### 4.1. MATERIALS

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Chemical</th>
<th>Manufacturer/ Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Acetonitrile (HPLC Grade)</td>
<td>Merck India Ltd. Mumbai, India</td>
</tr>
<tr>
<td>2.</td>
<td>Ammonia</td>
<td>Merck, Mumbai, India</td>
</tr>
<tr>
<td>3.</td>
<td>Capmul PG 8</td>
<td>ABITEC Corporation, Janesville, Wisconsin, USA</td>
</tr>
<tr>
<td>4.</td>
<td>Capryol PGMC</td>
<td>Gattefosse., Germany</td>
</tr>
<tr>
<td>5.</td>
<td>Capryol 90</td>
<td>Gattefosse., Germany</td>
</tr>
<tr>
<td>6.</td>
<td>Cetostearyl alcohol</td>
<td>Alpha Chemika, Mumbai, India</td>
</tr>
<tr>
<td>7.</td>
<td>Cetyl alcohol</td>
<td>Alpha Chemika, Mumbai, India</td>
</tr>
<tr>
<td>8.</td>
<td>Chloroform</td>
<td>S.D. Fine chemicals., Mumbai, India</td>
</tr>
<tr>
<td>9.</td>
<td>Compritol ATO-5 (Glyceryl behenate)</td>
<td>Gattefosse., Germany</td>
</tr>
<tr>
<td>10.</td>
<td>Diethyl ether</td>
<td>Merck, Mumbai, India</td>
</tr>
<tr>
<td>11.</td>
<td>EDTA</td>
<td>Merck, Mumbai, India</td>
</tr>
<tr>
<td>12.</td>
<td>Ethanol (Absolute alcohol) AR 99.9%</td>
<td>Jiangsu Huax Co., Ltd., Jiangsu, China</td>
</tr>
<tr>
<td>13.</td>
<td>Gellucire 50/13</td>
<td>Gattefosse., Germany</td>
</tr>
<tr>
<td>14.</td>
<td>Glacial acetic acid</td>
<td>Merck, Mumbai, India</td>
</tr>
<tr>
<td>15.</td>
<td>Glyceryl monostearate (Immwitor)</td>
<td>Gattefosse., Germany</td>
</tr>
<tr>
<td>16.</td>
<td>Hydrogenated castor oil – 7675, 7007</td>
<td>Kisan Agro Product Ind., Gujrat, India</td>
</tr>
<tr>
<td>17.</td>
<td>Hydrochloric acid</td>
<td>Merck, Mumbai, India</td>
</tr>
<tr>
<td>18.</td>
<td>Labrafac PG</td>
<td>Gattefosse., Germany</td>
</tr>
<tr>
<td>19.</td>
<td>Labrafil M 2125 CS</td>
<td>Gattefosse., Germany</td>
</tr>
<tr>
<td>20.</td>
<td>Labrafil M 1944 CS</td>
<td>Gattefosse., Germany</td>
</tr>
<tr>
<td>21.</td>
<td>Labrasol</td>
<td>Gattefosse., Germany</td>
</tr>
<tr>
<td>22.</td>
<td>Lauric acid (Decanoic acid)</td>
<td>Alpha Chemika, Mumbai, India</td>
</tr>
<tr>
<td>23.</td>
<td>Lauroglycol FCC</td>
<td>Gattefosse., Germany</td>
</tr>
<tr>
<td>24.</td>
<td>Lauroglycol – 90</td>
<td>Gattefosse., Germany</td>
</tr>
<tr>
<td>25.</td>
<td>Maisine 35-1</td>
<td>Gattefosse., Germany</td>
</tr>
<tr>
<td>26.</td>
<td>Mannitol</td>
<td>S.D. Fine chemicals., Mumbai, India</td>
</tr>
<tr>
<td>27.</td>
<td>Methanol HPLC Grade</td>
<td>Merck, Mumbai, India</td>
</tr>
<tr>
<td>28.</td>
<td>Miglyol 810</td>
<td>SASOL Germany GmbH</td>
</tr>
<tr>
<td>29.</td>
<td>1-Octanol</td>
<td>Spectrochem Pvt. Ltd., Mumbai, India</td>
</tr>
<tr>
<td>30.</td>
<td>Oleic acid</td>
<td>S.D. Fine chemicals., Mumbai, India</td>
</tr>
<tr>
<td>31.</td>
<td>Olive oil</td>
<td>Suyash Herbs export Pvt. Ltd. Gujarat, India</td>
</tr>
<tr>
<td>32.</td>
<td>n-butanol</td>
<td>S.D. Fine Chem., Mumbai, India</td>
</tr>
<tr>
<td>33.</td>
<td>Paraffin wax</td>
<td>S.D. Fine Chem., Mumbai, India</td>
</tr>
<tr>
<td>34.</td>
<td>Peccol</td>
<td>Gattefosse., Germany</td>
</tr>
<tr>
<td>35.</td>
<td>Plurol oleique</td>
<td>Gattefosse., Germany</td>
</tr>
<tr>
<td>Chapter 4</td>
<td>Experimental</td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>---------------</td>
<td></td>
</tr>
<tr>
<td>36. Poloxamer 188</td>
<td>Merck, Germany</td>
<td></td>
</tr>
<tr>
<td>37. Polyene glycol</td>
<td>Polype Polyene Group Company, Guindy, Chennai</td>
<td></td>
</tr>
<tr>
<td>38. Polyethylene glycol 400</td>
<td>Merck, Mumbai, India</td>
<td></td>
</tr>
<tr>
<td>40. Polyvinyl alcohol (PVA)</td>
<td>Merck, St. Louis, MO, US</td>
<td></td>
</tr>
<tr>
<td>41. Potassium bromide</td>
<td>Merck, Mumbai, India</td>
<td></td>
</tr>
<tr>
<td>42. Potassium dihydrogen ortho phosphate</td>
<td>S.D. Fine Chem., Mumbai, India</td>
<td></td>
</tr>
<tr>
<td>43. Precirol ATO-5</td>
<td>Gattefosse, Germany</td>
<td></td>
</tr>
<tr>
<td>44. Sodium hydroxide</td>
<td>S.D. Fine Chem., Mumbai, India</td>
<td></td>
</tr>
<tr>
<td>45. Sodium sulphide</td>
<td>Merck, Mumbai, India</td>
<td></td>
</tr>
<tr>
<td>46. Sodium taurocholate</td>
<td>Merck, Germany</td>
<td></td>
</tr>
<tr>
<td>47. Stannous chloride</td>
<td>Merck, Mumbai, India</td>
<td></td>
</tr>
<tr>
<td>48. Stearic acid</td>
<td>Qualikems Fine, Chemicals Pvt. Ltd., India</td>
<td></td>
</tr>
<tr>
<td>49. Sulphuric acid</td>
<td>Merck, Mumbai, India</td>
<td></td>
</tr>
<tr>
<td>50. Trichloro acetic acid</td>
<td>Merck, Mumbai, India</td>
<td></td>
</tr>
<tr>
<td>51. Triethylamine</td>
<td>S.D. Fine Chem., Mumbai, India</td>
<td></td>
</tr>
<tr>
<td>52. Tween – 20, 80</td>
<td>S.D. Fine Chem., Mumbai, India</td>
<td></td>
</tr>
<tr>
<td>53. Unitop – 100 (Polyoxyethylene Alkyl Phenol Ether)</td>
<td>Unitop, Mumbai, India</td>
<td></td>
</tr>
<tr>
<td>54. Water (Ultrapurified)</td>
<td>Millipore, Molsheim, France</td>
<td></td>
</tr>
</tbody>
</table>

All other reagents used were of analytical reagent grade.
## 4.2. EQUIPMENTS

### Table 4.2. List of equipments

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Equipment</th>
<th>Model / Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Centrifuge</td>
<td>REMI International, Mumbai, India</td>
</tr>
<tr>
<td>2.</td>
<td>Dialysis tube (flat width 25 mm)</td>
<td>Sigma Aldrich, St. Louis, MO, USA</td>
</tr>
<tr>
<td>3.</td>
<td>Differential scanning calorimeter</td>
<td>Pyris DSC, Perkin Elmer, Shelton, CT, USA</td>
</tr>
<tr>
<td>4.</td>
<td>Electronic balance</td>
<td>Mettler Toledo Inc., OH, USA</td>
</tr>
<tr>
<td>5.</td>
<td>Fourier Transform Infrared (FT-IR) spectrometer</td>
<td>Biotechnologies Inc., New Delhi, India</td>
</tr>
<tr>
<td>6.</td>
<td>Freeze dryer</td>
<td>Heto Drywinner, Birkerod, Denmark</td>
</tr>
<tr>
<td>7.</td>
<td>Gamma counter dose calibrator</td>
<td>Scientific Equipment, Delhi, USA</td>
</tr>
<tr>
<td>8.</td>
<td>Hot air oven</td>
<td>Scientific Equipment, Delhi, India</td>
</tr>
<tr>
<td>9.</td>
<td>Hot plate</td>
<td>Shimadzu, Kyoto, Japan</td>
</tr>
<tr>
<td>10.</td>
<td>HPLC (Quaternary)</td>
<td>(API 3000) Biosystems, CA, USA</td>
</tr>
<tr>
<td>11.</td>
<td>LC/MS/MS</td>
<td>5 MLH DX, Remi instruments Ltd., Mumbai, India</td>
</tr>
<tr>
<td>12.</td>
<td>Magnetic stirrer</td>
<td>Nirmal International, Delhi, India</td>
</tr>
<tr>
<td>13.</td>
<td>Melting point apparatus</td>
<td>(HB43) Halogen, Kolkata, India</td>
</tr>
<tr>
<td>14.</td>
<td>Moisture Analyzer</td>
<td>Axiva Sichem Biotech, New Delhi, India</td>
</tr>
<tr>
<td>15.</td>
<td>Nylon 0.45 and 0.22 μm syringe filter</td>
<td>Zetasizer Nano ZS, Malvern Instruments, Worcestershire, UK</td>
</tr>
<tr>
<td>16.</td>
<td>Particle size analyzer</td>
<td>Pan analytical, Model no. PW 18130, Germany</td>
</tr>
<tr>
<td>17.</td>
<td>Powder X-ray diffractometer (PXRD)</td>
<td>Microprocessor pH System, Punjab, India</td>
</tr>
<tr>
<td>18.</td>
<td>pH meter</td>
<td>LEO, 435 VP, Cambridge, USA</td>
</tr>
<tr>
<td>20.</td>
<td>Transmission electron microscope</td>
<td>PRAMA Ultrasonicator, Mumbai, India</td>
</tr>
<tr>
<td>21.</td>
<td>Ultrasonicator</td>
<td>REMI International, Mumbai, India</td>
</tr>
<tr>
<td>22.</td>
<td>Ultracentrifuge</td>
<td>(UV -1601) Shimadzu, Kyoto, Japan</td>
</tr>
<tr>
<td>23.</td>
<td>U.V – Vis spectrophotometer</td>
<td>VDA-DR, Veego, Mumbai, India</td>
</tr>
<tr>
<td>24.</td>
<td>USP dissolution apparatus</td>
<td>Nirmal International, Delhi, India</td>
</tr>
<tr>
<td>25.</td>
<td>Vortex mixer</td>
<td>Nirmal International, Delhi, India</td>
</tr>
<tr>
<td>26.</td>
<td>Water bath shaker</td>
<td>Nirmal international, Delhi, India</td>
</tr>
</tbody>
</table>
4.3. METHODS

4.3.1. Physical Characterization and Identification

The sample of DLX was characterized on the basis of its physiochemical properties such as colour, odour, physical form (amorphous or crystalline), taste, melting point, partition coefficient, loss on drying and solubility in solvents including water, chloroform, methanol, and ethanol.

4.3.1.1. Organoleptic properties

The organoleptic properties that were determined included physical form, colour, taste and odour.

4.3.1.2. Solubility

The solubility was determined by "Mechanical shaker method". The saturated solution of DLX was obtained by placing excess amount (2.0 g) of drug in distilled water (10 mL) at 25 °C in mechanical shaker for 72 h to attain the equilibrium (Higuchi and Connors, 1965). The solution was filtered through Whatman filter paper (0.45 µm) and quantified by UV spectrophotometry at 289 nm. Similarly, the solubility was determined in different solvents including chloroform, methanol and acetonitrile.

4.3.1.3. Partition coefficient

The partition coefficient of the DLX was determined by "Shake flask method". Equal volume of chloroform and distilled water was taken in a conical flask and then excess quantity of drug was added. This mixture was then kept in a mechanical shaker at 100 rpm for 48 h. The mixture was removed from the mechanical shaker, transferred into a separating funnel and then allowed to stand for 24 h in order to separate the two solvents. The two solvents were separated with separating funnel, filtered, diluted and absorbance were recorded at its specific λ_max with the help of spectrophotometer against the corresponding blank solvents.

4.3.1.4. Loss on drying

An accurately weighed amount of drug sample (about 2 g) was taken in a clean and dried aluminum pan. The moisture content was determined on HB43 Halogen Moisture Analyzer.
(METTLER TOLEDO, Columbus, OH, USA) at 105 °C for 3 min. After 3 min when the sample was fully dried, the percentage moisture content was analyzed on the instrument.

4.3.1.5. Melting point
Melting point of DLX was determined by capillary method. DLX was dried over self-indicating silica gel for 24 h. It was then introduced into a capillary glass tube. A sufficient quantity of the dried drug powder formed a compact column of 4 to 6 mm height. This capillary tube was then inserted into HICON melting point apparatus Hicon (Zhouxiang, China) along with thermometer. The temperature at which drug melted was recorded. The melting point of DLX was also confirmed by DSC.

4.3.1.6. Identification tests/Spectral analysis
4.3.1.6.1. UV spectral analysis
A sufficient amount of DLX sample was dissolved in 100 mL of double distilled water and scanned for UV-absorption using UV-spectrophotometer in the range of 200 to 400 nm and the \( \lambda_{\text{max}} \) was determined using the Shimadzu UV - 1601 spectrophotometer (Tokyo, Japan). Similarly, the \( \lambda_{\text{max}} \) was determined in different solvents including methanol, phosphate buffer, chloroform and ethanol.

4.3.1.6.2. Fourier transform infrared (FTIR) spectral analysis
The FTIR spectral analysis of DLX was carried out by FTIR spectrometer (Varian 7000, Agilent Technologies, NY, USA). Infrared spectrum of DLX was taken using potassium bromide (KBr) disc method. DLX was taken in a ratio of (100:1) with respect to KBr and then pellets were made. It was scanned between 400 – 4000 cm\(^{-1}\). Furthermore, the spectrum of DLX was compared with reference spectrum.

4.3.1.6.3. Differential scanning calorimetry (DSC) analysis
DSC analysis of DLX was performed by Pyris 6 DSC instrument (Perkin Elmer, Massachusetts, USA). Differential Scanning Calorimetry (DSC) was carried out in order to determine the melting point out of the drug sample and to confirm its purity. DLX was accurately weighed (3.1 mg) and sealed into an aluminium pan with a DSC loading puncher. The sample was
scanned between the temperature ranges of 40 – 300 °C at a heating rate of 10 °C/minute, under nitrogen atmosphere with a flow rate of 20 ml/minute.

4.3.1.6.4. X-ray diffraction (XRD) analysis
The XRD data analyses of DLX samples were carried out by Powder X-Ray Diffractometer (PW-1830, Philips, Netherland) using monochromatic Cu Kα radiation (8.04 keV and λ = 0.154 nm) and scanned from 2° to 70°, 2θ at a step size of 0.045° and a step time of 0.5 s.

4.3.2. COMPATIBILITY STUDIES
A physical compatibility study was designed to determine the interaction of the drug with various excipients. These samples were kept at accelerated conditions of 60°C in sealed glass vials, and 40°C/75 % RH in open glass vials (punctured to enable exposure to RH conditions for 30 days). These samples were then periodically examined against a control sample kept at 4°C. DSC of the control samples was also performed to check molecular interaction with any of the excipients. Accurately weighed amount of the drug (100 mg) was triturated well with the required amount of the excipients in a glass mortar. The sample was transferred to butter paper and packed as mentioned below:

- Control: Sealed vials
- 40°C/75 % RH (Open): Open vials
- 40°C/75 % RH (Close): Sealed vials
- 60°C (Open): Open vials
- 60°C (Close): Sealed vials

4.3.3. ANALYTICAL METHODOLOGY FOR DLX

4.3.3.1. Preparation of working solutions: Various working solutions were prepared freshly when needed according to USP 2000, 2006, BP 2001 and I.P. 1996.

- Potassium dihydrogen phosphate solution (0.2 M): 27.2 gm of KH₂PO₄ (M.W. 136.09) was dissolved in 1000 ml of distilled water to give 0.2 M potassium dihydrogen phosphate.
- Sodium hydroxide solution (0.2 M): 8 gm of NaOH (M.W. 40) was dissolved in 1000 ml of distilled water to give 0.2 M sodium hydroxide solution.
5 > Phosphate buffer (pH 3.0): Phosphate buffer (pH 3.0) was prepared by dissolving 34 g of Potassium dihydrogen orthophosphate in 250 ml of water (double distilled). The pH of the solution was adjusted to 3.0 with orthophosphoric acid (British Pharmacoepoeia, 2001).

> Phosphate buffer (pH 7.4): 50 ml of 0.2 M potassium dihydrogen phosphate was added to 39.1 ml of 0.2 M Sodium hydroxide solution in a 200 ml volumetric flask and volume was made up to 200 ml with distilled water.

> Phosphate buffer (pH 6.4): 50 ml of 0.2 M potassium dihydrogen phosphate was added to 11.6 ml of 0.2 M Sodium hydroxide solution in a 200 ml volumetric flask and volume was made up to 200 ml with distilled water.

> Phosphate buffer (pH 6.0): Potassium dihydrogen orthophosphate (KH$_2$PO$_4$) (6.80 g) was taken in a 250 ml volumetric flask and the volume was made with double distilled water. This solution was placed in a 1000 mL volumetric flask, and then adjusted to pH to 6.0 ± 0.05 by adding 5.7 mL of NaOH solution (0.2M), and finally the volume was made upto 1000 mL. The solution was filtered through 0.45 μm membrane filter and degassed prior to use (USP 2006).

> Normal Saline solution (0.9% NaCl): 0.9 gm of sodium chloride (NaCl) was dissolved in 100 ml of distilled water to give 0.9% NaCl solution.

### 4.3.3.2. Determination of $\lambda_{\text{max}}$ in different media

To observe the $\lambda_{\text{max}}$ of DLX in different media, a solution of DLX was prepared in various solvents including Phosphate buffer (pH 6.0), distilled water, chloroform, methanol and ethanol & chloroform (1:1 v/v). The UV scan was done between 200- 400 nm and $\lambda_{\text{max}}$ was observed.

### 4.3.3.3. Preparation of calibration curves

(A) Preparation of calibration curves in different solvents

DLX (10 mg) was weighed accurately using an electronic weighing balance. The weighed sample of DLX was dissolved in 100 ml of respective solvent. The resulting mixture gave a stock solution of 100μg/ml. From this stock solution, serial dilutions were prepared in the concentration range of 2 - 20 μg/ml using the same solvent. The absorbance values were taken at
the respective $\lambda_{\text{max}}$ using the spectrophotometer (UV-1601 Shimadzu, Tokyo, Japan) against the pure solvent as blank. The calibration curves were prepared in the following solvents:

- Phosphate buffer (pH-6.0)
- Distilled (double) water
- Methanol
- Chloroform
- Ethanol & Chloroform (1:1 v/v)

4.3.3.4. UV spectral analysis

UV method was employed for routine analysis of DLX, for the determination of encapsulation efficiency of NLC, drug loading and in vitro release studies, by making suitable changes in the sample preparation.

(A) Preparation of buffer (Phosphate buffer pH 6.0)

Phosphate buffer (pH 6.0) was prepared as described in section 4.3.3.1.

(B) Preparation of calibration curve

The calibration curve was prepared as described in section 4.3.3.1.

(C) Method Validation

The method was validated according to ICH guidelines, Q2 (R1). The method was validated with respect to linearity (calibration model) and range, precision, accuracy, limit of detection (LOD) and limit of quantitation (LOQ).

(i) Linearity and range

The calibration curve was plotted in the concentration range of 2 - 20 $\mu$g/mL. Accurately measured volumes of stock solution of DLX (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0 mL) of concentration 100 $\mu$g/mL were transferred into a series of 10 mL volumetric flasks and diluted to the mark. The absorbance of the solutions was determined at $\lambda_{\text{max}}$ of 289.6 nm. A calibration curve was constructed by plotting absorbance vs. concentration of standard solution and the regression equation was calculated. Each reading was mean of three determinations.
(ii) Precision

Three concentrations of drug solution were prepared near the test concentration of DLX. The precision method was assessed by analyzing DLX in three different concentrations at 6, 12 and 18 μg/ml of DLX.

(a) Repeatability

Repeatability (intraday) was assessed by analyzing DLX in three different concentrations (6, 12 and 18 μg/ml) of DLX three times a day. The % RSD was calculated for absorbance thus obtained, to obtain the intraday variation.

(b) Intermediate precision

Intermediate precision (interday) was established by analyzing three different concentrations (6, 12 and 18 μg/mL) of DLX three times a day for three different days. The % RSD was calculated for absorbance thus obtained, to obtain the interday variation.

(iii) Accuracy as recovery studies

Accuracy was determined by recovery studies using standard addition method. The pre-analyzed samples were spiked with extra 50, 100 and 150% of the standard DLX and the mixtures were analyzed by the proposed method. The experiment was conducted in triplicate. This was done to check for the recovery of the drug at different concentration levels in the formulations.

(iv) Limit of detection (LOD) and Limit of quantitation (LOQ)

The limit of detection (LOD) and the limit of quantification (LOQ) of the drug were calculated using the following equations as per International Conference on Harmonization (ICH) guidelines.

\[
LOD = 3.3 \times \sigma/S
\]

\[
LOQ = 10 \times \sigma/S
\]

Where \( \sigma \) = the standard deviation of the response; S = the slope of the regression line.
(v) Preparation of DLX sample solutions

Accurately weighed samples (20 mg) were transferred into a 100 ml volumetric flask, and then DLX was extracted from the NLC formulations by using 60 ml of phosphate buffer (pH 6.0) and sonicated for 5 min with intermittent shaking. Further it was diluted up to the mark with phosphate buffer and mixed. The resulting solution was taken in ultrafiltration centrifugation tube (Nanosep®) and centrifuged at 3000 rpm for 15 min. Further 0.5 ml of the clear filtrate solution was diluted to 5 ml with phosphate buffer. The solution was filtered through 0.45μm millipore filter and DLX content was determined spectrophotometrically.

(D) Sample preparation

(i) For encapsulation efficiency:

The supernatant of formulations after centrifugation were taken as such without further processing and filtered through 0.45 μm filter, and DLX content was determined spectrophotometrically.

(ii) For drug loading:

The pellet formed after centrifugation was dissolved to form drug solution. It was filtered through 0.45 μm filter and DLX content was determined spectrophotometrically.

(ii) For in vitro release studies:

Samples at pre-determined various time intervals were taken as such without further dilution and filtered through 0.45 μm filter and DLX content was determined spectrophotometrically.
4.3.3.5. HPLC method for analysis of samples of in vitro studies

Chromatographic conditions: The chromatographic system consisted of high performance liquid chromatography (HPLC), Shimadzu (Tokyo, Japan). HPLC equipped with quaternary LC-10A VP pumps, variable wavelength programmable UV/VIS detector SPD-10AVP and column oven was used. SCL 10AVP system controller, Rheodyne injector fitted with a 20-mL loop and Class-VP 5.032 software were used. The HPLC column used was a reverse phase 25 x 0.46 mm ID SUPELCO 516 C18 DB, 5 μm. The whole system was kept at ambient conditions. The mobile phase was composed of a mixture of acetonitrile and a pH 3.0, 20 mM phosphate buffer containing 0.3% (v/v) triethylamine (40:60 v/v) with a flow rate of 1 mL/min. The injection volume was 20 μL and the elute was analyzed at 230 nm. The chromatographic conditions were used as follows:

- **Column:** SUPELCO 516 C18 DB (25 mm x 0.46 mm x 5 μm)
- **Mobile phase:** Mixture of acetonitrile and a pH 3.0, 20 mM phosphate buffer containing 0.3% (v/v) triethylamine (40:60 v/v)
- **Flow rate:** 1.00 mL/min
- **Temperature:** 25°C
- **Wavelength:** 230 nm
- **Injection Volume:** 20 μL

**Calibration curve of DLX**

A stock solution of DLX (100 μg/ml) was prepared by diluting a concentrated solution (500 μg/ml). The concentrated solution (500 μg/ml) was prepared by dissolving 25 mg of DLX in 50 ml of mobile phase. The solution (2 ml) was transferred to 10 ml volumetric flask and volume was made to obtain a 100 μg/ml solution. Different concentrations (2 - 20 μg/ml) were made for the preparation of calibration curve from the prepared stock solution. The mobile phase after filtration through 0.22 μm membrane filter was delivered at 1.00 ml/min for column standardization and baseline was continuously monitored during the process. The wavelength of detection was selected at 230 nm. The prepared dilutions were injected serially and areas under the peaks were recorded for each dilution. The stability of drug in solution during analysis was
determined by repeated analysis of samples during the course of experimentation on the same
day and also after 48 h storage of DLX solution at laboratory bench conditions and in the
refrigerator.

Method Validation

Linearity
Various concentrations of DLX from 2 - 20 µg/ml were prepared from stock solution (100
µg/ml) and areas under peak were calculated. The graph was plotted between concentration and
area under peak for linearity.

Accuracy as recovery
Accuracy was determined by standard addition method. The pre-analyzed samples of DLX were
spiked with the extra 0, 50, 100 and 150 % of the standard DLX and the mixtures were analyzed
by the proposed method. The experiment was performed in triplicate. The % recovery of
samples, % RSD, % bias and standard error of mean (SEM) were calculated at each
concentration level.

Precision
Precision was considered at two levels of ICH, Q2 (R1), suggestions i.e. repeatability and
intermediate precision. Repeatability of sample application was determined as intraday variation
whereas intermediate precision was determined by carrying out inter-day variation for the
determination of DLX at four different concentration levels of 4, 8, 12 and 16 µg/ml in triplicate.

Reproducibility
Reproducibility of the method was investigated by obtaining precision on a different instrument,
which was analyzed by another person in different laboratory. Both intraday and interday
precision was calculated at four different concentration levels i.e., 4 µg/ml, 8 µg/ml, 12 µg/ml
and 16 µg/ml. This was done in triplicate.
Detection (LOD) and quantification (LOQ) limits

LOD and LOQ were determined by standard deviation (S_y/x) method. For the determination of LOD and LOQ, blank samples were injected in triplicate to the chromatograph, and then peak area of this blank was calculated. The LOD and LOQ were determined using the slope of the calibration curve and S_y/x of the blank sample by following formulae:

\[
LOD = 3.3 \times \frac{S_y/x}{S} \\
LOQ = 10 \times \frac{S_y/x}{S}
\]

Where S_y/x is the standard deviation of the blank response and S is the slope of the calibration curve.

4.3.3.6. HPLC method for analysis of plasma samples containing duloxetine (in-vivo studies)

Preparation of working solution

Phosphate buffer (PB) (pH 3.5) was prepared as per formula given below (British Pharmacopoeia, 2001);

\[
\text{Potassium dihydrogen ortho-phosphate: } 68 \text{ g} \\
\text{Distilled water (q.s.): } 1000 \text{ ml}
\]

The pH of the solution was adjusted to 3.5 with ortho-phosphoric acid.

Equipment: A Shimadzu model HPLC equipped with quaternary LC-10A VP pump, variable wavelength programmable UV/VIS detector SPD-10AVP column oven (Shimadzu), SCL 10AVP system controller (Shimadzu), Rheodyne injector fitted with a 20 µL loop and Class-VP 5.032 software was used.
**Experimental**

**Chromatographic conditions:**

The chromatographic conditions used were:

- **Column:** SUPELCO 516 C18 DB (25 mm x 0.46 mm x 5 µm)
- **Mobile phase:** Mixture of acetonitrile and a pH 3.5, 20 mM phosphate buffer containing 0.3% (v/v) triethylamine (50:50 v/v)
- **Flow rate:** 1.00 ml/min
- **Temperature:** 25°C
- **Wavelength:** 230 nm
- **Injection Volume:** 20 µl

**HPLC method for analysis of DLX**

The reported method for the determination of plasma concentration of DLX used a mixture of acetonitrile and a pH 3.0, 20 mM phosphate buffer containing 0.3% (v/v) triethylamine (40:60 v/v) as mobile phase and loxapine \((2\text{-chloro}-11-(4\text{-methyl}-1-piperazinyl)\text{dibenzo}[b,f][1,4]\text{oxazepine})\) as internal standard (Mercolini et al., 2007). The method was modified in terms of mobile phase (30 mM phosphate buffer and acetonitrile (50:50, v/v), pH (3.5) and an internal standard (Venlafaxine HCl). The suitability of the solvent system was decided by sensitivity of the assay, time required for the analysis and use of easily available cost effective solvents. Same solvent system was used for the extraction of the drug from the formulation containing excipients which was used for quantification.

**Sample preparation**

Blood samples were collected from Wistar rats into the tubes containing disodium EDTA. Plasma was obtained from the blood by centrifugation at 5000 rpm for 20 min at room temperature, thawed and allowed to reach at room temperature. An aliquot of plasma (500 µl) was placed into a test tube, standard solution of DLX (50 µl), phosphate buffer (50 µl) (pH 3.5, 20 mM) and chloroform (4 ml) were added respectively. The tubes were vortexed for 1 min and centrifuged for 20 min at 5000 rpm. Upper layer was discarded and the chloroform layer was transferred to a clean test tube and evaporated to dryness at 40°C. The residue was reconstituted.
in 100 μl of mobile phase, mixed well and 20 μl of the final clear solution was injected into the HPLC system.

The internal standard (50 μl of 10 μg/ml of Venlafaxine HCl in methanol) was added to each 500 μl of rat plasma samples and vortexed for 10 s. Phosphate buffer (50 μl) (pH 3.5, 20 mM) was added to it, followed by vortexing (10 s). Chloroform (4 ml) was added, followed by vortexing for 2 min and centrifuged at 5000 g for 5 min. Upper layer was discarded and organic layer was transferred to another set of labelled test tubes. Organic layer was evaporated to dryness under nitrogen. The residue was reconstituted in 100 μl of mobile phase, mixed well and 20 μl of the final clear solution was injected into the HPLC system.

Quantification and calibration curve

Calibration curve of DLX was prepared by spiking 50 μl of DLX standard solutions to 500 μl of blank rat plasma to give final concentrations over the range of 10–1000 ng/ml (working dilutions). These dilutions were done to encompass linearity range of the drug. An aliquot of 50 μl of the internal standard (Venlafaxine) in methanol (10 μg/ml) was added to each sample. The sample extraction and HPLC analysis was performed as described above. The calibration curves were constructed by plotting the peak area ratio of DLX to that of internal standard' versus 'their respective concentrations. The calibration curves were obtained by least square linear regression analysis.
4.4. FORMULATION DEVELOPMENT

4.4.1. SELECTION OF INGREDIENTS FOR NLC PREPARATION

The different ingredients required for NLC preparation include solid lipid (fat), liquid lipid (oil), active ingredient (drug), stabilizer and an aqueous phase. These ingredients were selected based on the parameters discussed below.

4.4.1.1. Screening of lipids (fat & oil) for NLC

The selection of lipid for the preparation of NLC was based on the solubility of DLX in solid lipid as well as in liquid lipid. An increased amount of DLX was gradually added with continuous stirring using vortex mixer in the melted solid lipid and the presence of DLX crystals was visually analyzed both at the melted condition and after cooling to room temperature during 24 h. The end point of the solubility study was the formation of a clear, pale yellow solution of molten lipid.

The solubility of DLX in liquid lipid was determined by gradually adding DLX in liquid lipid with continuous stirring using vortex mixer and the presence of DLX crystals was visually analyzed. The maximum amount of DLX that was dissolved without visual presence of DLX crystals was taken as the end point i.e., maximum solubility.

4.4.1.2. Screening of surfactant and co-surfactant for NLC preparation

The screening of surfactant and co-surfactant was based on HLB value of lipids (fat & oil) and their physicochemical properties suitable for nose to brain drug delivery system. According to the HLB System, all lipids including fats and oils have a required HLB value to be emulsified with aqueous phase in the presence of surfactant and co-surfactant for stable formulation. The HLB value of surfactant and co-surfactant needed to emulsifying the given lipid to form a stable formulation should be equal to or greater than the HLB value of the lipids. The calculation of HLB value was based on the following formula given by William C. (Bill) Griffin (1949):

\[
HLB_{\text{required}} = \frac{\text{Concentration } \% \times HLB}{100}
\]

The physicochemical properties of stabilizers (surfactant and co-surfactant) also need to be taken into consideration during the selection for nose to brain drug delivery systems. In addition to
emulsify the system, the stabilizers are desired to possess the following properties for the delivery of neurotherapeutics to the brain by intranasal administration (Hirai et al., 1981; Machida et al., 1994):

- Enhancing the permeability (across the nasal mucosa/BBB)
- Inhibiting P-glycoprotein and
- Inhibiting multidrug-resistance associated protein efflux systems on BBB.

4.4.1.3. Screening of cryoprotectant for lyophilization of NLC dispersion

NLCs are prepared in an aqueous environment and the aqueous dispersions have thermodynamic-driven tendency to lower their interfacial surface area with the environment and thus to aggregate. Lyophilization increases the stability and the shelf life of the finished product by preserving it in a relatively more stable dry state as lyophilized cake to be reconstituted with water immediately prior to administration. Freeze drying is a technique that consists of 3 separate steps. First the product is frozen, then sublimation (primary drying) starts which is followed by secondary drying process (Fransen et al., 1986). NLC integrity on freeze drying may be protected against aggregation and fusion in the presence of properly selected cryoprotectants which is an important factor is governing the efficiency of the process of freeze drying of NLC. The most popular cryoprotectants encountered in the literature for freeze-drying (lyophilization) are sugars like trehalose, sucrose, glucose and mannitol.
4.4.2. OPTIMIZATION, PREPARATION AND EVALUATION OF NANOSTRUCTURED LIPID CARRIER (NLC) SYSTEM FOR INTRANASAL ADMINISTRATION

4.4.2.1. Optimization of Lipid and DLX concentrations by Box-Behnken experimental design

In order to develop DLX-NLC (DLX loaded NLC) for nose to brain route of drug delivery, glyceryl monostearate (GMS) as fat and capryol PGMC as oil were selected on the basis of solubility and physicochemical parameters and NLCs were prepared using homogenization followed by ultrasonication method. The preliminary investigations were carried out to determine the value range of independent variables (lipid and DLX concentrations) that were based on the parameters including the formation of homogeneous mixture and phase separation of aqueous and lipid phases, and particle size. Furthermore, the response surface methodology (RSM) using the Box-Behnken design model was used to optimize the lipid and DLX concentrations for the preparation of DLX–NLC.

The formulation variables including drug concentration, lipid concentration, and ratio of liquid lipid (oil) to total lipid were optimized using Box-Behnken statistical design. This design evaluates the main effects, interaction effects and quadratic effects of the ingredients on the formulation characteristics. Initial trials indicated that the above mentioned variables during preparation were the main factors that affected the encapsulation efficiency, drug loading and particle size of the DLX-NLC. Therefore, a Box-Behnken design–response surface methodology was employed to systematically examine the effect of these three formulation variables on particle size (PS) (nm), encapsulation efficiency (EE) (% w/w) and drug loading (DL) (% w/w) of the prepared NLC. The response surface regression analysis generated the mathematical relationship between factors and parameters using a 3-factor, 3-level design by means of Design-Expert® software (8.0.4.1, Stat-Ease Inc., Minneapolis, MN). A total of 17 tests were conducted. The non-linear software-generated quadratic model is given as:

\[ \text{Response} = x_0 + x_1 A + x_2 B + x_3 C + x_{12} AB + x_{13} AC + x_{23} BC + x_{11} A^2 + x_{22} B^2 + x_{33} C^2 \]
Where “Response” is measured in association with each factor level combination; $x_0$ is an intercept; $x_1$ to $x_{33}$ are regression coefficients estimated from the observed experimental values of “Responses”; and A, B and C are the coded levels of independent variables. The terms AB, AC or BC and $A^2$, $B^2$ or $C^2$ represent the interaction and quadratic terms, respectively. The independent variables chosen, together with their low (-1), medium (0) and high levels (+1) are shown in Table 4.3. For each factor, the experimental range was selected on the basis of the results of initial trials. The value range of the independent variables used to prepare the 17 formulations was 1 - 3 g/l for DLX concentration (A), 1- 3 % for lipid concentration (B) and 0.9 - 1 for the ratio of liquid lipid to total lipid (C). The respective observed responses are given in Table 4.4.

| Table 4.3: Independent variables and their matching levels for NLC preparation |
|----------------------------------|---------------------------------|----------------------------|
| Factor | Name | Levels |                      |
|        |      |        | Low (-1) | Medium (0) | High (+1) |
| A      | DLX concentration (g/l) | 1 | 2 | 3 |
| B      | Lipid concentration (%) | 1 | 2 | 3 |
| C      | Ratio of liquid lipid to total lipid | 0.9 | 0.95 | 1 |

Dependent variables

<table>
<thead>
<tr>
<th>Goals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y1 Particle size (nm)</td>
</tr>
<tr>
<td>Y2 Drug encapsulation (%)</td>
</tr>
<tr>
<td>Y3 Drug loading (%)</td>
</tr>
</tbody>
</table>
Table 4.4: Box-Behnken design consisting of experiments for the study of three experimental factors with coded as well as actual values

<table>
<thead>
<tr>
<th>Standard Order</th>
<th>Coded value variables</th>
<th>Actual value variables</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>1</td>
<td>-1</td>
<td>-1</td>
</tr>
<tr>
<td>2</td>
<td>+1</td>
<td>-1</td>
</tr>
<tr>
<td>3</td>
<td>-1</td>
<td>+1</td>
</tr>
<tr>
<td>4</td>
<td>+1</td>
<td>+1</td>
</tr>
<tr>
<td>5</td>
<td>-1</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>+1</td>
<td>-1</td>
</tr>
<tr>
<td>7</td>
<td>-1</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>+1</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>-1</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>+1</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>-1</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>+1</td>
</tr>
<tr>
<td>13</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>17</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

4.4.2.2. Optimization of stabilizers (surfactant and co-surfactant) for the preparation of NLC

The amount of stabilizers including F-68 (Poloxamer 188) as a surfactant and bile salt (sodium taurocholate) as a co-surfactant was varied from 0.5 - 2 (% w/w) and 0.25 - 0.75 (% w/w) respectively, to emulsify the selected lipids (Glyceryl monostearate and capryol PGMC) for the preparation of NLC. The optimum amount of stabilizers required for stable NLC formulation
were screened on the basis of their effects on particles size (PS), polydispersity index (PI), and encapsulation efficiency (EE).

4.4.2.3. Optimization of speed of homogenizer
The different formulations of same composition were prepared at different speed of homogenizer (5000 and 6000 rpm). The physical parameters including the degree of milkiness and phase separation of homogenized mixture as visual parameters and the effects on particles size (PS), polydispersity index (PI), encapsulation efficiency (EE) and zeta potential (ZP) were considered for the selection of speed of homogenizer.

4.4.2.4. Optimization of duration of homogenization
The aqueous and lipid phases were homogenized for 15, 20, 25 min at a constant speed. The physical parameters including the degree of milkiness and phase separation of homogenized mixture as visual parameters and the effects on particles size (PS), polydispersity index (PI), encapsulation efficiency (EE) and zeta potential (ZP) were considered for the selection of duration of homogenization.

4.4.2.5. Optimization of duration of sonication
The homogenized mixture was ultrasonicated for different durations (5, 10 and 15 min). The duration of sonication was selected on the basis of effects on particles size (PS), polydispersity index (PI), encapsulation efficiency (EE) and zeta potential (ZP) of NLC obtained after sonication.

4.4.2.6. Optimization of cryoprotectant
An optimum amount of cryoprotectant was needed to maintain the integrity, particle size distribution and stability of NLC formulations. Mannitol was selected as cryoprotectant as it decreases the osmotic activity of water and crystallization and favors the glassy state of the frozen sample, thus preventing aggregation. The amount of cryoprotectant was varied from 1 - 4% and its effect on particles size (PS), polydispersity index (PI), encapsulation efficiency (EE) and zeta potential (ZP) was studied as optimization parameter in order to get stabilized NLC.
Moreover, the time required for re-dispersion (re-dispersion speed) of lyophilized NLC was observed for different formulations.

4.4.3. Preparation of DLX loaded NLC (DLX-NLC)

The preparation of aqueous NLC dispersions was carried out by homogenization (Rotor-stator homogenizer) (Heidolph, Diax 900, Schwabach, Germany) followed by ultrasonication (Altrasonics India, Mumbai, India) (Figure 4.1). The lipid phase was melted at 80 °C and dispersed in a hot surfactant aqueous solution heated at the same temperature using the homogenizer for 20 min. The obtained primary emulsion was ultrasonicated for 10 min. The lipid phase consisted of glyceryl monostearate (GMS) (1 g) as fat, capryol PGMC (1ml) as liquid lipid and DLX (100 mg) dissolved in it. The surfactant solution consisted of pluronic F-68 (poloxamer) (750 mg) and bile salt (sodium tauroglycholate) (250 mg) in water (50 ml). The obtained nanoemulsion was cooled down to form DLX-NLC dispersion.

Mannitol was used in the freeze-drying process as a cryoprotectant at a concentration of 3 % (w/w). The NLC dispersions were frozen in an aqueous mannitol solution at -20 °C overnight, and then the samples were transferred to the freeze-dryer (Heto Drywinner, Denmark) at -70 °C. After 72 h the lyophilized DLX-NLC powder was collected for further studies. The prepared NLCs were evaluated for physicochemical, *ex vivo*, *in vitro* and *in vivo* studies.

**Figure 4.1. Schematic representation of preparation technique for NLC.**
4.4.4. Characterization of optimized DLX-NLC formulation

The optimized DLX-NLC formulation for nasal drug delivery was analyzed by various characterization techniques as follows:

4.4.4.1. Particle size, size distribution and zeta potential

The particle size analysis of aqueous NLC formulations was performed by photon correlation spectroscopy (PCS) with a Zetasizer (Malvern Instruments, Malvern, UK). The PCS provides the mean particle size (z-average) and the polydispersity index (PI) as a measure of the width of the distribution. The analysis was performed after dilution with double distilled water followed by filtration through Whatman filter paper.

4.4.4.2. Surface morphology

Morphology and structure of the NLCs were studied using transmission electron microscopy (TEM) (Morgagni 268D, FEI, Hillsboro, USA) and scanning electron microscopy (SEM) (Leo 435 VP, Leica, Cambridge, UK).

4.4.4.2.1. Transmission electron microscopy (TEM)

The TEM was equipped with digital imaging and 35 mm photography system. The combination of bright field imaging at increasing magnification and diffraction modes were used to reveal the morphology and size of the NLCs. To perform the TEM observations, the lyophilized NLCs samples were suspended directly into the MilliQ water (5-10 µl) and were placed on a paraffin sheet. Subsequently, copper grid coated with carbon film was placed over the drop of NLC and was left for 1 min to allow the NLCs to adhere on the carbon substrate. The remaining NLC suspension was removed by absorbing the drop with the corner of a piece of filter paper. The grid was placed on a drop of 2% phosphotungstic acid solution for staining for 10 s. The remaining solution was removed by absorbing the liquid with a piece of filter paper and sample was air dried. The sample was examined by TEM and the photomicrographs were captured and analyzed by Soft Imaging Viewer software.
4.4.2.2. Scanning electron microscopy (SEM)
The size and the shape of NLC were further confirmed and evaluated by SEM. The microscope
is competent to work in low and high vacuum mode and equipped with digital imaging and 35
mm photography system. Prior to analysis, samples were diluted with ultrapurified water and
sonicated to obtain a suitable concentration. Then, the samples were spread on a sample holder
and dried using vacuum. Gold sputter coating (SCD 040) was carried out under reduced pressure
in an inert argon gas atmosphere (Agar Sputter Coater P7340) on the dried sample. After sputter
coating the sample on the carbon coated grid was examined under scanning electron microscope
operated at 15–25 KV and photomicrographs were recorded.

4.4.4.3. Crystallinity
X-ray diffraction (XRD) and differential scanning calorimetry (DSC) were employed to identify
the crystal form of DLX dispersed in the lipid matrix.

4.4.4.3.1. X-ray powder diffraction (XRD) analysis
Crystalline structures of the particles were investigated by means of an X-ray diffractometer (Pan
analytical, PW-1830, Germany) using monochromatic CuKα (8.04 keV and λ = 0.154 nm)
radiation. Samples used for XRD studies were DLX, GMS, DLX-NLC, lyophilized DLX-free
NLC (PNLC-M) and mannitol.

4.4.4.3.2. Differential scanning calorimetry (DSC) analysis
The thermograms were recorded by means of a differential scanning calorimeter (DSC) (Perkin-
Elmer, Uberlingen, USA) for the identification of crystallinity. For calorimetric measurements,
standard aluminium pans with accurately weighed 4 mg samples were tightly sealed. Samples
were heated at a scanning rate of 10°C/min over a temperature range between 40°C and 400°C.
An empty pan was used as reference. An inert atmosphere was maintained by purging with
nitrogen. A chiller unit was used in conjunction with the calorimeter to attain the lower
temperatures.
4.4.4. Encapsulation efficiency (EE) and Drug-loading capacity (DL)

The encapsulation efficiency (EE) of NLC was determined as the amount of drug encapsulated in percent related to the total amount of drug added to the system. However, the DL of NLC was determined as amount of loaded drug in percent related to the lipid phase (matrix lipid and drug). These were determined by ultrafiltration-centrifugation technique. A known volume of DLX-NLC dispersion and lyophilized DLX-NLC in double distilled water (500 µl) were transferred to the upper chamber of Nanosep® centrifuge tubes fitted with an ultrafilter (MWCO100KD, Pall Life Sciences, Mumbai, India). The Nanosep® was centrifuged at 4000 rpm (Remi centrifuge IEC-61010, Mumbai, India) for 15 min. The separated NLC was solubilized in a mixture of ethanol and chloroform (1:1 v/v). The organic solution was filtered through 0.45 µm PTFE (polytetrafluoroethylene) membrane filter. The aqueous and organic filtrates were diluted appropriately and the amount of DLX was estimated. The EE and DL were determined by the following equations:

\[
EE (\%) = \frac{\text{Total amount of DLX} - \text{Free amount of DLX}}{\text{Total amount of DLX}} \times 100
\]

\[
DL (\%) = \frac{\text{Amount of DLX detected}}{\text{Amount of lipid} + \text{Amount of DLX detected}} \times 100
\]

PhD (Pharmaceutics), Jamia Hamdard 133
4.4.4.5. In vitro studies of DLX-NLC

In vitro studies comprised of in vitro release and permeation studies which were performed in dissolution apparatus and modified Franz diffusion cell respectively and the samples were analyzed for the release and permeation of DLX respectively.

4.4.4.5.1. In vitro release studies

4.4.4.5.1.1. Dialysis membrane specification

The dialysis membrane used in the study was cellulose membrane (Sigma, St. Louis, Mo., USA). Tubing as such without treatment was stored at room temperature. Its capacity was 60 mL/feet; average flat width was 2.5 mm, and diameter was 16 mm. Its molecular weight cut off was 12000 g/mole.

4.4.4.5.1.2. Treatment of dialysis bag

Treatment of the dialysis bag was done in accordance with the directions written on the package to remove glycerin and sulphur compounds so that pores can be opened.

1) Removed glycerin by washing in running water for 3-4 h.
2) Removed sulphur compounds by treating it with 0.3% w/v sodium sulphide solution in water at 80 °C for 1 min.
3) Washed with hot water at 60 °C for 2 min.
4) Acidified the procured dialysis bag with 0.2% v/v H₂SO₄ in distilled water.
5) Rinsed it with hot water to remove acid.
6) Stored the dialysis bag in the dissolution medium in refrigerator in which the dissolution experiments were to be performed so that the pores remained open.

4.4.4.5.1.3. Procedure for in vitro drug release studies

In vitro release studies were performed using the dialysis bag method (Yang et al., 2010). It was modified to maintain sink conditions and achieve satisfactory reproducibility. A volume containing equivalent amount of DLX in DLX-NLC dispersion; lyophilized DLX-NLC in double distilled water or DLX solution in double distilled water was first poured into the dialysis bag (MWCO 12000, Sigma, MO, US) with the two ends fixed by thread and placed into the release media, containing 250 ml of phosphate buffer solution (pH 6.0). It was stirred with paddle rotating at 50 rpm to uniformly distribute the released DLX in the media at 37 ± 0.5 °C. A volume of 1 ml of the sample was withdrawn at fixed time intervals of 1, 2, 4, 6, 8, 10, 12, 24 h.
and the same volume of fresh medium was added accordingly. Samples were analyzed to determine the release of DLX. The different kinetic models including zero order equation, first order equation, Higuchi release and Korsemeyer-Peppas were used for the determination of release mechanism of DLX from NLC.

4.4.4.5.2. In vitro testing for nasal drug delivery (Permeation studies)
The study was performed to evaluate the potential to permeate through natural membrane to predict the real drug release characteristics (Pisal et al., 2004a; Pisal et al., 2004b). The in vitro testing model of excised porcine nasal mucosal tissue was employed for nasal delivery of DLX or NLC-DLX. The fresh nasal tissue removed from the nasal cavity of porcine was used for this purpose and the experiments were conducted using fabricated diffusion cell. The cumulative amount of drug permeated was used for the determination of the effective permeability coefficients across mucosal membrane was calculated. The histology of treated nasal mucosa membranes also was investigated after the completion of the experiments. Intranasal administration can be useful only if it does not result in either histological or functional toxicities (Wang et al., 2006).

4.4.4.5.2.1. Fabrication of diffusion cell
Franz diffusion cells were fabricated from the local fabricator for the permeation studies. It consisted of two half cells, the upper half part known as donor compartment and the lower half part (body) known as the receiver compartment. The area of Franz diffusion cell between two half cells was 1 cm² and the capacity of the receiver compartment was 15 ml. The diffusion cell was maintained at 37 °C by circulating water at 37 °C through the water jacket of the cell using a re-circulating water bath. The solution in the receiver compartment was stirred continuously at 200 rpm with magnetic stirrer. Nasal mucosal membrane along with the formulation was sandwiched between the two compartments with mucosal side facing the donor compartment.

4.4.4.5.2.2. Selection of animal model
A fairly good number of species have been reported as animal models that can be used for in vitro testing (nasal mucosa permeation studies) of nasal drug delivery systems including pig,
sheep and rabbit (Osth et al., 2002). In the present work due to easy availability porcine nasal mucosa was used (Sintov et al., 2011).

4.4.4.5.2.3. Procurement of nasal mucosa
The porcine nasal mucosa was selected for the study which was obtained from a local slaughter house (New Delhi, India). The morphology of pig mucosa is more comparable to that of humans because of the presence of ciliated and non-ciliated cells, basal cells, goblet cells, serous glands and expression of P-glycoprotein (Pgp) (Wadell et al., 1999). Relatively larger area of respiratory mucosa in the snout of the porcine makes it easier to handle the tissue without the chances of getting damaged and strained piece of mucosa. Furthermore the accessibility of the porcine nasal mucosa at local slaughter house avoided the need for getting ethical committee approval for the sacrifice of animal for a small piece of tissue. After sacrifice of the animal, the cavity mucosa was carefully cut with a scalpel, separated from the sub-layer bony tissues and immediately immersed in saline solution. It was then washed carefully to remove any extraneous particle adhering to the mucosal surface.

4.4.4.5.2.4. Stabilization of the membrane
The nasal tissue was cut to appropriate size and mounted between the donor and receptor compartments of a vertical diffusion cell, with the mucosal side facing the donor compartment and the receiver compartment was filled with saline solution and magnetically stirred at 200 rpm for proper mixing. The diffusion cell was thermostated at 37 ± 0.5°C. The buffer solution was replaced after every half an hour to stabilize the membrane. The skin was stabilized for 4-5 h. After stabilization, UV spectrum was taken and skin was considered stabilized when no UV absorption bands were visible.

4.4.4.5.2.5. Procedure for in vitro testing studies
The in vitro nasal permeation study was performed in modified Franz diffusion cell (Figure 4.2). The cell was fabricated in glass by a local fabricator consisting of a water-jacketed receiver compartment and a donor compartment. The cell was made with a receiver compartment volume of 15 ml and the effective diffusion area of 1 cm². The nasal mucosa was mounted in the diffusion cell with the mucosal and serosal sides facing the donor and receiver compartments,
respectively. The mucosa was placed in such a way that it just touched the diffusion medium in receiver compartment of the cell. A pre-incubation period of 30 min was used after filling both the chambers with saline solution for electrophysiological equilibrium at 37 ± 0.5 °C (Wadell et al., 1999). For permeation studies, a volume containing equivalent amount of DLX in DLX-NLC dispersion; lyophilized DLX-NLC in double distilled water or DLX solution in double distilled water was replaced in the donor compartment, while the fluid in the receiver compartment of the cell was replaced with buffer (pH 6.0). The receiver solution was constantly stirred and kept at a temperature of 37 ± 0.5 °C. A sample of 0.2 ml was taken from the receiver solution at predetermined time intervals of 0.5, 1, 2, 4, 6, 8, 10, 12, 20, and 24 h and replaced with an equal amount of fresh buffer. The collected samples were estimated for drug content. The histological studies were performed to evaluate the signs of severe disruption or damage on the integrity of the nasal mucosa (safety of the preparation) after performing the permeation studies.

4.4.4.5.2.6. Data analysis
The cumulative amount of DLX permeated through the porcine nasal mucosa (Q, μg/cm²) was plotted as a function of time (h). The drug flux (permeation rate) at the steady state (Jss, mg/cm²/h) and lag time were calculated from the slope and intercept of the straight line obtained by plotting the amount of DLX permeated versus time in steady state condition. Permeability coefficient (kp) was calculated by dividing the flux by initial drug concentration (C₀) in the donor portion of cell.

\[
\text{Cumulative amount of drug permeated} = \frac{\text{Concentration (μg/ml)} \times \text{Volume of diffusion cell}}{\text{Area (cm}^2\text{)}}
\]

Volume of diffusion cell = 15 ml
Area of diffusion cell = 1 cm²

\[
\text{Flux} = \text{Slope of steady state portion of the plot between cumulative amount of drug permeated per cm}^2 \text{ Vs time (mg/cm}^2\text{/h)}
\]

\[
\text{Permeability coefficient (Pb)} = \frac{\text{Flux}}{\text{Drug concentration in donor compartment}}
\]
Figure 4.2: Schematic diagram of a Franz diffusion cells.
4.4.4.6. Adhesion properties

The optimized formulation was developed as mucoadhesive NLC using chitosan. It increases the residence time on nasal mucosa and also acts as penetration enhancer (Braso et al., 2003). Different concentrations of chitosan (0.1 – 0.9 % w/w) were prepared in dilute lactic acid solution (2 % w/w). NLC was incorporated in different concentrations of chitosan gel and evaluated for viscosity and mucoadhesion force.

4.4.4.6.1. Determination of Viscosity

Rheological experiments were performed to examine the viscous and elastic properties of the different formulations. The viscosity of chitosan gel was determined at 25±2°C using a Brookfield R/S plus cone and plate Rheometer with spindle C50-1, (Brookfield Engineering Laboratories Inc., Middleboro, MA, USA), at 50 rpm. All viscosity measurements were performed in triplicate.

4.4.4.6.2. Determination of mucoadhesive force

The mucoadhesive forces of the solutions were determined by means of the mucoadhesive force-measuring device (adhesion weight method) shown in Figure 4.3 and according to the previously reported methods (Choi et al., 1998; Mikos and Peppas 1990), using tissues cut from the porcine nasal mucosa. The pieces of tissues were stored frozen in phosphate buffer pH 7.4 and thawed to room temperature before use (Wong et al., 1999). At the time of testing a section of tissue (E) was secured, keeping the mucosal side out, on to each glass vial (C) using a rubber band and an aluminum cap. The diameter of each exposed mucosal membrane was determined. The vials with the nasal mucosa tissue were stored at 37°C for 10 min. Next, one vial with a section of tissue (E) was connected to the balance (A) and the other vial was fixed on a height-adjustable pan (F). To the exposed tissue on this vial, a constant amount of the gel (D) was applied. The height of the vial was adjusted so that the gel could adhere to the mucosal tissues of both vials. A constant (preload) force was placed on the upper vial and applied for 2 minutes (Han et al., 1999), after which it is removed and the upper vial was then connected to the balance. Weights (B) were added at a constant rate to the pan on the other side of the modified balance of the used device until the two vials were separated. The bioadhesive force, expressed as the detachment
stress in dyne/cm², was determined from the minimal weights that detached the two vials using the following equation (Ch'ng et al., 1985):

\[
\text{Detachment stress (dyne/cm}^2) = \frac{mg}{A}
\]

Where, \( m \) = the weight added to the balance in gram.

\( G = \) acceleration due to gravity taken as 980 cm/sec².

\( A \) = area of tissue exposed and is equal to \( \pi r^2 \) ("r" is the radius of the circular hole in the aluminium cap).

Figure 4.3: Bioadhesive Force Measuring Device: (A) modified balance; (B) weights; (C) glass vial; (D) Chitosan gel; (E) Nasal tissue; (F) height-adjustable pan.
4.4.4.7. Hydrophilicity/hydrophobicity

Hydrophobicity/ Hydrophilicity were determined by partitioning in system of two immiscible liquids including chloroform and water (100% and 50% v/v). With the aim to increase the accuracy of result, the measurement was performed at different amounts of DLX-NLC and different volume ratios of partitioned mixture (chloroform-water).

Different mixtures of chloroform, water and DLX-NLC were prepared into separation funnels following the Table 4.5. The separating funnels were shaken gently with prepared mixtures for 20 min. The funnels were put to the holders. After few minutes the mixture apparently separated into two clear layers i.e., water layer (W), and chloroform layer (O). Both the phases were separated out into different beakers using the funnel’s valve. The separated chloroform was mixed with ethanol (50:50 v/v). Amount of DLX was estimated in both the phases.

<table>
<thead>
<tr>
<th>Funnel No.</th>
<th>Amount of NLC (mg)</th>
<th>Volume of water (ml)</th>
<th>Volume of chloroform (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>-</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>10</td>
<td>15</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>15</td>
<td>-</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td></td>
<td>15</td>
</tr>
</tbody>
</table>
4.4.4.8. In vivo studies for intranasal drug delivery

In vivo testing is often employed over in vitro because it is better suited for observing the overall effects of an experiment on a living subject. The use of animal models to assess the potential of a pharmaceutical/biopharmaceutical is a required part of validation and safety of the product and product test effects on humans.

Approval to carry out in vivo studies was obtained from the institutional animal ethics committee (IAEC), Jamia Hamdard, New Delhi (India) and their guidelines were followed throughout the studies. The in vivo studies were performed using adult Swiss albino wistar rats weighing between 200 to 250 gm.

4.4.4.8.1. Dose calculation for rats

Dose for the rats was calculated taking into consideration ratio of body surface area of rat to that of human. The dose to be given to a 200 g rat on the basis of surface area ratio was determined by multiplying the human dose by a factor of 0.018 (Freireich, et al., 1996; Ghosh, 2005).

i. **Intranasal dose of DLX**: - For intranasal administration DLX was dissolved in double distilled water.

   Calculation:

   \[
   \text{Oral human dose of DLX} = 60 \text{ mg/day}.
   \]

   \[
   \text{Oral bioavailability of DLX} = 50\%
   \]

   \[
   \text{Intranasal dose of DLX for human} = \text{Oral dose} \times \text{Bioavailability} = 60 \text{ mg/day} \times \frac{50}{100} = 30 \text{ mg/day}.
   \]

   **Conversion factor from human to rat dose** = 0.018

   \[
   \text{Therefore rat intranasal dose of DLX} = \text{Human oral dose} \times \text{Bioavailability} \times 0.018 = 60 \times \frac{50}{100} \times 0.018 = 0.54 \text{ mg/day}.
   \]

   13.5 mg of drug was dissolved in 1 ml of double distilled water (0.0135 mg/µl) and from this solution 20 µl was administered intranasally in each nostril of rat (20 µl + 20 µl = 40 µl) with the help of a micropipette.
ii. Oral dose of DLX: Oral administration of DLX solution to rats was achieved by using the gavage technique (administration of DLX solution directly into the lower esophagus or stomach using a feeding needle introduced into the mouth and threaded down the esophagus). DLX solution was prepared by dissolving in double distilled water.

\[
\text{Oral rat dose of DLX} = 60 \text{ mg} \times 0.018 = 1.08 \text{ mg/day}.
\]

iii. Intranasal dose of DLX-NLC. A suspension of DLX-NLC was made in double distilled water one hour prior to intranasal administration. DLX-NLC (138.75 mg) was suspended in 1 ml of double distilled water to give a concentration of 13.5 mg/ml of DLX. From this solution 20 μl was administered intranasally in each nostril of rat with the help of a micropipette i.e. the total volume administered was 40 μl (Table 7).

Calculation:

\[
9.73 \text{ mg DLX was present in } 100 \text{ mg of DLX-NLC (DL } = 9.73 \pm 3.22\%)
\]

\[
13.5 \text{ mg DLX was present in } 100 \times 13.5/9.73 = 138.75 \text{ mg of DLX-NLC}
\]

\[
138.75 \text{ mg of DLX-NLC was suspended in 1000 μl of water containing 13.5 mg/ml (or 0.0135 mg/μl) of DLX.}
\]

Therefore, 40 μl will contain 0.54 mg/ml of DLX.
In vivo studies for intranasal drug delivery comprise the following studies:

1. Pharmacodynamic studies
2. In vivo nasal permeation studies
3. Pharmacoscintigraphic studies

4.4.4.8.2. Pharmacodynamic studies
The antidepressant effect of the optimized duloxetine loaded NLC (DLX-NLC) was evaluated by:

1. Locomotor activity test
2. Forced swim test (FST)

Animals were randomly divided into four groups each containing three animals. Group-1 was given DLX solution orally. To the group-2 DLX solution prepared in double distilled water was given intranasally. To the group-3 DLX loaded NLC (DLX-NLC) was given intranasally. The group-4 was control group (untreated) (Table 4.6). Oral doses were given with the help of 16 gauge ball-tipped feeding needle with syringe. For intranasal administration the preparations were instilled in each nostrils (20 μl) with the help of micropipette (100 μl) attached with the polyethylene tube at the delivery site (posterior part of nasal cavity of rat). The rats were held from the back in slanted position during administration of the preparations through nasal route. The locomotor activity test and forced swimming test were performed for 5 min (300 sec).

4.4.4.8.2.1. Locomotor activity Test
Locomotor activity measurement is commonly used to study sensitivity to locomotor activating or depressing effects of a drug. In rats, locomotor activity has been used to discriminate drug effects, to elucidate the functional roles of specific neurobiological systems, and to screen drugs for potential psychoactivity (Paulus et al., 1999).

The test was performed in a digital photoactometer. The photoactometer was validated before each test session by manually interrupting the infrared beams and verifying the correspondence between the actual number of beam breaks and the number recorded by the system. Rats were first habituated to handling and dosing in the locomotor activity chambers where the behaviour was measured by infrared beam breaks, and then a baseline activity measurement was taken following vehicle administration.
Rats were individually placed in the photoactometer for five minutes. Each and every rat was administered (oral and intranasal doses) the dose as per the treatment schedule given in Table 4.6. After 60 minutes the rats were placed individually in the photoactometer for recording the basal activity score. Mean change in the locomotor activity was recorded for each rat. Each animal was observed in a closed square (30 x 30 cm²) arena equipped with infrared light sensitive photocells. A continuous beam of light was made to fall on corresponding photoelectric cells mounted on opposite wall of the photoactometer. The photoelectric cell got activated when an animal crossed the beam of light and thereby cuts off the rays of light falling on it. These cutoffs were counted automatically for a period of 5 min and the figure was taken as a measure of the locomotor activity of the animal. Locomotor activity was expressed in terms of total photobeam counts for 5 min per animal. The apparatus was placed in a darkened, light and sound attenuated, and ventilated testing room. Comparisons are made among treatment groups and/or between behaviour after drug administration and baseline behaviour.

4.4.4.8.2.2. Forced swim test (FST)
The procedure to carry out the FST was followed as described in literature (Porsolt et al., 1977; Cryan et al., 2002) (Figures 4.4 and 4.5). Rats were placed in a cylindrical transparent plastic tank (40 cm tall x 15 cm in diameter) filled with fresh water to a depth of 30 cm for a 15-min pretest. After that they were removed and dried before returning to their cage. Rats were given three oral and intranasal dose of the preparations (Table 4.6) before test swim period of 5-min. The first dose was administered 15 min after pretest (i.e., after removal of the rats from the water on the first day). It was followed by the second and third doses on the subsequent morning, 5 h and 1 h prior to the second immersion in the water for 5-minute test swim. The water depth of 30 cm allowed the rats to swim or float without their hind limbs touching the bottom of the tank. The behavioural analysis of rats was recorded at the end of each 5-minute period during the test session. The effect of treatment on mean time of immobility, climbing and swimming was observed (Kirby et al., 1997). Immobility was scored when the animal was making the minimum movements necessary to stay afloat. Climbing was scored when the animal made vigorous thrashing movements with its forepaws, usually directed against the sides of the tank. Swimming was defined as an active behavior when the animal actively swam around the tank.
Rats were forced to swim in a cylindrical transparent plastic tank filled with fresh water to a depth of 30 cm for 15-min (Pretest period) and then removed and dried before returning to their cage.

Rats were given three doses before test swim period of 5-min. The rats were held from the back in slanted position during administration through the nasal route.

The first dose was administered 15 min after pretest period. It was followed by the second and third doses on the subsequent morning, 5 h and 1 h prior to the second immersion in the water for 5-minute test swim.

Figure 4.4: Stepwise FST procedure.

Figure 4.5: Schematic FST procedure (Swim periods were separated by 24 h).
### Table 4.6: Number of groups with their treatment schedule

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Group Size</th>
<th>Treatment Schedule</th>
<th>Route</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLX solution 3</td>
<td>3</td>
<td>1.08 mg/day</td>
<td>Oral</td>
</tr>
<tr>
<td>DLX solution 3</td>
<td>3</td>
<td>0.54 mg/day</td>
<td>Intranasal</td>
</tr>
<tr>
<td>DLX-NLC 3</td>
<td>3</td>
<td>0.54 mg/day</td>
<td>Intranasal</td>
</tr>
<tr>
<td>Control</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

#### 4.4.4.8.2.2.1 ESTIMATION OF DLX IN BRAIN AND BLOOD

After FST rats were sacrificed and brain & blood samples were collected for DLX estimation. Rats were sacrificed by cervical dislocation method and brain samples were collected.

*Method of Brain Collection in rat*

The anaesthetized live rats were sacrificed by decapitation quickly and cleanly. The cut was made as close to the skull as possible. Using the scalpel, a single cut was made along the length of the head, starting at a point between the eyes, continuing along the dorsal (top) of the head, all the way through to the posterior aspect (back end) of the head. Using the ronguers, the two flaps of skin and muscle was pulled back to expose the skull underneath. It was followed by a cut of thick neck musculature surrounding the base of the skull to expose the entire dorsal/posterior aspect of the skull, with the base of the brain stem visible at the posterior-most point. To obtain a stable grasp of the skull during the process, the left or right mandible was clamped with a suitable sized hemostat. Rongeurs with a 2-3 mm jaw face were used to remove the bone. Beginning at upper edge of the foramen magnum, small bites of bone progressing upward (dorsally) and laterally were taken away. It was ensured that the slippage of the rongeurs will not penetrate the brain by maintain pressure away from the brain surface. The remaining bone was removed along the dorsal surface up to the sinus lying between the olfactory bulbs and the frontal pole of cortex. The bone lying above the bulbs was easily broken free and removed at this point. Using a small bladed spatula, the brainstem was elevated and cut any remaining attached blood vessels and optic nerves. The extracted brain was placed in saline solution (Figure 4.6, 4.7 & 4.8).
collection techniques are practiced on anesthetized or freshly euthanized rat. Blood was collected by the technique of terminal procedure including cardiac puncture (Figure 4.9). It is a suitable technique (cardiac puncture) to obtain a single, large, good quality sample from a euthanized rat or a rat under terminal anaesthesia if coagulation parameters, a separate arterial or venous sample or cardiac histology are not required. It is appropriate for all strains. A sample of 10 - 15 ml of blood can be obtained depending on the size of the rat. Blood samples are taken from the heart, preferably the ventricle either by a thoracotomy, the left side of the chest, through the diaphragm, or from the top of the sternum. Deep surgical anaesthesia is necessary unless the rat is dead. Blood should be withdrawn slowly to prevent the heart collapsing. If blood doesn’t appear immediately, withdraw 0.5 cc of air to create a vacuum in the syringe. Withdraw the needle without removing it from under the skin and try a slightly different angle or direction. When blood appears in the syringe, hold it still and gently pull back on the plunger to obtain the maximum amount of blood available. Pulling back on the plunger too much will cause the heart to collapse. If blood stops flowing, rotate the needle or pull it out slightly.

Figure 4.9: Schematic diagram for blood collection by cardiac puncture.
In vivo nasal permeation studies

The animal models employed for nasal permeation studies can be of two types (Chien et al., 1985),

1. *In vivo* nasal absorption studies (Whole animal or *in vivo* model)

2. *Ex vivo* nasal perfusion studies (Isolated organ perfusion or *ex vivo* model)

Healthy Wistar rats weighing 200 - 250 g were selected for the study and randomly divided into two groups for intranasal infusion of formulations (dose = 0.54 mg/ml), each consisting of 3 animals. One group of rats received intranasal infusion of DLX solution, and another group received intranasal infusion of DLX-NLC suspension. The surgical preparation of rat for *in vivo* nasal permeation studies was carried out as follows:

The rats were anaesthetized by intraperitoneal (i.p.) injection of chloral hydrate (3.5 mg/kg of body weight). An incision was made in the neck and the trachea was cannulated with a polyethylene tube for breathing. Another tube was inserted through the oesophagus towards the posterior region of the nasal cavity. During the perfusion studies, a funnel (if needed) was placed between the nose and receiver (beaker) to minimize the loss of drug preparations. The drug preparation (DLX solution or DLX-NLC suspension) was placed in a reservoir (Marriott bottle) maintained at 37°C and was circulated through the nasal cavity of the rat with the help of a peristaltic pump after maintaining the flow rate at 0.5 ml/min (Figure 4.11). A schematic presentation for *in vivo* nasal permeation studies is shown in Figure 4.12. The drug preparation in the reservoir was continuously stirred. The perfusion solution/suspension passing out from the nostrils was collected at 5, 10, 15, 30, 45 and 60 min of the study in the beaker. The amount of drug absorbed was estimated by measuring the residual drug concentration in the collected samples. The passage of nasopalatine was sealed to prevent the drainage of the drug preparation from the nasal cavity through the mouth. Since all the possible outlets in the rat model were blocked, the only possible passage for the drug to be absorbed and transported into the body was penetration and/or diffusion through the nasal mucosa.

For nasal absorption studies (*in vivo* model), the blood samples and brain (*ex vivo* nasal perfusion) were collected at the end of the study.
4.4.4.10. Pharmacoscintigraphy

Approval to carry out animal studies was obtained from the Institute of Nuclear Medicine and Allied Sciences Animal Ethics Committee, New Delhi and their guidelines were followed throughout the study. The biodistribution studies were performed on Swiss albino Wistar rats (200 to 250 gm) and they were obtained from the Central Animal House Facility of Jamia Hamdard, New Delhi, India.

Pharmacoscintigraphic technique has been widely used to study the in vivo behaviour of drug and drug delivery systems. This technology has proven to be of great value in the assessment of a wide range of pharmaceutical formulations and new drug delivery systems. The radiometric detection of drugs labeled with a suitable radiotracer is the best technique for the detection of concentration of the drugs given through nasal route. Pharmacoscintigraphy study includes gamma-counts of different organs and gamma-imaging of intact animal, after administering the calculated dose of drug. Gamma-counts of gamma-radiation emitted by the deposited radiolabelled DLX and NLC in different organs were done by the gamma-counter. Moreover, the gamma-imaging was done by the gamma-camera which gave images to provide the functional map of physiological processes.
The non-invasive imaging technique of gammascintigraphy was developed originally for use in diagnostic tests in nuclear medicine (Newman and Wilding, 1998). Specific radiopharmaceuticals which localize in different organs and which are visualized by gamma camera are used to provide vital information about the structure and function of various body systems. The technique has been extended to the evaluation of pharmaceutical dosage forms delivered by the oral (Billa et al., 2000), rectal, pulmonary (Saari et al., 1999; Bondesson et al., 2002), nasal (Soane et al., 1999; Ugwoke et al., 1999; Soane et al., 2001), parenteral, ophthalmic and vaginal (Richardson et al., 1996) routes. This method enables direct visualization and quantification of fate of drug which has been delivered, and confirms where the formulation has behaved according to its proposed rationale (Newman and Wilding, 1998). When gammascintigraphy is used in the assessment of nasal drug delivery, the formulation is usually labelled with the gamma-ray emitting radionuclide $^{99m}$Tc (technetium), which has ideal radiation energy (140 keV) for use with a gamma-camera (Newman and Wilding, 1998). The short half-life of $^{99m}$Tc (6 h), coupled with a very clean and safe radiation emission profile which contains few beta-particles, results in very low radiation doses, so that satisfactory scintigraphic data can be obtained using only a fraction of the radiation dose required for diagnostic X-ray procedures (Newman and Wilding, 1998).

4.4.4.10.1. Procedure for radiolabeling

Radiolabeling was performed using sodium pertechnetate. Radioactivity was eluted out from Mo-Tc generator in saline. Ethanol was chosen as a solvent to extract out the radioactivity. A suitable method of radiolabeling was chosen by which DLX was labeled with $^{99m}$Tc. The method of radiolabeling was standardized by gamma-imaging technique so as to visualize the distribution of radiolabeled drug in animal models.

Properties of $^{99m}$Tc-pertechnetate

- It is cost effective
- It has a short half-life of 6.02 h
- It can be easily eluted from the generator
- It is soluble in solvents like acetonitrile and methyl ethyl ketone (MEK)
- The dried form of activity is easily leached out from the glass beaker with the help of acetonitrile.
4.4.4.10.2. Radiolabeling of DLX or DLX-NLC with pertechnetate ions

Radiolabeling was done using $^{99m}$Tc by direct labeling method. DLX or DLX-NLC was accurately weighed (5 mg) in a vial and water (distilled) was added to make a solution. Stannous chloride was dissolved in alcohol to make a solution of 1 mg/mL and 100 μL of this solution was added to the vial containing DLX or DLX-NLC. To the resultant mixture $^{99m}$Tc pertechnetate (200 μCi) was added carefully with continuous mixing and incubated at 25°C for 30 min. Quality control of radiolabeling was done using ITLC-SA strips with acetone to determine radiolabeling efficiency (%).

4.4.4.10.3. Optimization of radiolabeling parameters

The various radiolabeling parameters required to optimize the radiolabeling of DLX or DLX-NLC included incubation time, pH and temperature and reducing agent (SnCl$_2$), to achieve the desired reaction condition for radiolabeling.

Effect of stannous chloride (SnCl$_2$) strength

The solution of concentration 1mg/ml was made by dissolving an accurately weighed sample of SnCl$_2$ in alcohol. The concentrations of SnCl$_2$ were varied from 50-500 μL of 1 mg/ml solution. The radiochemical purity was determined on instant thin layer chromatography (ITLC) using ITLC strips, with acetone as mobile phase.

Effect of pH

The radiochemical purity was determined for pH range of 3.0, 5.5, 7.4 and 10.0 and the pH was optimized for determining the radiochemical purity of the DLX or DLX-NLC complex using ITLC with acetone as a mobile phase.

Effect of temperature

Different temperatures of 20, 25, 30 and 35 °C, were optimized for determining the radiochemical purity of the DLX or DLX-NLC complex using ITLC with acetone as a mobile phase.
Effect of incubation time

The radiolabeled complex of DLX or DLX-NLC with the optimized strength of SnCl\textsubscript{2} solution, pH and temperature were incubated at room temperature for different time intervals of 10, 20, 30, 40, 50, and 60 min. The radiochemical purity of the DLX or DLX-NLC complex was determined using ITLC with acetone as a mobile phase.

4.4.4.10.4. Radiochemical stability of the radiolabeled complexes (DLX or DLX-NLC)

a) In saline - In vitro stability study was performed by mixing 200 μl of radiolabeled DLX or DLX-NLC with 1 ml of saline. Small aliquots were withdrawn at different time intervals up to 24 h and radiochemical stability of DLX or DLX-NLC was evaluated by ITLC using acetone as mobile phase. The developed strips were cut into 7:3 ratios and radioactivity in each part was measured to calculate the stability of the product.

b) In serum - An in vitro serum stability study was performed by mixing 200 μl of radiolabeled DLX or DLX-NLC with 1 ml of serum. Small aliquots were withdrawn at different time intervals up to 24 h and radiochemical stability of DLX or DLX-NLC was evaluated by ITLC using acetone as mobile phase. The developed strips were cut into 7:3 ratios and radioactivity in each part was measured to calculate the stability of the product.

4.4.4.10.5. Biodistribution studies of nasal NLC formulation

ETHICAL CLEARANCE FOR PERFORMING BIODISTRIBUTION STUDIES WAS TAKEN FROM INSTITUTIONAL ANIMAL ETHICS COMMITTEE, JAMIA HAMDARD, NEW DELHI AND THE STUDY WAS PERFORMED AT INMAS, NEW DELHI. THE ETHICS COMMITTEE APPROVED ONLY THREE TIME POINTS FOR THE STUDY.

The biodistribution studies were carried out using Wistar rats of either sex (200 - 250 g). Just before the experiment, the rats were weighed and restrained in rat restrainers. The animals were kept under standard laboratory conditions, temperature at 25 ± 2 °C and relative humidity of 55 ± 5%. The animals were housed in polypropylene cages, six per cage with free access to standard laboratory diet (Lipton feed, Mumbai, India; providing 3630 kcal/g energy and containing 22.10% crude protein, 4.10% crude oil, 4.05% crude fiber, 10.05% ash, 0.75% sand silica) and water ad libitum. All the rats were marked with picric acid solution for identification and
randomly divided into three groups consisting of three rats in each group. Group 1 was given DLX-loaded NLC (DLX-NLC) intranasally, group 2 received DLX solution intranasally and group 3 received DLX solution intravenously (Table 4.7). DLX-NLC was administered intranasally to rats, compared to intranasal and intravenous administration of DLX solution. Radiolabeled formulation, (200 μCi/100 μl) was administered in each nostril and intravenously. Formulations containing DLX solution, and DLX-NLC suspension (DLX-NLC was suspended in water containing chitosan - 0.6% w/w) was instilled into the nostrils with the help of micropipette (100 μl) fitted with micro tip at the delivery site (olfactory region of the nose). The rats were held from the back, in upright position during nasal administration. These were anaesthetized with dietyl ether and sacrificed at different time intervals (6, 12, 24h) and the blood was collected by cardiac puncture. Moreover, the brain and other organs (heart, liver, lungs, spleen, intestine, and kidney) were collected, washed twice using normal saline, made free from adhering tissue/fluid, dried and weighed. Radioactivity present in each tissue/organ was measured using shielded well-type gamma scintillation counter. Radiopharmaceutical uptake per gram in each tissue/organ was calculated as a fraction of administered dose using the following equation (Saha, 1993).

\[
\text{% Radioactivity/g of tissue} = \frac{\text{Counts in sample}}{\text{Weight of sample} \times \text{Total counts injected}} \times 100
\]

Table 4.7: Type of groups with their treatment schedule

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>Treatment</th>
<th>DOSE</th>
<th>ROUTE OF ADMINISTRATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DLX solution</td>
<td>0.54 mg/day</td>
<td>Intravenous</td>
</tr>
<tr>
<td>2</td>
<td>DLX solution</td>
<td>0.54 mg/day</td>
<td>Intranasal</td>
</tr>
<tr>
<td>3</td>
<td>DLX loaded NLC</td>
<td>0.54 mg/day</td>
<td>Intranasal</td>
</tr>
</tbody>
</table>
4.4.4.10.6. Pharmacokinetic studies for intranasal administration

Pharmacokinetic studies were carried out on the same rats used for biodistribution studies. Various pharmacokinetic parameters including $C_{\text{max}}$, MRT, $\text{AUC}_{0-24}$, and $\text{AUMC}_{0-24}$ were calculated.

The pharmacokinetic parameters were calculated using Kinetica software® (Thermo Fisher Scientific, Berman, Germany). The pharmacokinetic data among different formulations were compared for statistical significance by one way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison tests using GraphPad Instat software (GraphPad Software Inc., CA, USA). The brain targeting efficiency (DTE %) and nose to brain direct transport percentage (DTP %) were also calculated (Kumar et al., 2008).

$$DTE \, (\%) = \frac{(\text{AUC}_{\text{brain}}/\text{AUC}_{\text{blood}})_{\text{i.n.}}}{(\text{AUC}_{\text{brain}}/\text{AUC}_{\text{blood}})_{\text{i.v.}}} \times 100$$

$$DTP \, (\%) = \frac{(B_{\text{AUC}_{\text{brain}}} - B_{X})}{B_{\text{AUC}_{\text{brain}}}} \times 100$$

Where, $B_{X} = (B_{\text{i.v.}}/P_{\text{i.v.}}) \times P_{\text{i.n.}}$

$B_{x}$ is the brain AUC fraction contributed by systemic circulation through the BBB following intranasal administration.

$B_{\text{i.v.}}$ is the $\text{AUC}_{0-24}$ (brain) following intravenous administration

$P_{\text{i.v.}}$ is the $\text{AUC}_{0-24}$ (blood) following intravenous administration

$P_{\text{i.n.}}$ is the $\text{AUC}_{0-24}$ (brain) following intranasal administration

$B_{\text{i.n.}}$ is the $\text{AUC}_{0-24}$ (blood) following intranasal administration

$\text{AUC}$ is the area under the curve.

Dose for the rats was calculated as described in section 4.4.4.8.1. Once the dose for a rat (say, 200 g of rat) was known, dose for rats of other body weights was calculated. Blood samples were withdrawn via cardiac puncture at 0 (pre-dose), 6, 12, 24 h in microcentrifuge tubes in which 8 mg of EDTA was added as an anticoagulant. The collected blood was mixed properly with the anticoagulant and centrifuged at 4000 rpm for 20 min. The plasma was separated and stored at -21 °C until drug analysis was carried out.
4.4.4.10.7. Gamma scintigraphic studies for intranasal formulations (DLX-NLC, DLX solution)

The healthy New Zealand rabbits weighing between 2.00-2.50 kg (female) were selected for the study. The rabbits were anaesthetized using ketamine hydrochloride intramuscular injection (1 ml of 50 mg/ml) and held from the back in slanted position during intranasal administration. The radiolabeled formulations (DLX-NLC and DLX solution) was administered via nasal route and placed on the imaging platform. The localization of DLX was visualized using Single Photon Emission Computerized Tomography (SPECT) gamma camera, provided by GE healthcare system (Hawkeye Millennium VG, GE Medical Systems, Milwaukee, WI, USA) and the images were recorded using the eNTEGRA software.

4.4.5. Stability studies of optimized DLX loaded NLC (DLX-NLC) formulation

Stability of a lipid system refers to the chemical and physical integrity of the system. The stability studies were carried out to determine the effect of the presence of formulation additives on the stability of drug and also to determine the physical stability of the prepared formulation under conditions of storage temperature. The effect of the storage temperature on the stability of NLC dispersion (5 ml) and lyophilized NLC powder (10 mg) was assessed by storing at different temperatures (4°C and room temperature - RT) in the dark over a period of 90 days. The storage conditions were selected according to the reported method (Bhaskar et al., 2009; Wang et al., 2012). The optimized NLC formulations were subjected to stability studies in triplicate. Changes in particle size, zeta potential and encapsulation parameters against storage time were studied. The lyophilized NLC powders were re-dispersed in double distilled water by vortexing for 1 min before the assessment. The stability testing was carried out for the estimation of drug content in optimized DLX-NLC formulation, using stability indicating HPLC method.

4.4.5.1. Stability studies of DLX loaded NLC (DLX-NLC) formulation using Arrhenius equation (Estimation of residual drug content)

For the determination of shelf life by conventional method, DLX-NLC formulations were kept at 30 ± 2°C / 50 ± 5% RH, 35 ± 2°C / 50 ± 5% RH and 40 ± 2°C / 50 ± 5% RH for three months (90 days). Samples were withdrawn after specified time intervals (0, 30, 60, and 90 days) and the remaining drug content was determined using stability indicating HPLC method. Zero time samples were used as controls. All the samples were passed through 0.22 μm filter before being...
injected into the HPLC system. Logarithm of percent drug remaining versus time (in days) was plotted. The degradation rate constant ‘k’ was determined from the slope of the lines at each elevated temperature using the equation:

\[
\text{Slope} = - \frac{K}{2.303}
\]

Arrhenius plot was constructed between the logarithms of k values (log K) at various elevated temperatures against the reciprocal of absolute temperature (1/T) to determine the shelf life of optimized NLC formulations. The degradation rate constant at 25°C (K_{25}) was determined for each formulation by extrapolating the value at 25°C from Arrhenius plot. The shelf life (T_{0.9}) for each formulation was determined by substituting the value of K_{25} in the following equation:

\[
t_{0.9} = \frac{0.1052}{K_{25}}
\]

Where, \(t_{0.9}\) is the time required for 10% degradation of the drug and is referred to as shelf-life.
Figure 4.6: Dissected skull showing different parts of brain.

Figure 4.7: Dissection of skull for the collection of brain.
Extraction of DLX from brain

The brain was extracted from the treated rats (DLX solution or DLX-NLC), washed twice using normal saline, made free from adhering tissue/fluid, dried and weighed. The whole brain was homogenized for 5 min using a Tissue Homogenizer (Fisher Scientific, Germany) after adding saline at a ratio of 1:4 (w/v). The homogenate (500 µl) was mixed with venlafaxine (1 ml) as internal standard and n-hexane (2ml) as organic solvent and vortexed for 1 min followed by centrifugation at 5000 rpm for 5 min. Upper layer was transferred to a clean test tube and evaporated to dryness. The residue was reconstituted in 100 µl of methanol, mixed well, filtered by 0.2 µm filter and 20 µl of the final clear solution was injected into the HPLC system.

Method of Blood Collection in rat

Collecting blood from rat was necessary for DLX extraction. It is important to remember that blood collection, because it can stress the animals, may have an impact on the outcome of research data. To minimize pain and stress in the animal, it is recommended that all blood
Experimental

Extraction of DLX from blood

The blood (2 ml) was collected in precoated EDTA tubes from the rats (DLX treated), and then centrifuged for 15 minutes for separation of plasma from blood. Liquid-liquid extraction technique was used to separate the drug from plasma (Figure 4.10). The plasma (0.5 ml) was taken in a clean test tube and mixed with internal standard (venlafaxine - 1 ml) and chloroform (5 ml). It was then vortexed and centrifuged at 5000 rpm for 10 min to bring the drug in chloroform. Upper layer was discarded and the chloroform layer was transferred to a clean test tube and evaporated to dryness. The residue was reconstituted in 100 μl of methanol, mixed well, filtered by 0.2 μm filter and 20 μl of the final clear solution was injected into the HPLC system.

Figure 4.10: Collected blood and centrifuged blood (separated plasma).
Figure 4.11: Experimental set-up for *in vivo* nasal permeation studies (dose = 0.54 mg/ml).