \],

Where, \( C_t \) total Drug concentration and \( C_f \) free Drug concentration

- **Differential scanning calorimetry (DSC)**

It was carried out by using NETZSCH DSC 200F instrument. For calibration of DSC indium and zinc were used as standard. The lyophilized drug loaded niosomes and drugs are enveloped in crucible made by aluminium and heated at 10°C/min in temperature range 50 – 450°C. Purge nitrogen gas for maintaining inert environment during DSC study. Lyophilized drug loaded and blank niosomes were used for analysis along with drug. DSC graph of Drug, Blank niosome and drug loaded niosome are indicated as figure 3.8 & 3.16.

- **X-ray diffraction analysis (XRD)**

The XRD patterns were recorded on X-ray diffractometer. Cu-Ka radiation were used for irradiation of sample (1.542A °) and analyzed from 50 to 500 2θ. The 30 kV voltages and 30mA current were applied. Lyophilized drug loaded and blank niosomes were used for analysis along with drug. XRD spectra of Drug, blank niosome and drug loaded niosome are revealed in fig 3.9 & 3.17.

- **In- vitro drug diffusion**

The design and development of drug delivery system are greatly aided by in- vitro diffusion studies. To obtain qualitative and quantitative drug release pattern of API through niosomal dispersion and determined correlation among release mechanism and drug loaded niosomal dispersion and experimental data was determined using dialysis method. [Reddy L, 1997]

In this technique sacks were soaked in saline solution for one day. A 5 ml of drug loaded niosome was transferred into dialysis sack respectively. These drug loaded niosome dialysis bag was placed in a beaker at 37°C. One mL samples were analysed
by replacing with buffer medium using UV spectrophotometer at intervals 0.5, 1, 2, 3, 4, 5, 6, 12 & 24 hours. The results are shown in figure 3.10 & 3.18.

- **Ex-vivo diffusion study**

**Stabilization of the skin**

Fraz diffusion cell was used to carry out Ex-vivo skin permeation studies. For that porcine skin was used as model membrane. Initially porcine skin was shaved to remove the fat carefully. Shaved skin was placed on diffusion cell having capacity 15 mL. Receptor compartment contained phosphate buffer solution (pH 6.8) as diffusion medium. Dermal side of the skin was kept facing to receptor side and epidermal surface towards donor compartment.

Shaved porcine skin was placed between receiver and donor compartment. Diffusion assembly should be protected from direct sunlight. Preparation of skin used as membrane in diffusion study protocol was approved by Institutional Animal Ethics Committee form B (IAEC/TKCP/2012/10). Initially stabilization of skin was carried out. In that 1mL sample was withdrawn and analyzed for any absorbance showed by residual component. This study was repeated after 30 minutes of interval till sample show negligence absorbance. Stabilization of the skin was successfully completed when sample showed absorbance zero.

**Ex-vivo diffusion study** [Valenta, C 2003 & Vrhovnik 1998]

After stabilization of the skin Ex-vivo diffusion study was carried out. Phosphate buffer solution having pH 6.8 was used as diffusion medium. Diffusion assembly was placed on magnetically stirred plate and study was carried out at 37°C ± 2°C. Test samples i.e. niosomal dispersion and conventional dispersion was rest on dermal side separately. Donor compartment output element was covered with aluminium foil to avoid evaporation of diffusion medium. One mL sample was withdrawn at 30 min, 1 hr, 2 hrs, 3 hrs, 4 hrs, 5 hrs, 6 hrs and 24 hrs time intervals. At each withdrawal time intervals withdrawn sample was replaced with same amount of phosphate buffer solution pH 6.8 as a blank. Withdrawal sample was analysed for concentration determination using UV spectrophotometry. Diffusion graphs were shown in figure 3.11 & 3.19.
2.2.2.8 Effect of Variables

Statistical data
Niosomes were prepared using Ether injection method which was suited for the production of niosomes without aggregation in the selected region of factorial design. Obtained data were placed in multiple regression analysis employing PCP Disso software and obtained values were put in equation 3.1

\[ Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1X_1 + \beta_{22} X_2X_2 + \beta_1\beta_2 X_1 X_2 \ldots \ldots \text{eq.3.1} \]

The observed coefficients for the different parameters obtained after applying multiple regression analysis of Triamcinolone Acetonise niosome & Nystatin niosome are representing in table 3.14 & 3.17. Analysis of variance was used to check the adequacy of fitted model. Microsoft excel was used to generate response surface plot.

Effect of Variables on Vesicle Size
The most important parameter, which needs to monitor during niosomes preparation its best performance, is the vesicle size of niosomes. From the number of reports it was observed that effect of niosomes size on the drug diffusion also determined the skin deposition of a drug. Thus for the effective delivery the selected method should result in optimum size range and homogeneous population. In the present study, Ether injection was found to produce uniform niosomes, which indicates obtained niosome dispersion has narrow size of distribution. Effect of variables on Vesicle size of TA niosome and Nystatin niosome are revealed in fig 3.12 & 3.20 respectively.

Effect of Variables on Entrapment Efficiency
Determination of percentage entrapment efficiency is an important parameter in case of niosomes as it may affect the drug diffusion. Percentage entrapment means amount of drug trapped in to niosome corresponding to total amount of drug. Entrapment efficiency of niosome was decreased with increase in concentration of cholesterol. Effect of variables on Entrapment efficiency of TA niosome and Nystatin niosome are shown in figure 3.13 & 3.21 respectively.

2.2.3 Niosomal Gel

2.2.3.1 Introduction
Niosomal dispersion showed sustained released but further topical applicability of the developed formulation was enhanced by development of gel formulation. Carbopol 934 was selected as polymer for gel preparation.

2.2.3.2 Preparation of Niosomal carbopol gel

Carbopol at different concentration (1%, 1.5%, 2% w/w) were added to water and kept in a dark for humectation up to 24 hrs. To obtain a gel gel pH was adjusted within the range of 6.8 – 7.2 by addition of triethanolamine.

The optimized batch of the niosomal dispersion was centrifuged at 35000 rpm and the pellet was separated and again dispersed in to the each concentration of the carbopol gel.

2.2.2.8 Characterization of Drug loaded Niosomal gel

Rheological characterization

The Rheological studies were carried out on drug loaded carbopol gel (Batch code 1%, 1.5% & 2% w/w).

Rheological characterizations of all samples were conducted using a controlled stress Viscotech Rheometer For determination of rheology cone-plate geometry was used with specifications like 25 mm diameter with cone angle $10^\circ$. The gap was maintained at 0.5 mm. all the Rheological measurements were performed at 30$^0$C. [Bousmina M, 1999]

Rheological Tests

The Rheological characterizations of drug loaded niosomal carbopol gel of different concentration of carbopol were obtained by performing the following set of Rheological tests. [Islam M et.al. (2004), Biradar S et.al. (2009) & Laurati M et.al. (2011)]

- Oscillation stress sweep

To determine LVR linear viscoelastic region Dynamic oscillation stress sweep test was carried out. Critical stress was obtained during experiment therefore it gives idea
regarding the stress value which was used in other oscillation tests like creep recovery test and frequency sweep test. In this test gel was exposed to increasing stress. Stress was increasing in such a manner that structure of the test sample was break down. Frequency was kept constant 0.1 Hz while stress was increasing from 0.1 to 100 Pa. From this test we find out G i.e. storage modulus, G” loss modulus and tanδ loss tangent.

- **Oscillation Frequency sweep**

It was used to determine the ability of selected sample under the increased frequency subjected to increased frequency level it resist structural changes. The samples were exposed to a stepwise increasing frequency in the range of 1 to 100 Hz at a selected averaged stress of the stress sweep mode that falls well within the LVR of test samples

- **Creep recovery**

The creep recovery test was used to determine the viscoelastic properties of the selected gel samples. The samples were exposed to the selected averaged stress of the stress sweep mode for 100 s. It was followed by relaxation period for 200 s for recovery. Creep compliance Jc is a measured strain to the applied stress ratio. Creep compliance was checked against time.

<table>
<thead>
<tr>
<th>Test</th>
<th>Test conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oscillation stress sweep</td>
<td>0.1 to 200 Pa at constant frequency of 0.1 Hz</td>
</tr>
<tr>
<td>Oscillation Frequency sweep</td>
<td>0.1 to 100 Hz; at a selected average stress of the stress sweep mode that falls well within the LVR of each sample</td>
</tr>
<tr>
<td>Creep recovery</td>
<td>The samples were exposed to the selected averaged stress of the stress</td>
</tr>
</tbody>
</table>
sweep mode within LVR of each sample for 100s. and relaxation time 200s

The results are shown in Fig. 3.22 – 3.26.

Physical parameters characterization
Gel formulations were evaluated for the Colour, pH, Viscosity and Microscopic Apperance. [Martin A, 1993]

Spreadability of gel
For gel dosage form good spreadability value is one of the important properties. Spreadability means gel ability to spread on skin part. Spreading value decide the therapeutic efficiency of gel.

Procedure:
To determine spreadability value spreadability apparatus was used. Spreadability apparatus contains wooden block having two glass plates. Initially gel sample was placed between the two glass plates. Weight near about 300 g was putted on top plate which expelled the air and to form uniform gel layer. Afterwards 100 g weight was put to drag top plate by 10 cm using stringe attached to hook. Time required to move upper plate by 10 cm distance was noted, lesser the time required for dragging the upper plate better is the spreadability value. Speradability value was determined with the help of formula

\[ S = \frac{M \times L}{T} \]

Where, S is the Spreadability value, L is the Length of the glass slide, M is the Weight tied to the upper plate, and T is the Time taken to separate the glass slides.

Content Uniformity
The content uniformity of the optimized gel was determined by analyzing drug content by analyzing gel samples from five different points from the container. From the container 500 mg of gel sample was withdrawn and dissolved it into distilled
water. Sample was stirred at 200 rpm by addition of methanol to disrupt the niosomes make a require dilution and drug concentration was determined using UV-spectrophotometer.


The diffusion study of the niogel and conventional gel were carried out in Franz diffusion cell. Gel sample (0.5 g) was taken on cellulose nitrate membrane diffusion studies were carried out at 37±1° C using 15 ml phosphate buffer (pH 6.8) as the dissolution medium. At time intervals of 0.5, 1, 2, 3, 4, 5, 6, 12 and 24 hours and replaced by fresh medium 2 mL sample were withdrawn and drug content was determined by UV spectrophotometer. The results are shown in figure 3.28.

**Ex-vivo diffusion study** [Vanhal D, 1996]

Ex vivo diffusion study for niogel and conventional gel were carried out as per procedure in niosomal dispersion. It was performed on the porcine skin using Franz Diffusion cells. [Khamanga S et.al., 2011] The results are shown in figure 3.29.

**Deposition study** [Fang J, 2001]

Drug deposition study was performed on the excised skin of porcine skin using Franz diffusion cell. Full thickness porcine skin over which topical gel was applied was removed. The skin loaded with 500mg of gel formulations was cleaned with water and then swabbed with cotton immersed in phosphate buffer. Stratum corneum was placed towards donor compartment and dermal towards receptor compartment. Phosphate buffer solution (pH 6.8) used as diffusion medium (37 ± 0.5°C). Diffusion cells were protected from the light. For determination of drug deposition diffusion cell was dismantled after 48 hrs, skin was removed. Excess drug present on the skin was removed. Afterwards skin was cut into small pieces which were placed in methanol undergo sonication for 1 hr. Drug deposition in the skin was checked by using UV spectrophotometer. The results are shown in figure 3.33.

**2.2.4 Skin irritation study**

**Introduction**
Skin reversible damage subsequent using of a gel sample known as skin irritation. Primary irritation is due to irritant which cause inflammation. These irritant involve the interaction of chemicals with sensory receptors in the skin at the site of application. Secondary irritation cause progressive inflammation on repeated contact. [Thomas J, 2008]

A variety of substances are used in the manufacture of topical gels. Therefore, finished products when used on the human body have the potential for several types of adverse reactions. The adverse effects that may be caused include skin irritation and allergic conditions, contact urticaria and photoallergy. [Sarfaraz N, 2004]

The Draize patch test using rabbit as an animal model was used to evaluate the skin irritation index of gel sample on abraded and intact skin area. [Berger R, 1982]

**Evaluation of dermal reactions**

Edema and erythema scores were added at 24 and 72 hours for abraded and intact skin. Total scores were divided by 24 means two scoring intervals multiplied by two test parameters multiplied by six rabbits recognized as Primary Irritation Index.

<table>
<thead>
<tr>
<th>Table 2.9: Draize evaluation of dermal reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin reactions</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Skin reactions</th>
<th>Score</th>
</tr>
</thead>
</table>


**Erythema formation**

- No erythema 0
- Very slight erythema 1
- Well-defined erythema 2
- Moderate to severe erythema 3
- Severe erythema 4

eschar formation (injuries in depth)

**Edema formation**

- No edema 0
- Very slight edema 1
- Slight edema (edges of area well defined by definite raising) 2
- Moderate edema (raising about 1 mm) 3
- Severe edema (raise above 1 mm) 4

---

**Table 2.10: Evaluation of Primary Irritation Index**

<table>
<thead>
<tr>
<th>Index</th>
<th>Evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>No irritation</td>
</tr>
<tr>
<td>0.04-0.99</td>
<td>Irritation barely perceptible</td>
</tr>
<tr>
<td>1.00-1.99</td>
<td>Slight irritation</td>
</tr>
<tr>
<td>2.00-2.99</td>
<td>Mild irritation</td>
</tr>
<tr>
<td>3.00-5.99</td>
<td>Moderate irritation</td>
</tr>
<tr>
<td>6.00-8.00</td>
<td>Severe irritation</td>
</tr>
</tbody>
</table>
Procedure

Primary skin irritation study of niogel carried out as per the guidelines of the Consumer Product safety Commission with the help of albino rabbit.

- The back of the animals were shaven to remove the fur, at least four hours before application of the test sample.
- Two square patches (3×3) were made on each rabbit. Just prior to formulation application, on test site four stratum corneum scratches were made on each rabbit. This is required because the maximum effect of an irritant is expected to be observed with the abraded skin.
- Skin kept intact on opposite site for comparative study with that of the abraded skin.
- The measured amount of the test formulation (0.5 gm) applied on intact and abrasion site on each rabbit.
- The patches were enclosed with gauze layer. Formulation was removed after 24 hour exposure.
- Residue test sites were cleaned with water also to take out any residue of the formulation.
- Dermal reactions were observed on each test sites as per Federal Hazardous Substance Act (FHSA) after one day and third day of gel application on sites.
- Gel formulation Primary irritation index was determined.
- Formalin and placebo gel formulation were used as positive control and negative control respectively.

Total scores were divided by 24 means two scoring intervals multiplied by two test parameters multiplied by six rabbits recognized as Primary Irritation Index. The gel formulation proved no erythema or edema on the two aites like intact and abraded rabbit skin as shown in figure 3.34 & 3.39 respectively.

2.2.5 Antifungal activity

Introduction
The antifungal activity of a drug is studied by observing growth of the fungi when contact with the drug. In agar diffusion method, the drug solution is placed in a cup of solid nutrient medium. The nutrient medium is seeded entirely or only on the surface with the test organisms. The drug diffuses through the nutrient medium and affects the growth of the fungi around it. This procedure is conducted by two techniques. [Finegold S, 1992]

a) Ditch plate technique
b) Cup plate technique

Nystatin is broad spectrum antifungal active produced by *streptomyses naurse*, shows high level of activity against *candida albicans* [United States Pharmacopoeia, 2002]

**Microbiological evaluation of antifungal agent**

- **Sterilization of Apparatus, Media and solvent used**
  All the apparatus viz. petriplate, pipettes and test tube were washed, wrapped in a brown paper and sterilized at 160°C for 2 hours. The agar media, buffer solution and saline solution were sterilized at 121°C at 15 psi for 20 minutes in an autoclave. All ingredient utilized during experiment were of analytical grade.

- **Maintenance of fungal strains**
  Cultures were maintained by streaking a loopful of microbes on agar slant and incubating at 25°C (48 hrs) for *candida albicans (36082) *. The slants were then kept in refrigerator. This procedure was repeated every week to maintain culture in pure state.

- **Preparation of test organism**
  Forty eight hours old of *C.albicans* was used for study. The microorganisms were suspended in 0.9% w/v NaCl. This suspension was further diluted with saline solution to obtain 1X10^6 CFU/ml. It matched with 1 McFarland standards. 0.5 mL of this suspension was used for inoculation of the agar plates.

- **Sample preparation**
  - Niosomal drug dispersion (100µg/ml)
  - Niosomal gel (100µg/ml)
• **Cup plate technique**

Sterile sabouraud dextrose agar was used as a culture media for fungi. Twenty ml of sterile molten agar was poured into sterile petriplate to form basal layer and allowed to solidify. To 4 mL of molten agar 0.5 mL of the culture suspension containing $1 \times 10^6$ CFU/mL was added, mixed well and poured over the basal layer. The plates were allowed to solidify. Cups were bored in the solidified agar plates using sterile cork bore to give cups of 9 mm diameter. A uniform volume (0.15 mL) of the test solutions were added to each cup. All operation was carried out in sterile area. The plate was then incubated at $25^0C$ (48 hrs). Finally after completion of incubation period, the zone of inhibition in plate was measured. A negative control of methanol and phosphate buffer solution pH 6.8 (1%) used as a solvents for the test preparation, was also included in studies. The testing was carried out and zone of inhibition, exclusively of the cup diameter, for each sample preparation is given in table 3.21 & figure 3.40.

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**2.2.6 Stability study**

**Introduction**

To determine shelf life of a developed formulation stability studies were carried out. Stability testing gives idea regarding temperature, humidity other environmental condition impact on product quality. Drugs and their formulations are exposed to variable storage conditions throughout their shelf life, during storage, shipment and handling. In addition to this, diversity of conditions with respect to temperature and humidity, in various countries, also propel us to investigate the stability of drugs and their formulation under influence of various storage conditions. [Padamwar M, 2006]
The product is evaluated at different time points for various formulation parameters such as physicochemical characters. [Arsic I (1999] Stability studies of a drug product are carried out in the same container closure as it is going to be packed in.

**Selection of formulation**

Optimized Niosomal dispersion and Niogel were filled in amber coloured bottle and aluminium collapsible tube respectively. Stability study was carried out for three months at two different temperatures (4-8°C and room temp.)

**Protocol for stability studies**

“Stability testing of new drug substances and products was carried out using International conference on harmonization (ICH) Q1A (R2)” [ICH, 2003]. The guideline recommends following time period and condition was used during stability evaluation.

<table>
<thead>
<tr>
<th>Study</th>
<th>Storage condition</th>
<th>Time period</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long term</td>
<td>5°C ± 3°C</td>
<td>12 months</td>
</tr>
<tr>
<td>Accelerated</td>
<td>25°C ± 2°C/60% RH ± 5% RH</td>
<td>6 months</td>
</tr>
</tbody>
</table>

The present study involves investigation of the stability of the formulated niosomal gels under influence of 25°C ± 2°C/60% RH ± 5% RH and 4°C ± 2°C storage conditions for a period of 3 months. The study was carried out to evaluate the effect of storage conditions on essential attributes of gels such as appearance, entrapment, vesicle size, viscosity and drug content after specified time intervals.

The results are shown in table 3.22 & 3.23.