CHAPTER – V

METODOLOGY

5.1 LIST OF CHEMICALS

Table 6: List of Chemicals

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Materials</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Zidovudine</td>
<td>Aurobindo Labs, Hyderabad</td>
</tr>
<tr>
<td>2</td>
<td>Cholesterol</td>
<td>Loba Chemie, Mumbai</td>
</tr>
<tr>
<td>3</td>
<td>Sorbitan monolaurate (Span 20)</td>
<td>Loba Chemie, Mumbai</td>
</tr>
<tr>
<td>4</td>
<td>Sorbitan monopalmitate (Span 40)</td>
<td>Fine Chemicals, Chennai</td>
</tr>
<tr>
<td>5</td>
<td>Sorbitan monostearate (Span 60)</td>
<td>Central Drug House, New Delhi</td>
</tr>
<tr>
<td>6</td>
<td>Sorbitan monooleate (Span 80)</td>
<td>Loba Chemie, Mumbai</td>
</tr>
<tr>
<td>7</td>
<td>Polysorbate 20 (Tween 20)</td>
<td>Hi-media laboratories, Mumbai</td>
</tr>
<tr>
<td>8</td>
<td>Polysorbate 40</td>
<td>Hi-media laboratories, Mumbai</td>
</tr>
<tr>
<td>9</td>
<td>Polysorbate 60</td>
<td>Hi-media laboratories, Mumbai</td>
</tr>
<tr>
<td>10</td>
<td>Polysorbate 80 (Tween 80)</td>
<td>Hi-media laboratories, Mumbai</td>
</tr>
<tr>
<td>11</td>
<td>N propanol</td>
<td>Loba Chemie, Mumbai</td>
</tr>
<tr>
<td>12</td>
<td>Triton X 100</td>
<td>Loba Chemie, Mumbai</td>
</tr>
<tr>
<td>13</td>
<td>Chloroform AR</td>
<td>Loba Chemie, Mumbai</td>
</tr>
<tr>
<td>14</td>
<td>Methanol AR</td>
<td>SD Fine Chem Ltd., Mumbai</td>
</tr>
<tr>
<td>15</td>
<td>Potassium dihydrogen phosphate</td>
<td>Himedia, Mumbai</td>
</tr>
<tr>
<td>16</td>
<td>Disodium hydrogen phosphate</td>
<td>Himedia, Mumbai</td>
</tr>
<tr>
<td>17</td>
<td>Sodium chloride</td>
<td>SD Fine Chem Ltd., Mumbai</td>
</tr>
<tr>
<td>18</td>
<td>Dialysis bag (M. Wt 12,000-14,000)</td>
<td>Hi-media, Mumbai</td>
</tr>
<tr>
<td>19</td>
<td>Membrane filters</td>
<td>Millipore filter (0.85 μm)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nylon filter (0.45 μm)</td>
</tr>
<tr>
<td>20</td>
<td>Acetonitrile</td>
<td>Merck Laboratories, Mumbai</td>
</tr>
<tr>
<td>21</td>
<td>Glacial acetic acid</td>
<td>RFCL Limited, Delhi</td>
</tr>
<tr>
<td>22</td>
<td>Lactose</td>
<td>Loba Chemie, Mumbai</td>
</tr>
<tr>
<td>23</td>
<td>Betacyclodextrin</td>
<td>Hi-media, Mumbai</td>
</tr>
<tr>
<td>24</td>
<td>Trisodium citrate</td>
<td>Loba Chemie, Mumbai</td>
</tr>
<tr>
<td>25</td>
<td>Ethyl acetate</td>
<td>Loba Chemie, Mumbai</td>
</tr>
<tr>
<td>26</td>
<td>Marketed zidovudine oral solution</td>
<td>Cipla Pharmaceuticals, Mumbai</td>
</tr>
</tbody>
</table>
Table 7: List of Instruments

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Equipments</th>
<th>Model/Make</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Magnetic stirrer</td>
<td>2 MLH, Remi Instruments, Vasai</td>
</tr>
<tr>
<td>2.</td>
<td>Rotary Flash Evaporator</td>
<td>Super Fit Instruments, Mumbai</td>
</tr>
<tr>
<td>3.</td>
<td>Vacuum Pump</td>
<td>SH TID-75, Oil free diaphragm type</td>
</tr>
<tr>
<td>4.</td>
<td>Mechanical Stirrer</td>
<td>RQ- 122, Remi Instruments, Vasai</td>
</tr>
<tr>
<td>5.</td>
<td>Eppendorf Centrifuge</td>
<td>5415, Germany</td>
</tr>
<tr>
<td>6.</td>
<td>UV spectrophotometer</td>
<td>UV 1650 PC, Shimadzu, Philippines</td>
</tr>
<tr>
<td>7.</td>
<td>pH meter</td>
<td>Phan-PN 05141120, Lab India, Mumbai</td>
</tr>
<tr>
<td>8.</td>
<td>Optical Microscope</td>
<td>806253, Olympus</td>
</tr>
<tr>
<td>9.</td>
<td>Electronic balance</td>
<td>ELB 300, Shimadzu, Philippines</td>
</tr>
<tr>
<td>10.</td>
<td>Bath Sonicator</td>
<td>RP120, Ralsonics, Mumbai, India</td>
</tr>
<tr>
<td>11.</td>
<td>Sieves</td>
<td>Jayant Scientific, Mumbai</td>
</tr>
<tr>
<td>12.</td>
<td>Zetasizer</td>
<td>Nano ZS 90, Malvern Instruments, UK</td>
</tr>
<tr>
<td>13.</td>
<td>HPLC with binary pump and Spectrophotometric detector</td>
<td>Spinco Laboratory, Chennai</td>
</tr>
<tr>
<td>14.</td>
<td>Transmission Electron Microscope (TEM)</td>
<td>Jeol, 1200Ex II, Tokyo</td>
</tr>
<tr>
<td>15.</td>
<td>Tissue Homogenizer</td>
<td>Remi Instruments, Vasai</td>
</tr>
<tr>
<td>16.</td>
<td>Centrifuge</td>
<td>Rotex Instruments, Kerala</td>
</tr>
<tr>
<td>17.</td>
<td>Non-compartmental model pharmacokinetics</td>
<td>WinNonlin software version (4.1), US</td>
</tr>
<tr>
<td>18.</td>
<td>Statistics</td>
<td>GraphPad Prism software (version 3.0).</td>
</tr>
</tbody>
</table>
5.2 PREPARATION OF REAGENTS

Preparation of phosphate buffer saline pH 7.4 (I.P. 2007)
Disodiumhydrogen phosphate (2.38 g), Potassium dihydrogen phosphate (0.19 g) and Sodium chloride (8 g) was dissolved in 1000 ml of distilled water. The pH was adjusted to 7.4.

Treatment of cellulose membrane
The cellulose membrane with molecular weight cutoff (12,000-14,000) was used for the study. It was soaked in warm water for 10 min. Then it was soaked in normal saline solution overnight and then used for in vitro diffusion studies.

Preparation of mobile phase for HPLC analysis of zidovudine
HPLC grade acetonitrile and 0.1% glacial acetic acid were taken in the ratio of (25:75) in a dry glass bottle. The contents were subjected to sonication in a bath type sonicator for degassing the solution. The solution was filtered by passing through 0.45 µm membrane filter and the bottle was corked and stored under refrigeration.

5.3 PURITY PROFILE / VALIDATION OF RAW MATERIALS
Zidovudine

State : Solid
Description : White powder.
Solubility : Sparingly soluble in water. Soluble in ethanol (95 per cent)
Melting Point : 109˚C

Absorption spectrum of the zidovudine solution exhibits one maximum at about 267.4 nm.
Formulation Development and In vivo Evaluation of Zidovudine Niosomes

Fig: 5 IR Spectrum of Zidovudine

Table 8: IR Interpretation for Zidovudine

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Wave Number cm(^{-1})</th>
<th>Functional group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>3000-3200 cm(^{-1})</td>
<td>CH-Stretching Vibration</td>
</tr>
<tr>
<td>2.</td>
<td>1600-1300 cm(^{-1})</td>
<td>Hetero ring Stretching Vibration</td>
</tr>
<tr>
<td>3.</td>
<td>1600-1580 cm(^{-1})</td>
<td>C=C</td>
</tr>
<tr>
<td>4.</td>
<td>750-700 cm(^{-1})</td>
<td>CH out of plane bending</td>
</tr>
</tbody>
</table>

The I.R spectrum of the drug zidovudine was compared with the reference spectrum given in Indian pharmacopoeia and was found to be similar. The functional groups assigned in the wave numbers exhibited same wave length and had similar intensities to that of the reference spectrum.
Cholesterol
State : Solid
Description : White
Solubility : Soluble in acetone, methanol, chloroform. Insoluble in water
Density : 1.052 g/cm³
Melting point : 148°C

Dicetyl Phosphate
State : Solid
Description : Off-white powder
Solubility : Soluble in chloroform
Melting point : 75°C

Beta cyclodextrin
State : Solid
Description : White
Solubility : Soluble in water
Melting point : 258°C

Table 9: Purity Profile of Tween

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Tween 20</th>
<th>Tween 40</th>
<th>Tween 60</th>
<th>Tween 80</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solubility</td>
<td>Soluble in water. Insoluble in mineral oil</td>
<td>Soluble in water. Insoluble in mineral oil</td>
<td>Soluble in water. Insoluble in mineral oil</td>
<td>Soluble in water. Insoluble in mineral oil</td>
</tr>
<tr>
<td>Surface Tension</td>
<td>57.46 dynes/cm</td>
<td>41.5 dynes/cm</td>
<td>42.5 dynes/cm</td>
<td>42.5 dynes/cm</td>
</tr>
<tr>
<td>Density</td>
<td>1.1 gm/ml</td>
<td>1.05 gm/ml</td>
<td>1.08 gm/ml</td>
<td>1.08 gm/ml</td>
</tr>
</tbody>
</table>

Formulation Development and In vivo Evaluation of Zidovudine Niosomes
5.4 CONSTRUCTION OF STANDARD CURVE

Preparation of Standard Stock Solution

Accurately, about 100 mg of Zidovudine was weighed and transferred to a 100 ml volumetric flask. The drug was dissolved in 100 ml of PBS pH 7.4 with shaking and then the volume was made up to the mark with PBS pH 7.4 to obtain a standard stock solution of a drug concentration, 1000 µg/ml.

Selection of Analytical Wavelength

With appropriate dilution of the standard stock solution with PBS pH 7.4, the solution was scanned using the double beam UV visible spectrophotometer (Model: UV - 1650 PC, SHIMADZU) in the spectrum mode between the wavelength range of 400 nm to 200 nm.

Standard Plot

Stock solution was further diluted with PBS pH 7.4 to get different concentrations 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 µg/ml for the determination of linearity range.

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Table 10: Purity Profile of Span

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Span 20</th>
<th>Span 40</th>
<th>Span 60</th>
<th>Span 80</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Soluble in petroleum ether, benzene</td>
<td></td>
</tr>
<tr>
<td>Surface Tension</td>
<td>28 dynes/cm</td>
<td>36 dynes/cm</td>
<td>46 dynes/cm</td>
<td>30 dynes/cm</td>
</tr>
<tr>
<td>Density</td>
<td>1.01 gm/ml</td>
<td>Solid</td>
<td>Solid</td>
<td>1.01 gm/ml</td>
</tr>
</tbody>
</table>
5.5 FORMULATION OF NIOSOMES (Alisagar et al., 2002)

Multilamellar niosomes were prepared by the thin-film hydration method. Accurately weighed quantities of drug, surfactant (Tween or Span), and CHOL were dissolved in chloroform in a round-bottom flask (Table 12). Different molar ratios of DCP were added to each formulation as a negative charge-inducing agent. The chloroform was evaporated at 60°C under reduced pressure using a rotary flash evaporator at various RPM. After chloroform evaporation, the flask was kept under vacuum overnight in a nitrogen atmosphere to remove residual solvent. The thin films were hydrated with 6 ml of phosphate buffered saline (PBS), pH 7.4 and the formulations were sonicated 3 times at 50 Hz in a bath-sonicator for 15 min with 5 min interval between successive times. Vesicle suspensions were also sonicated for 5 min and 2 min.

5.6 OPTIMIZATION OF PROCESS-RELATED VARIABLES IN NIOSOME FORMULATION

The process-related variables of sonication time, hydration medium, hydration time, speed of rotation of flask evaporator and charge-inducing agents were investigated in vesicle formation with 90 µM Tween 80 and 20 µM cholesterol with a fixed amount of zidovudine by trial and error method.

5.7 OPTICAL MICROSCOPY

A drop of niosomal suspension was placed on the microscopic glass slide. Photographs of sonicated and nonsonicated formulations were taken at 40x magnification using the digital camera (Olympus 8 mega pixel) attached to the eye piece of the microscope. Shape and lamellar nature of the nonsonicated vesicles was confirmed with the photographs.
Vesicle size determination

Vesicle size of nonsonicated formulation was determined by optical microscopy using a pre-calibrated eye piece. Eyepiece was calibrated using stage micrometer at 40x magnification. Size of each division of eyepiece micrometer was determined using the formula

\[
\text{Size of each division} = \frac{\text{Number of divisions of stage micrometer}}{\text{Number of divisions of eyepiece micrometer}} \times 100
\]

The average diameter of 100 vesicles was counted for 3 times at different time intervals after 4 hours for the prepared formulation.

5.8 TRANSMISSION ELECTRON MICROSCOPY

A drop of the sonicated niosomal sample was placed onto a carbon-coated grid to leave a thin film. Before drying of this film on the grid, it was negatively stained with 1% phosphotungstic acid (PTA). For this, a drop of staining solution was added onto the film and the excess of the solution was drained-off with a filter paper. The grid was allowed to air dry thoroughly and was then visualized using a Transmission Electron Microscope (Jain et al., 2004) (TEM) with an accelerating voltage of 80 kV.

5.9 DETERMINATION OF POLYDISPERSITY INDEX AND ZETA POTENTIAL

The polydispersity index of vesicles determined using Zetasizer, Nano ZS 90, (Malvern Instruments) working of which is based on dynamic light scattering (Perriea et al., 2004). For the measurement, 100 μl of the formulation was diluted with the appropriate volume of PBS (pH 7.4) and the vesicle diameter was determined. Zeta potential was determined by taking the appropriate diluted formulation in a disposable clear polycarbonate capillary cell.

5.10 DETERMINATION OF VISCOSITY
Viscosity of the formulations was determined using Ostwald viscometer. The time taken for water and formulations to flow from point A to B was calculated and substituted in the formula and the viscosity was calculated.

\[
\text{Viscosity of sample (}\eta_1) = \frac{\rho_1 x t_1}{\rho_2 x t_2} \times \eta_2
\]

Where,
- \(\rho_1\) – density of sample
- \(\rho_2\) – density of water
- \(\eta_1\) – viscosity of sample
- \(\eta_2\) – viscosity of water
- \(t_1\) – time taken by the sample to flow from point A to B
- \(t_2\) – time taken by water to flow from point A to B

5.11 OSMOTIC SHOCK

The effect of osmotic shock on niosomal formulations with and without cholesterol was investigated by monitoring the change in vesicle diameter after incubation of niosome suspensions in media of different tonicity: 1 M NaI (hypertonic), 0.9% NaCl (normal) and 0.5% NaCl (hypotonic). Suspensions were incubated in these media for 3 h and the change in vesicle size was measured by optical microscopy with a calibrated eyepiece micrometer (Vyas et al., 1999).

5.12 DETERMINATION OF DRUG ENTRAPMENT IN VESICLES

Zidovudine niosomal formulations were centrifuged at 15,700 \( \times \) g for 90 min at 4\(^\circ\)C using a refrigerated centrifuge (Eppendorf, 5415 R, Germany) to separate niosomes from non-entrapped drug. Concentration of the free drug in the supernatant was determined by measuring absorbance at 267 nm with a UV spectrophotometer (Shimadzu, model UV 1650 PC, Kyoto, Japan). The percentage of drug entrapment in niosomes was calculated using the following formula of Alsarra et al., 2005. This process was repeated thrice to ensure that free drug was completely removed.

\[
\% \text{ Drug entrapment} = \frac{\text{Total drug} - \text{Drug in supernatant}}{\text{Total drug}} \times 100
\]
5.13 **IN VITRO RELEASE STUDIES**

*In vitro* release was studied using a dialysis bag (Himedia dialysis membrane, 12,000-14,000 molecular weight cut-off) as a 'donor compartment'. Niosomes containing entrapped zidovudine obtained after centrifugation of 2 ml of the formulation were resuspended in 1 ml of PBS, pH 7.4, and used for the release study. The dialysis membrane was soaked in warm water for 10 min, one end was sealed with a clip, the niosome preparation or drug in solution was pipetted into the bag and the bag was sealed with another closure clip to prevent leakage. The dialysis bag was placed in 50 ml of PBS, pH 7.4, at 37 ± 2°C. The medium, which acted as the receptor compartment, was stirred at 100 rpm. Samples of medium (5 ml) were withdrawn hourly and replaced with fresh buffer and zidovudine absorbance at 267 nm was measured using PBS as blank (Zhang et al., 2007). Results were the mean values of three runs.

Percentage release of Zidovudine =

\[
\frac{\text{Concentration obtained from graph (μg/ml)} \times \text{Total volume of dissolution medium} \times 100}{\text{Amount of the drug present in 1 ml of niosomal formulation} \times 1000 \times \text{Dilution factor}}
\]

**Drug release kinetics**

In order to understand the mechanism and kinetics of drug release from niosomal formulations the results of the *in vitro* drug release study were fitted with various kinetic equations like zero order (% release Vs t), first order (log% release Vs t), Higuchi model \((M_t / M_\infty Vs t^{1/2})\).

In order to define a model which would represent a better fit for the formulation, drug release data was further analysed by Hixson-Crowel and Korsmeyer- Peppas models. In peppas equation, \(m_t / m_\infty = kt^n\),
where \( m_t \) is the amount of drug released at time \( t \) and \( m_\infty \) is the amount released at the amount released at time \( t \), \( K \) is the kinetic constant and \( n \) is the diffusional exponent, a measure of the primary mechanism of drug release. \( R^2 \) values were calculated for the given curves obtained by regression analysis of the above plots.

5.14 CALIBRATION CURVE OF ZIDOVUDINE IN SPIKED PLASMA BY HPLC TECHNIQUE

Accurately, about 10 mg of zidovudine was weighed and transferred to a 10 ml standard flask. The drug was dissolved in 5 ml of PBS pH 7.4 with shaking and then the volume was made up to the mark with PBS pH 7.4. Drug in stock solution was extracted with equal volume of ethyl acetate with vigorous shaking. Then the mixture was allowed to separate and the organic layer was completely evaporated at 100\(^\circ\) C. Then the residue was reconstituted with 10 ml of buffer which is considered as the standard stock solution containing 1000 µg/ml.

From the stock solution further dilutions was made to get different concentrations. Linearity was obtained in the concentration between 2 - 10 µg/ml. The standard samples were analyzed at 267 nm using HPLC with acetonitrile: 0.1% glacial acetic acid (25:75) as mobile phase. Retention time was found to be 4.9 minutes. Then the standard graph was plotted against concentration Vs area.

Analysis of samples

100 µl of plasma was taken and to this 1 ml of ethyl acetate was added, mixed well for 5 min and kept aside for 15 min for the separation of organic layer. The organic layer was separated using micropipette and allowed to evaporate at 100\(^\circ\) C to get the residue. The residue was then reconstituted with 1 ml of PBS pH 7.4. Buffer solutions were then injected into 250 x 4.6 mm, phenomenex Luna C18 (2) 100-A column and eluted with Acetonitrile: 0.1% glacial acetic acid (25:75) mobile phase. The peak area was determined at 267 nm.
Drug present in the supernatant layer obtained from different organs was extracted with equal volume of ethyl acetate and analysis was performed as described above.

The area obtained from the analysis of different organs was calibrated using the standard curve.

Drug distributed in different organs were calculated using the area obtained from the HPLC analysis.

Instrumental Model: HPLC with binary pump LC-10 ATVP
Mobile phase: Acetonitrile: 0.1% glacial acetic acid (25:75)
Detector: SPDM-10 AVP UV visible detector
Column: Phenomenex Luna C18 (2), (250 x 4.6 mm) 100 Å
Column temperature: Ambient temperature
Back Pressure: 190 kgf/cm²
Flow rate: 1 ml/min
Injection volume: 0 µl
Lambda max: 267 nm
Retention time: 4.9 min

5.15 TISSUE DISTRIBUTION STUDIES

Tissue distribution nature of prepared niosomal AZT formulations was compared with AZT in intravenous solution and the marketed oral solution formulation in mice. This study protocol was approved by the institutional ethical committee for the use of animals.

For the study, male mice with the body weight of 20–25 g were divided into 6 groups, each of which consisted of 3 animals to carry out tissue distribution profile. Animals were housed in individual, but identical laboratory cages supplied with food and water. Animal dose was calculated from AZT human dose according to the body surface area (BSA) of the animal (Ghosh, 2005). No
toxicity was found after the acute toxicity studies in mice (OECD guideline 423, 2008). AZT formulation that was stored in a refrigerator for 3 months was taken for the study. Mice received niosome formulations and drug in solution at a dose of 52 mg/kg intravenously via the tail vein. Group 1 received Tween 80 formulation, group 2 received Tween 80 formulation with DCP, group 3 received Span 20 formulation, group 4 received Span 20 formulation with DCP, group 5 received drug in solution and group 6 received oral solution formulation.

After 45 min of injection, mice were sacrificed under anaesthesia. By cardiac puncture blood was collected into tubes containing 30 μl of 10% trisodium citrate as anticoagulant. Plasma was separated from other blood components by centrifugation at 3000 rpm for 20 min and it was stored below 0°C. Different organs like heart, lungs, liver, spleen and kidney were removed from each animal and washed with PBS of pH 7.4.

Each organ was separately homogenized in 4 ml of PBS (pH 7.4) using tissue homogenizer at 13,000 rpm for 30 min. The clear supernatant solution was then separated and kept in a refrigerator until further analysis. Drug concentration in this solution obtained from an organ and plasma was analyzed by HPLC technique. Drug was extracted from plasma and other organs by liquid-liquid extraction technique using ethyl acetate as solvent. For this purpose, 200 μl of plasma was taken and to this 1 ml of ethyl acetate was added, mixed well for 5 min and kept aside for 15 min for the separation of organic layer. The organic layer was separated using micropipette and allowed to evaporate at 70–80°C to get the residue. The residue was then reconstituted with 1 ml of PBS (pH 7.4). Buffer solutions were then injected into Phenomenex Luna C18 (2), 250 x 4.6 mm, 100 Å column and eluted with acetonitrile: 0.1% glacial acetic acid (25:75) as mobile phase (Kuksal et al., 2007). The peak area was determined at 267 nm.

Drug present in the supernatant layer obtained from different organs was extracted with equal volume of ethyl acetate and analysis was performed as
described above. The method was validated for accuracy and repeatability (intraday, interday) was ensured.

5.16 PHARMACOKINETIC STUDIES

Pharmacokinetic evaluation was carried out using rabbits in order to determine the kinetics of Tween 80 niosome formulation with and without DCP, drug in solution formulation and the marketed oral solution formulation. Rabbits were kept in individual cages with food and water. Animals were maintained in a 12 h light/12 h dark cycle at room temperature. Rabbits weighing around 1.5 to 2.0 kg were divided into 4 groups, each of which consisted of 3 animals.

Rabbits received formulations and drug in solution at a dose of 18.6 mg/kg which was calculated based on human dose equivalent to 400 mg. Group 1 received Tween 80 formulation, group 2 received Tween 80 formulation with DCP, group 3 group received AZT in solution formulation and group 4 received marketed oral AZT solution formulation. Formulations described were not sterilized and had been tested neither for pyrogen content nor for immunogenicity. Niosome formulations and drug in solution were administered intravenously through marginal ear vein and marketed formulation was given orally to the rabbits.

Blood (0.5 ml) was collected from the marginal ear vein every 30 min time interval up to 2 h and every 1 h interval up to 8 h and 2 h interval till 24 h from the time of injection. The collected blood samples were centrifuged at 3000 rpm for 20 min to separate the plasma. 200 μl of the plasma samples was taken and treated with acetonitrile for extracting the drug from plasma. The plasma samples were centrifuged at 5000 rpm for 20 min in order to separate the organic layer. Then the drug concentration in acetonitrile layer was analysed using HPLC at 267 nm.

Concentration of the drug was calculated and the parameters such as Area Under the Curve (AUC), Area Under Mean Curve (AUMC), Volume of Distribution ($V_d$), Clearance (Cl), Half-life ($t_{1/2}$), Mean Residence Time (MRT) and
Elimination rate Constant ($K_{el}$) were determined (Callender et al., 1999; Wenli et al., 2006) based on non-compartmental model (WinNonlin 4.1 version).

**DETERMINATION OF RELATIVE BIOAVAILABILITY**

Relative bioavailability for the formulations was determined using the following formula,

$$\text{Relative bioavailability (Fr)} = \frac{(\text{AUC})_{\text{test}} \times D_{\text{std}}}{(\text{AUC})_{\text{std}} \times D_{\text{test}}}$$

Where,

- $(\text{AUC})_{\text{test}}$ – Area under the curve after niosomal administration
- $(\text{AUC})_{\text{std}}$ – Area under the curve after drug in solution administration
- $D_{\text{std}}$ – Standard dose
- $D_{\text{test}}$ – Test Dose

**Data analysis and statistics**

The data is expressed as mean ± SD. Statistical analysis of tissue distribution studies and pharmacokinetic parameters was performed by student’s t-test or ANOVA analysis followed by Turkey-Kramer multiple comparisons post-test using Graph Pad Prism software (version 3.0). The level of significance was taken at p values < 0.05.

**5.17 STABILITY STUDIES**

Stability studies were carried out at an accelerated condition as per ICH guidelines with minor modifications. 2 ml of each formulation was taken in a 10 ml ampoule, sealed and kept at room temperature (28±2°C) and at refrigerated condition (2–8°C). Relative humidity was noted each day which was found to be 65±5% during a study period of 90 days (Saranjit Singh., 2006; Agarwal et al., 2001; Alisagar et al., 2002). Under accelerated storage condition, the products were stored up to 3 months. The sampling was done at intervals of 15, 30, 60
and 90 days after storage. The samples were analyzed for the parameters such as colour, turbidity, pH, leakage and vesicle size.

**Colour**

The change in colour of the formulations was visually analyzed by keeping the sample at the dark background.

**Turbidity**

Percentage transmittance was analyzed to determine the turbidity nature of the formulation with different non-ionic surfactants. 100 μ litres of niosomes formulations were taken and diluted with PBS pH 7.4 (5 ml) in order to reduce the lipid concentration. After rapid mixing for 5 minutes, the percentage transmittance was measured as the absorbance at 267 nm with an ultraviolet – visible spectrophotometer. Higher the percentage transmittance shows less turbid the nature of formulation.

**pH**

pH changes on storage was measured at periodic intervals.

**Drug leakage from vesicles**

Drug leakage from vesicles on accelerated storage condition was determined by analyzing the supernatant solution after ultra centrifugation at 13,000 rpm for 90 minutes at 4° C.

**Vesicle size**

Changes in the vesicle size on storage were determined at 40 X magnification using optical microscopy with pre-calibrated eye piece micrometer.

5.18 **PREPARATION OF PRONIOSOMES**

Proniosomes were formulated using a slurry method with minor modification (Blazekwelsh and Rhodes, 2001) using β-cyclodextrin as the carrier.
One gram of β-cyclodextrin powder (sieved with a 100 mesh) was placed in a 200 ml round bottom flask. Niosomal formulation containing the entrapped drug equivalent to 300 mg was added directly to the flask to form slurry. The flask was attached to the rotary evaporator and vacuum was applied until the powder appeared to be dry and free-flowing. The dry powder was then removed from the flask and kept in a desiccator for 24 h for complete drying.

5.19 TISSUE DISTRIBUTION AND PHARMACOKINETIC STUDIES OF PRONIOSOMES WERE CARRIED OUT AS PER THE PROCEDURE FOR NIOSOMES SPECIFIED IN 5.15 AND 5.16.

5.20 DETERMINATION OF DRUG CONTENT IN PRONIOSOMES (The International Pharmacopoeia, 2009)

The drug content in proniosomes was determined by weighing accurately 100 mg of the AZT Tween 80 proniosomes in 10 ml of methanol: water (20:80) and was sonicated for 2 min. The resulting supernatant solution was filtered and analyzed using UV double beam spectrophotometer at 267 nm. Drug content was estimated after converting the niosomes to proniosomes and after storage of proniosomes for 90 days at room temperature (28±2°C). Data obtained were compared with the niosomal formulation stored at room temperature.