## Chapter - 4. Experimental Investigations

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CHAPTER 4

EXPERIMENTAL INVESTIGATIONS

4.1 FORMULATION AND EVALUATION OF TERBINAFINE HCl

NANOEMULSION

4.1.1 MATERIALS

Table 4.1: List of materials

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Materials</th>
<th>Source</th>
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<tbody>
<tr>
<td>1</td>
<td>Terbinafine HCl</td>
<td>Aurobindo Pharmaceuticals, Hyderabad</td>
</tr>
<tr>
<td>2</td>
<td>Olive oil</td>
<td>Central Drug House Pvt Ltd, New Delhi</td>
</tr>
<tr>
<td>3</td>
<td>Oleic acid</td>
<td>Ranbaxy fine chemicals, Delhi</td>
</tr>
<tr>
<td>4</td>
<td>Tween 80</td>
<td>S.D fine chemicals Ltd. Mumbai</td>
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<td>5</td>
<td>Brij 35</td>
<td>Sigma Aldrich, Mumbai</td>
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<td>6</td>
<td>Ethanol</td>
<td>S.D fine chemicals Ltd. Mumbai</td>
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<td>7</td>
<td>Potassium dihydrogen phosphate</td>
<td>Qualigen fine chemicals</td>
</tr>
<tr>
<td>8</td>
<td>Sodium hydroxide</td>
<td>Qualigen fine chemicals</td>
</tr>
<tr>
<td>9</td>
<td>Methanol</td>
<td>S.D fine chemicals Ltd. Mumbai</td>
</tr>
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<td>10</td>
<td>Hydrochloric acid</td>
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<td>11</td>
<td>Acetonitrile HPLC grade</td>
<td>Sigma chemical Ltd.</td>
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<td>12</td>
<td>0.5% Triethylamine</td>
<td>Sigma chemical Ltd.</td>
</tr>
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<td>13</td>
<td>Dialysis bag (MWCO-12,000 g/ml)</td>
<td>Himedia labs, Mumbai.</td>
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<td>14</td>
<td>Potassium bromide (IR Grade)</td>
<td>S.D. Fine Chemicals Ltd., Mumbai</td>
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<td>15</td>
<td>Sodium chloride</td>
<td>Qualigen fine chemicals</td>
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<td>16</td>
<td>Mannitol</td>
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<td>17</td>
<td>Polyethylene Glycol 6000</td>
<td>Oxford Laboratories, Mumbai</td>
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### 4.1.2 EQUIPMENTS

**Table 4.2**: List of equipments

<table>
<thead>
<tr>
<th>Sl. No.</th>
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<td>2</td>
<td>FTIR spectrophotometer</td>
<td>Perkin-Elmer-1600</td>
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<td>3</td>
<td>Vortex mixer</td>
<td>Remi motors</td>
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<td>4</td>
<td>HPLC</td>
<td>Shimadzu LC 2010A HT</td>
</tr>
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<td>5</td>
<td>Centrifuge</td>
<td>Remi Instruments</td>
</tr>
<tr>
<td>6</td>
<td>Digital pH meter</td>
<td>Systronics, Mumbai</td>
</tr>
<tr>
<td>7</td>
<td>Electronic balance</td>
<td>Sartorius, India, Bangalore</td>
</tr>
<tr>
<td>8</td>
<td>Sonicator</td>
<td>Bandelin RK 100 H, Germany</td>
</tr>
<tr>
<td>9</td>
<td>Viscometer</td>
<td>Brookfield, Inc</td>
</tr>
<tr>
<td>10</td>
<td>Conductivity meter</td>
<td>Systronic</td>
</tr>
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<td>Deep freezer</td>
<td>Labline</td>
</tr>
<tr>
<td>12</td>
<td>Isothermal shaker</td>
<td>IKA® KS 4001, Germany</td>
</tr>
<tr>
<td>13</td>
<td>Dissolution apparatus</td>
<td>Electro lab India, Mumbai</td>
</tr>
<tr>
<td>14</td>
<td>Water bath</td>
<td>SCIENTEC</td>
</tr>
<tr>
<td>15</td>
<td>Magnetic stirrer</td>
<td>REMI Equipments</td>
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<tr>
<td>16</td>
<td>Abbe Refractometer</td>
<td>Bausch and lomb optical, NY.</td>
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</table>
4.1.3 PREFORMULATION STUDIES

Preformulation may be described as a phase of the research and development process where the formulation scientist characterizes the physical, chemical and mechanical properties of a new drug substance, in order to develop stable, safe and effective dosage forms. Ideally, the Preformulation phase begins early in the discovery process such that appropriate physical, chemical data is available to aid in the selection of new chemical entities that enter the development process. During this evaluation possible interaction with various inert ingredients intended for use in final dosage form are also considered(5,40,51).

4.1.3.1 Solubility studies:

The solubility of the Terbinafine HCl was determined in various type of oils by adding excess quantity of Terbinafine HCl to 1 ml of selected oils (oleic acid, isopropyl myristate, olive oil, triacetin, castor oil and ground nut oil) in stoppered vials. The vials were kept for 72 hours at 25± 0.5°C in an isothermal shaker so that it can reach equilibrium. When the samples reached to equilibrium these samples were removed from the isothermal shaker and after that samples were centrifuged for 15 min at 3000 rpm. After centrifugation the supernatant of the sample was taken and filtered through Whatman filter paper and concentration of Terbinafine HCl was determined in the oils after dilution using UV-Visible spectrophotometer at 283 nm (5,10,12,50-52).
4.1.3.2 Compatibility Study:

Infrared spectra matching approach was used to detect any possible chemical interaction between the drug, oil, surfactant and the cosurfactant. Drug, oil, surfactant and cosurfactant was mixed physically and finally that mixture was mixed with required quantity of (KBr) potassium bromide. Hydraulic press was used to prepare a transparent pellet of that mixture (about 100 mg of this mixture was compressed) at 15 tons pressure. The prepared pellet was then scanned from 4000 to 400 cm\(^{-1}\) wave length using Perkin Elmer FTIR spectrophotometer. After that the physical mixture IR spectrum was compared with those of pure drug (terbinafine HCl), oil (Olive oil), surfactant(Tween 80) and cosurfactant (Ethanol) and matching was done to detect any appearance or disappearance of peaks. (6,10,12-15,53,54).

4.1.3.3 Development of calibration curve in methanol

50 mg of terbinafine HCl was accurately weighed and dissolved in 50 ml of methanol to give a stock solution of 1mg/ml (1000µg/ml) concentration. This was served as stock solution. From this 10ml of the solution was taken and diluted to 100ml to get a solution of 100 µg/ml and this was served as a standard solution. Into a series of 10 ml volumetric flask, aliquots of standard solution i.e. 0.5 ml, 1 ml, 1.5 ml, 2 ml and 2.5 ml were added and the volumes were made up to 10 ml using methanol. The absorbance of these solutions was measured against the reagent blank at 283 nm using UV- spectrophotometer. A
standard curve was plotted with concentration (X-axis) and absorbance (Y-axis) (48,49,55).

4.1.3.4 Development of calibration curve in phosphate buffer (pH 7.4)

50 mg of terbinafine HCl was accurately weighed and dissolved in 50 ml of phosphate buffer pH 7.4 to give a stock solution of 1mg/ml (1000µg/ml) concentration. This was served as a blank. From this 10ml of the solution was taken and diluted to 100ml to get a solution of 100 µg/ml and this was served as a standard solution. Into a series of 10 ml volumetric flask, aliquots of standard solution i.e. 0.5 ml, 1 ml, 1.5 ml, 2 ml and 2.5 ml were added and the volumes were made up to 10 ml using phosphate buffer. The absorbance of these solutions was measured against the reagent blank at 283 nm using UV-spectrophotometer. A standard curve was plotted with concentration (on X-axis) verses absorbance (on Y-axis) (49,55,56).

4.1.4 STUDY OF PSEUDO-TERNARY PHASE DIAGRAM:

To develop the pseudo-ternary phase diagrams aqueous titration method were used. Measured quantity of surfactant (Tween 80) and co-surfactant (Ethanol) were mixed (Smix) in different volume ratios (1:0, 1:1, 1:2, 1:3, 1:4, 2:1, 3:1, 4:1). Olive oil optimized as an oil phase based on the solubility study. For each phase diagram, oil (olive oil) and specific smix ratios were thoroughly mixed using magnetic stirrer in various volume ratios from 1:7 to 7:1. 13 different combinations of oil and smix (1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 2:1, 3:1,
4:1, 5:1, 6:1, 7:1) were made for the study to detect the boundaries of the phases precisely formed in the phase diagrams. By using water as aqueous phase slow titration was performed for different combination of oil (Olive oil) and smix (Tween 80 and ethanol) separately. The volume of aqueous phase used while constructing the pseudo ternary phase diagram was 50 ml, where as 5ml was added at each interval under magnetic stirring (200 rpm) and visually observed for phase clarity and flowability. Through visual screening following observation were made:

1. Transparent as well as easily flowable : o/w nanoemulsion (NE)
2. Transparent gel: nanoemulsion gel (NG)
3. Milky or cloudy: emulsion (E)
4. Milky gel: emulgel (EG)

In the similar manner, calculations for other various ratios of oil phase and smix (surfactant and cosurfactant mixture) were also done. The physical state was plotted on a pseudo-three-component phase diagram with one axis representing the aqueous phase, the second representing the oil phase, and third representing a mixture of surfactant and cosurfactant (SMIX) at a fixed volume ratio (4,5,12-21, 40,42,57,58).
**Fig. 4.1:** Preparation of Nanoemulsion by aqueous titration method

### 4.1.5 SELECTION OF FORMULATIONS

From the various constructed phase diagram different formulations were selected from Nanoemulsion region so that terbinafine HCl (Active compound) could be incorporated into the oil phase on the following basis. 10 mg of Terbinafine HCl was selected as the dose for incorporation into the oil phase. For convenience 2 mL was selected as the NE formulation. The oil conc. should be such that it solubilizes the drug (single dose) completely. 10 mg of Terbinafine HCl will dissolve easily in 0.2 mL of oil (10% of 2 mL). From each phase diagram, different concentration of oils were selected at a difference of 5% (10%, 15%, 20%, 25%, etc) from the NE region. For each 5% of oil selected, the formula that used the minimum
concentration of smix for its NE formulation was selected from the phase diagram (10,12,18-24, 59,60,61).

4.1.6 EVALUATION OF NANOEMULSION:

4.1.6.1 Thermodynamic stability tests:

Selected formulations of nanoemulsion were then subjected to various thermodynamic stability tests like heating cooling cycle, freeze thaw study etc.

4.1.6.2 Heating cooling cycle:

This study was performed by keeping the formulations six cycles in between refrigerator temperature which is 4°C and 45°C. During the study formulations were stored for not less than 48 h at each temperature. Those formulations, which were stable at these temperatures, were subjected to centrifugation (5,19,22,24-26,62,63,64).

4.1.6.3 Centrifugation:

Passed formulations were then centrifuged for 30 minutes at 3500 rpm. Formulations that did not show any phase separation after centrifugation were taken for freeze-thaw cycle test (5,19,22,24-26,65,66,67).

4.1.6.4 Freeze thaw cycle:

Selected formulation was then under gone three freeze thaw cycles temperature in between –21°C and +25°C with storage for not less than forty-eight hours at each temperature. Formulations, which
passed all these thermodynamic stress tests, were evaluated for the dispersibility tests for studying the efficiency of self-emulsification (5,19,22,68,69,70).

4.1.6.5 Dispersibility tests

The efficiency of self-emulsification of oral nanoemulsion was assessed using a dissolution apparatus II. 1 (One) ml of formulation from each batch was then added to 500 mL of water maintained at 37 ± 0.5°C. A dissolution paddle made up of standard stainless steel and rotates at 50 rpm provides gentle agitation in the formulation. Using the following grade system in vitro performance of the selected nanoemulsion formulation was visually assessed:

**Grade A:** Formulation forming rapid nanoemulsion that is within 1 minute and have a clear / bluish appearance.

**Grade B:** Formulation forming rapid but slightly less clear emulsion, with a bluish white appearance.

**Grade C:** Formulations forming fine milky emulsion which formed within 2 min time interval.

**Grade D:** Formulations formed dull and greyish white emulsion with slightly oily appearance.(more than two minutes)

**Grade E:** Formulations, which exhibits poor or minimal emulsification efficacy and presence of large oil globules on the surface.

Those formulations that passed the thermodynamic stability and dispersibility tests in Grade A and Grade B were then selected for
further evaluation studies. 10 mg (single dose) of drug (terbinafine HCl) in oil was dissolved (10%, 15%, 20%, 25% etc.) to prepare the desired formulations. Different smix ratio was then added to the oil and drug mixture and mixed uniformly using magnetic stirrer and aqueous phase was added in the above mixture slowly. The resulting mixture gave nanoemulsion (5,19,22,24-26,41,71,72,73,74).

**4.1.6.6 Viscosity determination:**

The viscosity of the formulations was determined using Brookfield viscometer (Servewell Instruments PVT Limited, Bengaluru) as such without doing any dilution using spindle 61 at 25 ± 0.5°C (5,19,75,76).

**4.1.6.7 Electroconductivity study:**

Electroconductometer was used to determine the electroconductivity of the formulated system. During the conductivity measurement study, the tested nanoemulsions were mixed with 0.01 N aqueous solution of sodium chloride (NaCl) inplace of distilled water (5,77,78).

**4.1.6.8 Refractive index and percent transmittance**

Abbe refractometer was used to determine the refractive index of the selected formulations by placing 1 drop of the formulation on the slide. The percent transmittance of the formulated system was then measured at 283 nm using a UV visible spectrophotometer (Analyticals, Mumbai). Distilled water was used as blank (5,79,80,81).
4.1.6.9 Drug content:

The dose of the drug used in the formulation was quite below the saturation point; therefore it is assured that the total amount of terbinafine HCl (drug) incorporated in the nanoemulsion formulation will be available for release from the formulation. Since surfactant (tween 80) and cosurfactant (eanolt) (Smix) were added in the nanoemulsion formulation, there were chances of precipitation. Therefore, the content of drug was quantified by UV visible spectrophotometer at 283 nm. The nanoemulsion formulation was then diluted to required concentrations by using solvent (methanol) and the absorbance of the various formulations were measured at 283 nm. Methanol was used as blank (5,48-50,24-26,82-87). The drug content was calculated as:

\[
\text{Drug content} = \frac{\text{ Analyzed content}}{\text{Theoretical content}} \times 100
\]

4.1.6.10 Globule size and Zeta potential

The size of the droplet of the nanoemulsions were determined using a method known as photon correlation spectroscopy which analyses the light scattering fluctuations in the formulation due to the Brownian motion of the droplets using an instrument named zetasizer ZS 90. Scattering of light was monitored at a temperature of 25°C at a 90° angle. Zeta potential was also determined for the nanoemulsion formulations (5,19,22,24-26,87).
4.1.6.11 Transmission electron microscopy (TEM)

Structure of the formulated nanoemulsion and its morphology were evaluated using Transmission electron microscopy (TEM). To perform the TEM analysis nanoemulsion formulation was diluted with distilled water (1/100). Then one drop of the diluted nanoemulsion was applied to a 300 mesh size copper grid and left for 1 minute. It was then observed after drying (5,40,41,52).

4.1.6.12 In vitro drug release:

The in vitro dissolution release test was performed using USP Dissolution test apparatus Type II (rpm maintained was 50 and temperature was maintained at 37±0.5°C) in 500 ml of Phosphate buffer pH 7.4. Two (2) ml of formulated nanoemulsion which contains a single dose 10mg of drug (terbinafine HCl) was kept in a dialysis bag. At various time intervals (0, 0.5, 1, 1.5, 2, 4, 6, 8 and 12 h) samples (5ml) were withdrawn and a required quantity of phosphate buffer pH 7.4 (maintained at 37±0.5°C) was replaced. The samples were filtered, diluted and analyzed for the amount of drug present using UV Visible spectrophotometer at 283 nm (2,5,28,29,35,85).

4.2 FORMULATION OF SOLID DISPERSION OF TERBINAFINE HCL

4.2.1 I.R SPECTROSCOPY TO PREDICT THE COMPATIBILITY OF POLYMER WITH DRUG:

I.R spectroscopy can be used to investigate and predict any physiochemical interaction between different components in a formulation and therefore it can be applied to the selection of suitable
chemical compatible excipients while selecting the ingredients, we would choose, those which are stable, compatible, cosmetically and therapeutically acceptable.

The aim of the study was to detect the possible interaction between the selected polymer mannitol and polyethylene glycol - 6000 and the drug terbinafine HCl and also to identify the compatibility between the drug and polymer.

10mg of the sample and 400mg of KBr were taken in a mortar and triturated. A small amount of triturated sample was taken into a pellet maker and was compressed at 10kg/cm² using a hydraulic press. The pellets were kept onto the sample holder and scanned from 4000 cm⁻¹ to 400 cm⁻¹ in Perkin Elmer FT-IR Spectrophotometer.

Samples were prepared for pure polymer, pure drug, physical mixture of drug and polymer and solid dispersion. The spectra obtained through those samples were compared and interpreted for the shifting of functional peaks and disappearance or appearance of new functional peaks (88-91).

4.2.2 DEVELOPMENT OF CALIBRATION CURVE IN DISTILLED WATER

50 mg of terbinafine HCl was accurately weighed and dissolved in 50 ml of distilled water with the help of methanol to give a stock solution of 1mg/ml (1000µg/ml) concentration. This was served as stock solution. From this 10ml of the solution was taken and diluted to 100ml to get a solution of 100 µg/ml and this was served as a
standard solution. Into a series of 10 ml volumetric flask, aliquots of standard solution i.e. 0.5 ml, 1 ml, 1.5 ml, 2 ml and 2.5 ml were added and the volumes were made up to 10 ml using distilled water. The absorbance of these solutions was measured against the reagent blank at 283 nm using UV- spectrophotometer. A standard curve was plotted using concentration (X-axis) and absorbance (Y-axis) (48,49,92,93).

**4.2.3 DEVELOPMENT OF CALIBRATION CURVE IN PHOSPHATE BUFFER (PH 7.4)**

50 mg of terbinafine HCl was accurately weighed and dissolved in 50 ml of phosphate buffer pH 7.4 to give a stock solution of 1mg/ml (1000µg/ml) concentration. This was served as a blank. From this 10ml of the solution was taken and diluted to 100ml to get a solution of 100 µg/ml and this was served as a standard solution. Into a series of 10 ml volumetric flask, aliquots of standard solution i.e. 0.5 ml, 1 ml, 1.5 ml, 2 ml and 2.5 ml were added and the volumes were made up to 10 ml using phosphate buffer. The absorbance of these solutions was measured against the reagent blank at 283 nm using UV- spectrophotometer. A standard curve was plotted using concentration (on X-axis) and absorbance (on Y-axis).

**4.2.4 PREPARATION OF SOLID DISPERSIONS**

Solid dispersion of terbinafine hydrochloride was prepared by melting method. The composition is shown in table no 4.3. In melting method the drug and carrier (polyethylene glycol 6000 & manitol) were
mixed in 1:1 to 1:6 ratios in a china dish and heated on a paraffin bath. The mixture was poured on a tile and cooled. The resulted solidified mass was dried pulverised and passed through sieve # 100 (87,88,94,95,96).

**Fig. 4.2:** Formulation of solid dispersion by fusion method
Table 4.3: Composition of Solid dispersion

<table>
<thead>
<tr>
<th>Formulation Code</th>
<th>Carrier</th>
<th>Drug : Carrier</th>
<th>Method Of Preparation</th>
</tr>
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<tbody>
<tr>
<td>M1</td>
<td>Mannitol</td>
<td>1:1</td>
<td>Melting method</td>
</tr>
<tr>
<td>M2</td>
<td></td>
<td>1:2</td>
<td></td>
</tr>
<tr>
<td>M3</td>
<td></td>
<td>1:3</td>
<td></td>
</tr>
<tr>
<td>M4</td>
<td></td>
<td>1:4</td>
<td></td>
</tr>
<tr>
<td>M5</td>
<td></td>
<td>1:5</td>
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</tr>
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<td>1:6</td>
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<td>Melting method</td>
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</tr>
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<td>P6</td>
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<td>1:6</td>
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</tr>
</tbody>
</table>

4.2.5 EVALUATION OF SOLID DISPERSION

4.2.5.1 Estimation of drug content:

The formulation equivalent to 50 mg of terbinafine hydrochloride was weighed and transferred to a 50 ml flasks. Formulations were kept in 25 ml of distilled water and sonicated for 72 hours, after 72 hours the content of each flasks were then filtered through whatman filter paper. Filtrate were diluted properly and absorbance of the samples were determined by UV Visible spectrophotometer using wave length at 283 nm (49, 50,88).
4.2.5.2 Dissolution Studies:

A quantity of solid dispersion, which will theoretically contain a drug content of 100 mg of drug, was calculated from each drug loaded batch. The invitro dissolution studies were done to compare the rate of dissolution of solid dispersions with that of pure drug Terbinafine hydrochloride and nanoemulsion. The test was performed in USP II apparatus using 900ml phosphate buffer solution at pH 7.4, 50 rpm and temperature 37±1°C. The drug content was analyzed by UV Spectrophotometer at 283 nm.

The change in amount of drug release after formation of solid dispersion and nanoemulsion of the drug were compared (48,49,95-99).

4.2.5.3 Scanning Electron Microscopy (SEM)

A scanning electron microscope (SEM) is a type of electron microscope that images a sample by scanning that with electron of a high-energy beam in a pattern of raster scan. During scanning process the electrons of high energy beam interact with the atoms of the sample and produces signals which contain information regarding the surface of the sample's topography, and other properties like composition, electrical conductivity etc.

The particles were placed on a gold-coated plate and maintained for at least for 12 hours at room temperature in a desiccator for complete dryness of the sample. The dried sample was coated with a thin layer of gold and the particle size was determined using scanning
electron microscope. The scanning electron microscopy was done for physical mixture of drug and carrier and prepared solid dispersion (84, 88, 89,95).

**4.3 IN VIVO ORAL BIOAVAILABILITY STUDIES OF THE FORMULATIONS**

To carry out in vivo study, approval was granted from the Institutional Animal Ethics Committee (IAEC) of Jagatguru Sri Sivarathri College of Pharmacy, Ottacamund (Proposal no. JSSCP/IAEC/Ph.D/PHARMACY/02/2012-13) and their guidelines were followed during the study period. The formulation of the nanoemulsion that is (NE2), which was selected as the best formulation among all the formulations in connection to its highest release profile of drug during in vitro studies, was selected for in vivo studies. Adult male Wistar albino rats of 200±20g body weight were selected as animals for in vivo experiments. Five groups of the animals were made for the study and 5 rats were kept in each group. Animals used for the study were kept under strict standard laboratory conditions during the study period, temperature maintained at 25±2°C and relative humidity was maintained 55±5%. The selected animals were accommodated in polypropylene cages, 5 animals per cage with standard laboratory diet and water. The formulations (Nanoemulsion, Marketed tablet, Solid dispersion and drug suspension) were given orally using oral feeding sonde (5,7,100).
**Group 1**: Control group

**Group 2**: Nanoemulsion treated group

**Group 3**: Solid Dispersion treated group

**Group 4**: Drug suspension treated group

**Group 5**: Marketed tablet (Terbiforce\textsuperscript{TM}, 250 mg) treated group

Dose of the drug for the rats was calculated based on the weight of the rats that is 0.18 mg of Terbinafine HCl per 200g body weight. The rats were anesthetized by ether and blood samples of 0.5 ml were withdrawn from the cyano-orbital puncture at various time intervals i.e 0 (pre-dose), 0.5, 1, 2, 3, 4, 6, 8 and 24 hours in eppendorf tubes which contains 0.2 mL of citrate solution as anticoagulant, it was mixed and centrifuged for 20 minutes at 5000 rpm. The plasma was then separated from the blood samples and stored at -21\degree C temperature until analysis of the plasma samples were carried out to determine the amount of drug present using HPLC (5,23,41,100,101,102).

### 4.3.1 BIOANALYTICAL METHOD DEVELOPMENT AND ANALYSIS

Reverse phase HPLC method is one of the most popular mode for analytical and preparative separations of the compounds in chemical, biological, pharmaceutical and food samples. In reversed phase mode, the stationary phase is non polar and the mobile phase is polar. As the mobile phase is polar, water soluble compounds eluted first in this mode and water insoluble compounds are retained for long period of time. In present study, methods for the estimation of the
Terbinafine HCl present in the blood plasma samples were developed and validated. For the estimation of Terbinafine HCl in blood plasma, the chromatographic variables, namely pH, solvent strength, solvent ratio, flow rate, addition of peak modifiers in mobile phase, nature of the stationary phase, detection wavelength and internal standard were studied and optimized for the separation and retention of the drug. The following are the optimized chromatographic conditions, preparation of standard and sample solutions and the methods used for the estimation of Terbinafine HCl in plasma (5,100,102, 103,104,105).

**Chromatographic conditions:** Shimadzu gradient HPLC system was used with following configurations:

- LC-20 AD 230V Solvent delivery system (Pump).
- Manual Injector 25µl (Rheodyne)
- SPD-M20A 230V Photo diode array detector
- LC solutions data station

**Stationary phase:** Phenomenex Gemini C18 (250 x 4.6 mm i.d., 5µ)

**Mobile phase:** 50% H₃PO₄ Solution: Acetonitrile

**Mobile phase ratio:** 40:60

**Flow rate:** 1.0 ml/min

**Sample volume:** 20 µl

**Detection:** 283 nm
The mobile phase was filtered through 0.22µ membrane and degassed using ultrasonicator. All the experiments were carried out at room temperature.

**Preparation of Terbinafine HCl standard stock solution**

10 mg of Terbinafine HCl was transferred into a 10 mL volumetric flask and dissolved in 1mL of methanol and the volume was made upto the mark with mobile phase to give 1mg/mL (1000µg/mL) solution. From this stock solution, 10 mL of 100µg/mL solution was prepared (100,104,106,107).

**Preparation of analytical calibration curve solutions**

From the standard stock solution, 5-25µg/ml standard solutions were prepared and stored below 8°C until analysis.

**Preparation of blank plasma**

Blank plasma (0.5 ml) was transferred into 2.0 ml centrifuge tube and 0.1 ml of mobile phase and 0.3 ml of precipitating agent (10% perchloric acid) were added. Above solution was then vortexed for a time period of 5 minutes and centrifuged at 4000 rpm for 10 minutes. The supernatant layer was separated and analyzed (104,108).

**Preparation of bioanalytical calibration curve samples**

0.1 ml of 5,10,15,20 and 25µg/ml of Terbinafine HCl solutions were transferred to 2.0 ml centrifuge tube respectively, to this 0.5 ml of plasma, 0.3 ml of 10% perchloric acid were added as precipitating
agent. The above solution was vortexed for five minutes and centrifuged at 4000 rpm for 10 minutes. The supernatant layer was separated and analyzed.

**Preparation of plasma samples**

Plasma samples (0.5 ml) obtained from study subjects was transferred into 2.0 ml centrifuge tube and 0.3 ml of precipitating agent was added. The resulting solution was vortexed for 5 minutes and centrifuged at 4000 rpm for 10 minutes. The supernatant layer was separated and analysed.

**Method of analysis**

The bioanalytical calibration curve samples and plasma sample solutions were injected with above chromatographic conditions and the chromatograms were recorded. The quantification of the chromatogram was performed using peak area (5,104,108).

**4.4 STATISTICAL ANALYSIS**

The pharmacokinetic data was analyzed using one-way analysis of variance that is ANOVA using Tukey test (5,7,40,41).

**4.5 STABILITY STUDIES ON OPTIMIZED NANOEMULSION**

Stability testing should be performed to the finished product to provide evidence of how the API quality varies with time under the various environmental factors such as light, temperature and humidity. It also includes the study of formulation related factors.
such as interaction of API with container, other drug, excipients and packaging materials.

Three batches of the stable nanoemulsion formulation (NE2) were prepared by aqueous titration method, by following the previous procedure. These batches were maintained at a temperature of 40±2°C and 75±5% RH for a period three months. Samples of the formulations were withdrawn during that three months period after specified time intervals that is (0, 30, 60 and 90 days) and then those samples were investigated visually for any changes physical in the formulation. Evaluation were also done for viscosity, refractive index, droplet size, remaining drug content and logarithm of percent drug remaining versus time (in days) was plotted to know the rate of order of degradation (12,78).