5.0 EXPERIMENTAL

5.1. Equipment and Materials

5.1.1. Equipment

<table>
<thead>
<tr>
<th>Equipment/Instrument</th>
<th>Manufacturer/Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double Beam spectrophotometer UV 1601</td>
<td>Shimadzu, Kyoto, Japan.</td>
</tr>
<tr>
<td>pH meter</td>
<td>Mettler Toledo, Langacher, Switzerland</td>
</tr>
<tr>
<td>HPLC</td>
<td>Shimadzu, Kyoto, Japan.</td>
</tr>
<tr>
<td>HPLC column LiChrospher®C18 (150 mm×4.6 mm i.d., 5µm particle size; Merck, Mumbai, India)</td>
<td></td>
</tr>
<tr>
<td>Electronic Weighing balance</td>
<td>Mettler Toledo, Langacher, Switzerland</td>
</tr>
<tr>
<td>Micropipettes Eppendorf micropipette, Chennai, India</td>
<td></td>
</tr>
<tr>
<td>Oven</td>
<td>Thermo Scientific, Delhi, India.</td>
</tr>
<tr>
<td>Water bath</td>
<td>Grover enterprises, Delhi, India.</td>
</tr>
<tr>
<td>Magnetic stirrer Mertex, Delhi, India.</td>
<td></td>
</tr>
<tr>
<td>Centrifuge Apparatus Remi Equipment, Delhi, India</td>
<td></td>
</tr>
<tr>
<td>Biological shakers Nirmal International, Delhi, India</td>
<td></td>
</tr>
<tr>
<td>Deep Freezer Vestfrost, Delhi, India.</td>
<td></td>
</tr>
<tr>
<td>Vortex mixer Nirmal International, Delhi, India</td>
<td></td>
</tr>
<tr>
<td>Particle size analyzer Malvern Instrument, Worcestershire, U.K.</td>
<td></td>
</tr>
<tr>
<td>Ultra sonicator Bio Technics, Mumbai, India</td>
<td></td>
</tr>
<tr>
<td>Transmission electron microscopy FEI, Eindhoven, Netherlands</td>
<td></td>
</tr>
<tr>
<td>FTIR spectrophotometer Shimadzu, Kyoto, Japan</td>
<td></td>
</tr>
<tr>
<td>DSC Perkin Elmer, Mumbai, India</td>
<td></td>
</tr>
<tr>
<td>Dissolution apparatus Hanson Research SR8 plus, California, USA</td>
<td></td>
</tr>
<tr>
<td>Dialysis bag (MWCO 1200 g/mole) Sigma Aldrich, Bangalore, India</td>
<td></td>
</tr>
<tr>
<td>Melting Point apparatus Adarsh Scientific, Ambala, India</td>
<td></td>
</tr>
</tbody>
</table>
### 5.1.2. Materials

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin</td>
<td>Sigma Aldrich, Bangalore, India</td>
</tr>
<tr>
<td>Tween 80</td>
<td>CDH, Mumbai, India</td>
</tr>
<tr>
<td>Tween 20</td>
<td>CDH, Mumbai, India</td>
</tr>
<tr>
<td>Transcutol HP®</td>
<td>Gattéfossè, Mumbai, India</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>Merck, Mumbai, India</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Merck, Mumbai, India</td>
</tr>
<tr>
<td>Methanol</td>
<td>Merck., Mumbai, India</td>
</tr>
<tr>
<td>Labrasol®</td>
<td>Gattéfossè, Mumbai, India</td>
</tr>
<tr>
<td>Labrafac Lipophile WL 1349®</td>
<td>Gattéfossè, Mumbai, India</td>
</tr>
<tr>
<td>Solutol HS 15®</td>
<td>Signet, Mumbai, India</td>
</tr>
<tr>
<td>Linseed oil</td>
<td>Expo Essential Oils, New Delhi, India</td>
</tr>
<tr>
<td>Fish oil</td>
<td>Coastal exports, Mangalore, India</td>
</tr>
<tr>
<td>Pancreatin (8×USP)</td>
<td>Sigma-Aldrich, Bangalore, India</td>
</tr>
<tr>
<td>L-a-phosphatidylcholine</td>
<td>Sigma-Aldrich, Bangalore, India</td>
</tr>
<tr>
<td>Tris maleate</td>
<td>Sigma-Aldrich, Bangalore, India</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>Sigma-Aldrich, Bangalore, India</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>Merck, Mumbai, India</td>
</tr>
<tr>
<td>Captex 500 (Glyceryl Triacetate)</td>
<td>Abitec, Indiana, USA</td>
</tr>
<tr>
<td>Captex 355 Low C6</td>
<td>Abitec, Indiana, USA</td>
</tr>
<tr>
<td>Unitop FFT 40</td>
<td>Unitop Chemicals, Mumbai, India</td>
</tr>
<tr>
<td>Peceol (Glycerol monooleates)</td>
<td>Gattefosse, Mumbai, India</td>
</tr>
<tr>
<td>PEG 400</td>
<td>Merck, Mumbai, India</td>
</tr>
<tr>
<td>Dulbecco's modified Phosphate Buffered Saline (PBS)</td>
<td>Sigma Aldrich, Bangalore, India</td>
</tr>
<tr>
<td>Material</td>
<td>Supplier</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>Dulbecco's Modified Eagle's Medium (DMEM)</td>
<td>Sigma Aldrich, Bangalore, India</td>
</tr>
<tr>
<td>Fetal calf serum (FCS)</td>
<td>Sigma Aldrich, Bangalore, India</td>
</tr>
<tr>
<td>Trypsin</td>
<td>Sigma Aldrich, Bangalore, India</td>
</tr>
</tbody>
</table>
5.2 Characterization of Curcumin

5.2.1 Physical Properties

Physical properties of curcumin such as nature, colour and odour was determined visually. The melting point and loss on drying of the drug was determined by melting point apparatus and IR moisture analyzer respectively.

5.2.2 Identification Tests

5.2.2.1 UV-Vis spectrum analysis

An accurately weighed quantity (5 mg) of curcumin was dissolved in methanol and the volume was made up to 50 ml with methanol in amber colour volumetric flask. 1 ml of the above solution was diluted with methanol to 10 ml in amber colour volumetric flask and the resulting solution was scanned for UV-Vis absorption using UV-Vis spectrophotometer in the range of 400 nm to 600 nm and $\lambda_{\text{max}}$ was determined. Furthermore the spectrum of curcumin ($\lambda_{\text{max}}$) was compared with reported $\lambda_{\text{max}}$ of the reference spectrum of curcumin.

5.2.2.2 Fourier transform infrared spectroscopy (FT-IR) spectral analysis

FT-IR spectrum of curcumin was obtained using Shimadzu Biorad FT-IR system (Kyoto, Japan). The sample was dispersed and triturated with dry potassium bromide (5 % wt of sample), grounded well in mortar and pestle and potassium bromide (KBr) disk was prepared at a pressure of 1,000 psig. The disk was placed in the FT-IR sample holder and IR spectra, in absorbance mode, were obtained in the spectral region 4,000 to 400 cm$^{-1}$ using the resolution 4 cm$^{-1}$. Furthermore the spectrum of curcumin was compared with reported reference spectrum of curcumin.

5.2.2.3 Differential Scanning Calorimetry (DSC) Analysis

Thermal investigation of curcumin was performed by using Perkin Elmer pyres 6 DSC (MA, USA). In an aluminum pan, 5–7 mg of curcumin was placed and crimped with a lid containing a pin hole and kept in the differential scanning calorimetry (DSC) unit along with a similar pan as a reference. The sample was heated at the rate of 10°C/min from the temperature range of 30–400°C. Nitrogen was used as a purge gas and flow was adjusted to 50 ml/min.
5.2.3 Analytical Methodology

5.2.3.1 Preparation of working solution: Various working solutions were prepared freshly when needed according to USP-NF 36, 2012.

5.2.3.1.1 Preparation of 0.2M potassium dihydrogen orthophosphate solution:
An accurately weighed quantity (27.22 g) of potassium dihydrogen orthophosphate was dissolved in small quantity of distilled water in a 1000 ml volumetric flask and volume was made up to 1000 ml with distilled water.

5.2.3.1.2 Preparation of 0.2M sodium hydroxide solution:
An accurately weighed 8 gm of sodium hydroxide was dissolved in distilled water in a volumetric flask and volume was made up to 1000 ml with distilled water.

5.2.3.1.3 Phosphate buffer (pH 6.8):
50 ml of 0.2M potassium dihydrogen orthophosphate was mixed with 22.4 ml of 0.2M sodium hydroxide and volume was made up to 200 ml with distilled water.

5.2.3.1.4 Phosphate buffer (pH 7.4):
50 ml of 0.2M potassium dihydrogen orthophosphate was mixed with 39.1 ml of 0.2M sodium hydroxide and volume was made up to 200 ml with distilled water.

5.2.3.1.5 Kreb’s Ringer Solution:
An accurately weighed quantity 1.8 g glucose, 0.0468 g magnesium chloride (anhydrous), 0.34 g Potassium chloride, 7.0 g sodium chloride, 0.1 g sodium phosphate dibasic (anhydrous), 0.18 g sodium phosphate monobasic (anhydrous) was dissolved in 900 mL water at room temperature and stirred up to complete solubilization. To this solution 1.26g of sodium bicarbonate was added and stirred up to complete solubilization. Remaining quantity of water was added to this solution to make 1 L solution (pH 7.4). pH of the buffer was maintained with 1N HCl or 1N NaOH.

5.2.3.2 UV-Vis spectral analysis
UV-Vis method was employed for in vitro release studies by making suitable changes in the sample preparation.
5.2.3.2.1 Determination of absorption maxima ($\lambda_{\text{max}}$) in ethanol

An accurately weighed quantity of curcumin (5 mg) was dissolved in ethanol and sonicated for 5 min. The volume was made up to 50 ml with ethanol in amber colour volumetric flask. 1 ml of the above solution was diluted to 10 ml with ethanol in amber colour volumetric flask. UV-Vis scan between 400 nm to 600 nm was taken.

5.2.3.2.2 Determination of absorption maxima ($\lambda_{\text{max}}$) in 0.1N HCl

An accurately weighed quantity (5 mg) of curcumin was dissolved in 10 mL ethanol and the volume was made up to 50 ml with 0.1N HCl in amber colour volumetric flask. 1 ml of the above solution was diluted with 0.1N HCl to 10 ml in amber colour volumetric flask and the resulting solution was scanned for UV-Vis absorption using UV-Vis spectrophotometer in the range of 400 nm to 600 nm and $\lambda_{\text{max}}$ was determined.

5.2.3.2.3 Determination of absorption maxima ($\lambda_{\text{max}}$) in phosphate buffer (pH 6.8)

An accurately weighed quantity (5 mg) of curcumin was dissolved in 10 mL ethanol and the volume was made up to 50 ml with phosphate buffer (pH 6.8) in amber colour volumetric flask. 1 ml of the above solution was diluted with phosphate buffer (pH 6.8) to 10 ml in amber colour volumetric flask and the resulting solution was scanned for UV-Vis absorption using UV-Vis spectrophotometer in the range of 400 nm to 600 nm and $\lambda_{\text{max}}$ was determined.

5.2.3.2.4 Calibration curve in release/QC medium

0.1N HCl (pH 1.2) and phosphate buffer (pH 6.8) were selected as the medium in which release studies were carried out as these pH simulate the pH of gastrointestinal tract. 0.1% w/v ascorbic acid was added to these medium to prevent the degradation of curcumin. Calibration plots were prepared in both the medium.

5.2.3.2.4.1 Calibration plot in 0.1N HCL

An accurately weighed quantity (5 mg) of curcumin was dissolved in 10 ml of ethanol in amber colour volumetric flask and sonicated for 5 min. 1 ml of the above solution was diluted with mixture of ethanol and 0.1N HCl (1:4 ratio) to 10 ml in amber colour volumetric flask. Further dilution was made with 0.1N HCl to obtain the required concentration in the range from 2 – 14 µg/ml. The absorbance values were taken at $\lambda_{\text{max}}$ 423 nm spectrophotometrically against the pure medium as blank. The regression analysis was performed.
5.2.3.2.4.2 Calibration plot in phosphate buffer (pH 6.8)

An accurately weighed quantity (5 mg) of curcumin was dissolved in 10 ml of ethanol in amber colour volumetric flask and sonicated for 5 min. 1 ml of the above solution was diluted with mixture of ethanol and phosphate buffer pH 6.8 (1:4 ratio) to 10 ml in amber colour volumetric flask. Further dilution was made with phosphate buffer (pH 6.8) to obtain the required concentration in the range from 3 – 15 µg/ml. The absorbance values were taken at λ<sub>max</sub> 424 nm spectrophotometrically against the pure medium as blank. The regression analysis was performed.

5.2.3.2.5 UV-Vis 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).

5.2.3.2.6.1 Linearity and range

Linearity of the method was evaluated at six concentration levels by diluting the standard stock solution with phosphate buffer (pH 6.8) to give solutions in the range of 3–15 µg/mL. The absorbance of the solutions was determined at λ<sub>max</sub> of 424 nm. The data of absorbance versus drug concentration were treated by linear least square regression analysis. Standard solutions were analyzed in triplicate.

5.2.3.2.5.2 Precision

Precision validation was performed in two stages, namely system repeatability and method repeatability (intermediate precision). For system repeatability, the repeatability of absorbance was carried out by analyzing six samples at two concentrations (3 and 6 µg/mL) on the same day. For method repeatability, intra and inter-day variations were studied at three different concentration levels of curcumin (3, 6 and 9 µg/mL). The acceptance criteria were less than 2% relative standard deviation (RSD) for peak area.

5.2.3.2.5.3 Limit of Detection (LOD) and Limit of Quantification (LOQ)

In order to determine the detection and quantification limits, curcumin concentrations in the lower part of calibration curve were used. Curcumin standard solutions of 2, 3, 5 and 6 µg/mL were prepared and absorbance taken at 424 nm. The amount of curcumin concentration versus absorbance was plotted and the equation for this curve was determined,
thereby obtaining an estimate of the target response: ybl. The ybl value corresponds to intersection of the curve. Subsequently, a second curve was graphed showing the concentration versus standard deviation of the area. From this equation we obtained an estimate of standard deviation of the target: sbl, which corresponds to the intersection of curve. Detection and quantification limits were calculated by using the following formula:

Detection limit \(=\ \frac{y_{bl} + 3 \cdot s_{bl}}{b}\)

Quantification limit \(=\ \frac{y_{bl} + 10 \cdot s_{bl}}{b}\) where \(b = \) slope of linearity curve

5.2.3.2.5.4 Accuracy or Recovery

Standard solution of 2 µg/mL curcumin was prepared and analyzed by proposed method. For the determination of the accuracy of the method standard addition and recovery method was used. The analyzed samples were spiked with an extra of 50, 100 and 150% of standard drug and then the mixtures were reanalyzed. The experiment for each recovery sample was carried out three times, to check the recovery of drug at different levels in the standard solution.

5.2.3.4 Intrinsic stability testing of drug solution

For testing the intrinsic stability of the drug in the working standard (ethanol), a known concentration of drug solution (5 µg/ml) was prepared and was divided into three parts. Each part was kept at different temperatures viz. refrigeration (2-8° C) and room temperature (25 °C) and deep freezer (-21 °C). UV-Vis spectra was taken initially and after 48 h again and observed for any change in \(\lambda_{\text{max}}\) and assayed in order to ascertain the intrinsic stability of the solution in working standard.
5.3 HPLC METHOD DEVELOPMENT FOR ANALYSIS OF CURCUMIN

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 5.033 software was used. Curcumin was assayed by reversed-phase high-performance liquid chromatography (RP-HPLC) method. The mobile phase was a mixture of eluant A, acetonitrile (75%) and eluant B, 3% acetic acid (25%), pumped at a flow rate of 1 mL/min. The eluents were filtered through nylon filter (pore size, 0.45 µm) before use and then degassed by sonication in an ultrasonic bath. The assays were performed at ambient temperature (25±1°C). The injection volume was 20 µL for a sample and detector wavelength was fixed at 425 nm. The chromatographic conditions were as follows:

- **Column**: LiChrospher® C18 (150×4.6 mm i.d., 5µm particle size; Merck, Mumbai, India)
- **Mobile phase**: Mixture of acetonitrile (75%) and 3% acetic acid (25%)
- **Flow rate**: 1.00 mL/min
- **Temperature**: 25°C
- **Wavelength**: 425 nm
- **Injection Volume**: 20 µL

5.3.1 Standard and sample preparation

Stock solutions of CU standard for HPLC was prepared by dissolving 5 mg curcumin in 10 mL methanol, and making-up the solution to 50 mL in a volumetric flask with mobile phase to obtain stock solution of concentration 100 µg/mL. The working standards were prepared by dilution of the stock solution with mobile phase, and all the solutions were stored at room temperature (25 ±1°C). Quantification was achieved by regression analysis of the peak areas against concentration and triplicate injections were made. Calibration standard solutions were prepared by diluting the working standard to concentrations of 100, 200, 400, 500 and 1000 ng/mL with mobile phase.
5.3.2 Method Validation

Validation of the developed HPLC method was done with respect to various parameters including precision, linearity, limit of detection (LOD), limit of quantification (LOQ), recovery (accuracy) and robustness according to the ICH guideline Q2 (R1).

5.3.2.1 Linearity and range

Linearity of the method was evaluated at six concentration levels by diluting the standard stock solution with mobile phase to give solutions in the range of 100–1000 ng/mL. The data of peak area versus drug concentration were treated by linear least square regression analysis. Standard solutions were analyzed in triplicate. The analytical range was established by the highest and lowest concentrations of analyte where acceptable linearity, accuracy and precision were obtained.

5.3.2.2 Precision

Precision validation was performed in two stages, namely system repeatability and method repeatability (intermediate precision). For system repeatability, the repeatability of sample injection and measurement of peak areas were carried out by analyzing six samples at two concentrations (400 and 500 ng/mL) on the same day. For method repeatability, intra and inter-day variations were studied at three different concentration levels of curcumin (100, 200 and 300 ng/mL). The acceptance criteria were less than 2% relative standard deviation (RSD) for peak area.

5.3.2.3 Limit of Detection (LOD) and Limit of Quantification (LOQ)

In order to determine the detection and quantification limits, curcumin concentrations in the lower part of calibration curve were used. Curcumin standard solutions of 100, 200, 400 and 500 ng/mL were prepared and injected in triplicate. The amount of curcumin concentration versus peak area was plotted and the equation for this curve was determined, thereby obtaining an estimate of the target response: $y_{bl}$. The $y_{bl}$ value corresponds to intersection of the curve. Subsequently, a second curve was graphed showing the concentration versus standard deviation of the area. From this equation we obtained an estimate of standard deviation of the target: $s_{bl}$, which corresponds to the intersection of curve. Detection and quantification limits were calculated by using the following formula:
Detection limit = \( y_{bl} + 3 \cdot s_{bl} \) \\
\( \frac{b}{b} \)

Quantification limit = \( y_{bl} + 10 \cdot s_{bl} \) \\
\( \frac{b}{b} \) where \( b \) = slope of linearity curve

5.3.2.4 Accuracy as Recovery

Standard solution of 100 ng/mL curcumin was prepared and analyzed by proposed method. For the determination of the accuracy of the method standard addition and recovery method was used. The analyzed samples were spiked with an extra of 50, 100 and 150% of standard drug and then the mixture was reanalyzed. The experiment for each recovery sample was carried out three times, to check the recovery of drug at different levels in the standard solution.

5.3.2.5 Robustness

The robustness of an analytical procedure is a measure of its ability to remain unaffected by small, but deliberate variations in method parameters and give an indication of its consistency during normal usage. To determine the robustness of the developed method, experimental conditions were deliberately altered and the effects on the result were examined. To study the effect of flow rate on the resolution, flow was changed by 0.2 units from 0.8 to 1.2 mL/min. The effect of the percent organic mobile phase on resolution was studied by varying ratio of composition by -5 to +5%. The acceptance criteria were less than 2% relative standard deviation (RSD) for peak area.

5.3.2.6 Specificity/Stability indicating method

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities or other component which may be expected to be present. The specificity of the developed HPLC method for curcumin was carried out in the presence of its degradation product. Forced degradation studies at a concentration of 5000 ng/mL of curcumin (CU) were performed to evaluate the stability indicating properties and specificity of the method.
Separately 1 ml of 50 µg/mL methanolic solution of curcumin was diluted to 10 ml with 1N HCl and 1N NaOH in 10 mL amber colour volumetric flask and kept at 50 ±2°C for 24 h in an oven.

In the same manner 1ml of 50 µg/mL methanolic solution of curcumin was diluted to 10 mL with 30% v/v H2O2 in 10 mL amber colour volumetric flask and refluxed. The solutions were kept for 24 h at room temperature (25±2°C).

Photo-stability test was performed in presence of UV light (wavelength 254 nm) and daylight. The methanolic solution having a concentration of 5000 ng/mL was kept for 3 days in UV light and for 7 days in daylight in a transparent volumetric flask.

The dry heat degradation study was performed using a high precision hot air oven (Narang Scientific Works, New Delhi, India) capable of controlling the temperature with in ±2 ºC. The methanolic solution having a concentration of 5000 ng/mL was kept for 8 h in an oven to expose it to dry heat (100 ºC).
5.4 HPLC Method Development for Analysis of Curcumin in Plasma

Curcumin in plasma was assayed by isocratic reversed-phase high-performance liquid chromatography (RP-HPLC) method using LiChrospher®C_18 (150×4.6mm i.d., 5µm particle size; Merck, Mumbai, India) column fitted with guard column LiChroCART®. β-estradiol was used as internal standard. The mobile phase employed composed of 75% acetonitrile and 25% acetic acid (2.5% v/v). The flow rate was 1.0 mL/min and the detection wavelengths were 420 and 280 nm, respectively, for curcumin and β-estradiol (internal standard, IS). The detection wavelength was changed from 280 to 420 nm automatically at 3.0 min after the elution of IS. Class VP (5.03 version) data acquisition software was used to process the chromatograms. The blood sample was taken from Wistar rats of either sex and was transferred to 10 ml vacutainer tubes containing EDTA to prevent coagulation. After gentle inversion of tubes, blood was centrifuged at 10,000 rpm for 10 min to separate red blood cells from plasma. Plasma was transferred to clean 10 ml polypropylene screw cap tubes and stored in a −21 °C freezer until use. The chromatographic conditions were as follows:

| Column           | LiChrospher®C_{18} (150×4.6 mm i.d., 5µm particle size; Merck, Mumbai, India) |
| Guard column     | LiChroCART® |
| Mobile phase     | Mixture of acetonitrile (75%) and 2.5% acetic acid (25%) |
| Flow rate        | 1.00 mL/min |
| Temperature      | 25°C |
| Wavelength       | 280 nm (For β-estradiol) 425 nm (For curcumin) |
| Injection Volume | 20 µL |

5.4.1 Stock and working standard solutions

10 mg of curcumin and β-estradiol was weighed separately and dissolved separately in 10 mL methanol in amber colour volumetric flask which gave a methanolic stock solution of curcumin (1 mg/mL) and β-estradiol (1 mg/mL). These solutions were protected from light and stored at −20°C between experiments. Different working solutions of curcumin were obtained by diluting the stock solutions with methanol. Calibration standards were prepared.
daily by spiking 90 µL of blank plasma with 10 µL of the appropriate working solutions resulting in concentrations of 200, 400, 500, 800, 1000 and 1500 ng/mL. The β-estradiol solution was subsequently diluted with acetonitrile to make a working IS solution of 200 ng/mL.

5.4.2 Sample preparation

The plasma samples (100 µL) were transferred to a 2 ml eppendorf tube and 50 µL (200 ng/ml) of β-esterdiol (IS) were added to it with through mixing. After mixing, 250 µL of acetonitrile was added, vortexes for 1 min and centrifuged at 10,000 rpm for 10 min. Following transfer to new Eppendorf tube, 20 µL of the supernatant was injected directly into the column of HPLC system for analysis.

5.4.3 Method Validation

A thorough validation of the method was carried out as per the US FDA guidelines (2001). The method was validated for linearity, precision, accuracy, selectivity, sensitivity, recovery, and stability.

5.4.3.1 Calibration curve and Linearity

A calibration curve consists of a blank sample (matrix sample processed without internal standard), a zero sample (matrix sample processed with internal standard), and six non-zero samples covering the expected range, including lower limit of quantification (LLOQ). Linearity of the method was evaluated at six concentration levels in the range of 200–1500 ng/mL. The data of the ratio of peak area for drug to IS versus drug concentration were treated by linear least square regression analysis. The samples were analyzed in triplicate.

5.4.3.2 Precision and accuracy

Precision validation was performed in two stages, namely system repeatability and method repeatability (intermediate precision). For system repeatability, the repeatability of sample injection (matrix sample) and measurement of ratio of peak areas (CU/IS) were carried out by analyzing six samples at concentrations (400 ng/mL) on the same day. For method repeatability, intra and inter-day variations were studied at three different concentration levels of curcumin (200, 500 and 1500 ng/mL). The acceptance criteria include accuracy within
±15% deviation (SD) from the nominal values and a precision of ±15% relative standard deviation (RSD).

5.4.3.3 Limit of Detection (LOD) and Limit of Quantification (LOQ)

In order to determine the detection and quantification limits, curcumin drug concentrations in the matrix at the lower part of calibration curve were used. Curcumin concentration of 200, 400 and 500 ng/mL in standard matrix sample were prepared and injected in triplicate. The amount of curcumin concentration versus ratio of peak area (drug/IS) was plotted and the equation for this curve was determined, thereby obtaining an estimate of the target response: ybl. The ybl value corresponds to intersection of the curve. Subsequently, a second curve was graphed showing the concentration versus standard deviation of the area. From this equation we obtained an estimate of standard deviation of the target: sbl, which corresponds to the intersection of curve. Detection and quantification limits were calculated by using the following formula:

Detection limit  =  \( \frac{y_{bl} + 3s_{bl}}{b} \)

\[
\text{Quantification limit} = \frac{y_{bl} + 10s_{bl}}{b}
\]

where \( b \) = slope of linearity curve

5.4.3.4 Selectivity

The selectivity of the method was assessed by analyzing six blank plasma matrix samples. The responses of the interfering substances at the retention time of the curcumin are acceptable if they are less than 20% of the response of the lowest standard curve point. The responses of the interfering substances at the retention time of the internal standard are acceptable if they are less than 5% of the response of the working internal standard.

5.4.3.5 Sensitivity

The sensitivity of the method was established from the response from six spiked LLOQ (lower limit of quantification) samples. The six replicates should have a precision of <20% and an accuracy of <20%.
5.4.3.6 Stability

Stability tests were conducted to evaluate the analyte stability in stock solutions and in plasma samples under different conditions. The stock solution stability at room temperature and refrigerated conditions (2–8 °C) was performed by comparing the area response of the analytes (stability samples) with the response of the sample prepared from fresh stock solution. The samples were stored for 24 h and then analyzed with developed method.

5.4.3.7 Recovery studies

To determine the recovery of curcumin with the developed method, direct injections of the methanolic standards were made. These direct injection samples contained the equivalent amount of curcumin as spiked in the extracted plasma standards. The ratios of the area of the extracted standards and the area of the direct injection samples were used to determine the recovery of curcumin from plasma. The recovery of curcumin was determined at three levels, low concentration (200 ng/mL), medium concentration (500 ng/mL) and high concentration (1500 ng/mL) as per US-FDA recommendation 2001.
5.5 Preformulation studies

5.5.1 Solid state stability at high temperature and humidity

According to ICH guidelines forced decomposition studies (stress testing) at temperatures in 10 °C increments above the accelerated temperatures should be carried out on the drug substance so as to establish the inherent stability characteristics and degradation pathways to support the suitability of the proposed analytical procedures. The solid state stability was performed to evaluate the effect of relative humidity and temperature on the degradation of CU. 200 mg CU was placed in each of open glass vials. The vials were placed in desiccators at different relative humidity (%RH) and temperature using saturated salt solution of lithium chloride (45°C/11% RH), magnesium nitrate (55°C/45% RH) and sodium chloride (65°C/75% RH) at dark place (Bakshi and Singh., 2002). After 1, 3, 5 and 7 days, the drug content was determined by HPLC method. Methanolic solution of CU (500 ng/mL) with mobile phase was prepared for HPLC analysis after recommended storage.

5.5.2 Effect of pH on the stability of CU

Accurately weighed 5 mg of CU was dissolved in 10 ml methanol and transferred to 50 mL of volumetric flask and diluted to volume with mobile phase to obtain 100 µg/mL of stock. The pH values of buffer used for measurement of degradation for CU were 0.1N HCl (pH 1.2), phosphate buffer (pH 6.8 & 7.4) and Krebs Ringer solution (pH 7.4) to simulate pH in GIT. These buffers were prepared as per pharmacopeia recommendation (USP 36, 2012). A solution of 5 µg/mL was made from the stock solution in respective buffer and stored in amber colour volumetric flask and kept at dark place for 24 h. The percentage amount of drug remaining at different time interval 1.0, 4.0, 8.0 and 24.0 h was measured. The desired pH was further adjusted using orthophosphoric acid for specific-acid catalyzed and using 0.1 M NaOH for specific-base catalyzed degradation.

To prevent the degradation of CU at different pH, ascorbic acid (an antioxidant) was added in different concentration level of 0.1% w/v and 1 % w/v to the CU solution at mentioned pH. The percentage amount of drug remaining at above mentioned time was also measured using validated HPLC method.
5.5.3 Excipient compatibility studies

The selection of excipients is vital in the design of a quality drug product. Excipients and their concentration in a formulation are selected based not only on their functionality, but also on the compatibility between the drug and excipients. The excipients which were selected for formulation development were assessed for compatibility studies with CU. Drug interaction studies were performed by screening the binary mixtures, kept at room temperature and ambient humidity, 40°C ± 75% RH for 12 week in open vials. Binary mixture was prepared by mixing CU maximum quantity (10 mg/mL) in oil and 50 mg/mL in surfactant and co surfactant. The samples were analyzed after 4 weeks and 12 weeks period for drug content (assay) and were also physically/visually observed for colour or physical form changes. The assay of CU was determined after the dilution of mixture with mobile phase.

5.5.4 pH solubility profile of CU

Solubility is defined as the amount of drug substance that passes into solution to achieve as saturated solution at constant temperature and pressure. The equilibrium solubility at different pH range (pH 1.0 to pH 6.8) containing 0.1% ascorbic acid and water was determined using shake flask method at 37 °C. An excess amount of CU was added in 2 mL of the selected vehicle in 5 mL stopper vials and mixed with the help of vortex mixer (Nirmal International, New Delhi, India). The mixture vial was then kept at 37 °C in an isothermal shaker (Nirmal International, New Delhi, India) for 72 h to get to equilibrium. The saturation was confirmed by observation of the presence of undissolved CU. The equilibrated samples were removed from the shaker and centrifuged at 5000 rpm for 15 minutes. The supernatant was taken and filtered through a 0.45µ membrane filter and the concentration of CU was determined using validated HPLC method.

5.5.5 Partition coefficient

Partition coefficient is the measure of drug lipophilicity and an indication of its ability to cross cell membrane. It is defined as the ratio of unionized drug distributed between organic and aqueous phases at equilibrium.

\[ \log P_{oct/wat} = \log \left( \frac{[solute]_{octanol}}{[solute]_{water}} \right) \]
The partition coefficient was determined using shake flask method. Octanol was saturated with water by keeping it on bath shaker for 24 h. Then 40 ml (20 ml octanol and 20 ml water) was taken in separating funnel and 10 mg of CU was added to this mixture. This mixture was shaken for 6 h on shaker in order to complete the distribution of CU in both layers. After 6 h the separating funnel was kept in a stagnant position for 2 hr to allow for settlement of the undissolved CU. Then the samples were withdrawn from both the layer and analyzed by HPLC method.
5.6 Formulation Development and characterization of nanoemulsion

5.6.1 Formulation of Nanoemulsion

5.6.1.1 Excipients selection

An important criterion for screening of components for high drug loading and more stability in nanoemulsions is the solubility of drug in oil and surfactant, and miscibility of oil with surfactant and co-surfactant. The other important criterion for selection of the materials include that all the components are pharmaceutically acceptable for oral application and fall under GRAS (Generally regarded as safe) category.

5.6.1.1.1 Oil Screening

Edible oils, which represent the logical and preferred lipid excipient choice for the development of nanoemulsions, are not frequently selected due to their poor ability to dissolve large amounts of lipophilic drugs (Kimura et al., 1994). Modified or hydrolyzed vegetable oils have been widely used since these excipients form good emulsification systems with a large number of surfactants approved for oral administration and exhibit better drug solubility properties. They offer formulative and physiological advantages and their degradation products resemble the natural end products of intestinal digestion (Hauss et al., 1998).

For determination of solubility of CU in different oils, an excess quantity of CU was added to each 5-mL capacity stoppered vial containing 2 mL of Fish oil, Labrafac Lipophile WL1349, Sesame oil, Sefsol 218, Linseed oil, Labrafac PG, Peceol, Maisine 35-1, Caprol 10 G 100, Captex GTO, Captex 500, Captex 355 low C8, Captex 355 E9, Precirol, Capmul MCM and Capryol 90 and mixed using a vortex mixer (Optics Technology, Delhi, India) at room temperature. The mixture vials were then kept at 37 °C±1 °C in an isothermal shaker (Nirmal International, New Delhi, India) for 72 h to get to equilibrium. The equilibrated samples were removed from the shaker and centrifuged at 5000 rpm for 15 min. The supernatant was taken and filtered through a 0.45 μm membrane filter and the concentration of CU was determined using validated high-pressure liquid chromatography (HPLC) method at 425 nm.
5.6.1.1.2 Surfactant Screening

The surfactant chosen must be able to lower interfacial tension to a very small value to help in the dispersion process during the preparation of the nanoemulsion, provide a flexible film that can readily deform around droplets and be of the appropriate lipophillic character to provide the correct curvature at the interfacial region for the desired nanoemulsion type (Anton and Vandamme, 2011). For determination of solubility of CU in different surfactant, an excess quantity of CU was added to each 5-mL capacity stopper vial containing 2 mL of Solutol HS 15, Tween 80, Labrasol, Tween 20 and Unitop FFT 40, and mixed using a vortex mixer (Optics Technology, Delhi, India) at room temperature. The mixture vial was then kept at 37 °C±1 °C in an isothermal shaker (Nirmal International, New Delhi, India) for 72 h to get to equilibrium. The equilibrated samples were removed from the shaker and centrifuged at 5000 rpm for 15 min. The supernatant was taken and filtered through a 0.45 µm membrane filter and the concentration of CU was determined using validated high-pressure liquid chromatography (HPLC) method at 425 nm.

5.6.1.1.3 Co-surfactant Screening

Transient negative interfacial tension and fluid interfacial film is rarely achieved by the use of single surfactant, usually necessitating the addition of a co-surfactant. The presence of co-surfactant decreases the bending stress of interface and allows the interfacial film sufficient flexibility to take up different curvatures required to form nanoemulsion over a wide range of composition (Lawrence and Rees, 2000). The basic criteria for selection of cosurfactant were the miscibility of co-surfactant with oils. For miscibility studies co-surfactants such as Lauroglycol FCC, Transcutol HP, PEG 400, Lauroglycol 90, Plurol Oleique and 1, 2 propylene glycol were considered.

For selection of suitable surfactant and co-surfactant, miscibility of oils (Labrafac Lipophile WL1349, Captex 500) was done with surfactants such as Solutol HS 15, Labrasol and Unitop FFT 40, Tween 80, tween 20 and co-surfactants such as Lauroglycol FCC, Transcutol HP, PEG 400, Lauroglycol 90, Plurol Oleique and 1, 2 Propylene glycol in 1:1 ratio. The criterion for miscibility was decided by performing transmittance of the sample mixture at 690 nm. The sample which showed transmittance more than 85% was considered clear, The sample which showed less than 85% and more than 70% was considered translucent and those have less than 70% transmittance was considered as turbid.
5.6.1.2 Process parameter optimization for construction of pseudo-ternary phase diagrams

On the basis of solubility/miscibility studies, different combinations of oil, surfactant and co-surfactant were selected. Double distilled water was used as an aqueous phase to avoid impurities. Surfactant and co-surfactant were mixed (S\text{mix}) in different weight ratios (1:1, 2:1, 3:1 and 1:0) with increasing and then decreasing (1:2) amount of surfactant with respect to co-surfactant.

5.6.1.2.1 Stirring Speed

The effect of stirring speed (200, 400, 600 and 1000 rpm) of vortex mixer (Optics Technology, Delhi, India) on turbidity was investigated by adding the aqueous phase to the mixture of oil and S\text{mix} (1:9 ratio) at 25 °C. For measuring turbidity, the formed NE sample was diluted 200 times with water. Turbidity was measured using UV-Vis spectrophotometer at 688 nm for LUP combination and 704 nm for LST and CST combination in terms of percent transmission or transmittance, varying from 0-100% and is based on the amount of light that is able to penetrate through the sample.

5.6.1.2.2 Stirring Time

The effect of stirring time (30, 45, 60, 90 and 120 sec) of vortex mixer (Optics Technology, Delhi, India) on turbidity was investigated by adding the aqueous content to the mixture of oil and S\text{mix} (1:9 ratio) at 25 °C. For measuring turbidity, the formed NE sample was diluted 200 times with water. Turbidity was measured using UV-Vis spectrophotometer at 688 nm for LUP combination and 704 nm for LST and CST combination in terms of percent transmission or transmittance, varying from 0-100% and is based on the amount of light that is able to penetrate through the sample.

5.6.1.2.3 Temperature

The effect of temperature on turbidity was investigated by incubating the oil phase and non-ionic surfactant or S\text{mix} at different temperatures (20, 30, 40 and 50 °C for 30 min) before titration. For measuring turbidity, the formed NE sample was diluted 200 times with water. Turbidity was measured using UV-Vis spectrophotometer at 688 nm for LUP combination and 704 nm for LST and CST combination in terms of percent transmission or transmittance,
varying from 0-100% and is based on the amount of light that is able to penetrate through the sample.

5.6.1.2.4 Process of addition of oil and surfactant or Smix

The effect of addition of surfactant and oil on turbidity was investigated using two different approaches. In first approach the Smix was added drop wise to the oil phase followed by addition of aqueous phase drop wise. In second approach oil was added drop wise to the Smix phase followed by addition of aqueous phase drop wise. For measuring turbidity, the formed NE sample was diluted 200 times with water. Turbidity was measured using UV-Vis spectrophotometer at 688 nm for LUP combination and 704 nm for LST and CST combination in terms of percent transmission or transmittance, varying from 0-100% and is based on the amount of light that is able to penetrate through the sample.

5.6.1.3 Construction of pseudo-ternary phase diagrams

For the development of CU-loaded NE, pseudoternary phase diagrams were constructed to recognize the zone of NE formation using aqueous titration method. Surfactant and co-surfactant were mixed (S\text{mix}) in different weight ratios (1:1, 2:1, 3:1 and 1:0) with increasing and then decreasing (1:2) amount of surfactant with respect to co-surfactant, and was used for detailed study of the phase diagrams.

Sixteen different combinations of oil and S\text{mix} (1:9, 1:8, 1:7, 1:6, 1:5 1:4, 1:3.5, 1:3, 3:7, 1:2, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1) were made so that maximum ratio could be covered for the study to mark out the boundaries of the phases formed precisely in the phase diagrams. Slow titration with the aqueous phase under moderate stirring (at 400 rpm for 60 sec) was done for each weight ratio of oil and S\text{mix}, and visual observations were made for transparent and easily flowable oil-in-water (o/w) nanoemulsions. Gels were claimed for those clear and highly viscous mixtures that did not show a change in the meniscus after tilted to an angle of 90°. The physical state of NE was marked on a pseudo three component phase diagram with one axis representing the aqueous phase, second representing oil and the third representing a mixture of surfactant and co-surfactant (S\text{mix}) at fixed weight ratio.

5.6.1.4 Selection of formulations from phase diagram

Formulations were selected from phase diagram, which formed maximum NE area. The criteria for the selection were based on the following points:
• The formulation having oil concentration such that it could dissolve maximum concentration of CU easily without any precipitation.
• For each oil percentage selected, the concentration of surfactant should be minimum for NE preparation.
• The formulation composition should be having maximum water content.

5.6.1.5 Physical Stability studies

To overcome the problem of metastable formulations, physical stability tests were performed. Selected formulations were subjected to physical stability stress tests as heating cooling cycle, freeze–thaw cycle and centrifugation.

5.6.1.5.1 Heating-cooling cycle

It was used to see the effect of variations in temperature on the stability of nanoemulsions. Six cycles between 25±2°C and 45 ± 2°C with storage at each temperature for not less than 24 h were performed. The formulations that were found to be stable at these temperatures were subjected to freeze thaw stress test.

5.6.1.5.2 Freeze thaw cycle (accelerated ageing)

In this study, formulations were subjected to six freeze thaw cycles at temperatures between −20±2°C and +25±2°C with storage at each temperature for not less than 24 h. The formulations that passed this test were subjected to centrifugation study.

5.6.1.5.3 Centrifugation study

The formulations which passed the freeze thaw cycle were centrifuged at 5000 rpm for 20 min and observed for phase separation, creaming or cracking. The formulations which did not show any instability (creaming, cracking, phase separation) were considered for further studies.

5.6.1.6 Formulation of CU loaded nanoemulsion

5.6.1.6.1 Optimization of process of addition of drug

The effect of addition of drug on turbidity and drug precipitation was investigated using two different approaches. In first approach the drug (10 mg/ml) was added with stirring to the oil phase followed by addition of S_{mix} and aqueous phase. In second approach the drug was added
to the formed nanoemulsion. In order to observe the drug precipitation formed nanoemulsion was centrifuged at 5000 rpm for 10 min. Turbidity was measured using UV-Vis spectrophotometer at 688 nm for LUP combination and 704 nm for LST and CST combination in terms of percent transmission or transmittance, varying from 0-100% and is based on the amount of light that is able to penetrate through the sample.

5.6.1.6.2 CU loaded Nanoemulsion

Nanoemulsifying properties of NE strongly depend upon the selected oil, surfactants, co-surfactants and their relative amounts. The utilization of oil and surfactant(s) mixtures gives the possibility to optimize the NE for a particular drug. CU was dissolved in required quantity of oily phase with stirring, $S_{\text{mix}}$ was added to the oily phase and finally required quantity of aqueous phase was added to the mixture. The mixture was vortexes for 60 seconds to form NE formulations. The developed NE formulations were subjected to dispersibility studies.

5.6.1.7 Dispersibility Studies

The in vitro process of emulsification of CU nanoemulsion was assessed using a standard USP dissolution apparatus. One mL of nanoemulsion was mixed with 500 mL of media (0.1N HCl) maintained at 37 ± 0.5 °C. The paddle was rotated at a speed of 50 rpm to provide gentle mixing. The in vitro performance of the NE formulations was visually assessed using the grading system:

- **Grade A** - Quickly forming NE (<1 min of dilution) with a clear, transparent appearance.
- **Grade B** - A slightly less clear and rapidly forming NE (<1 min of dilution) with a bluish-white appearance.
- **Grade C** - A fine milky-white emulsion formed within 2 min of dilution.
- **Grade D** - Gradually formed (>2 min of dilution) oily emulsion with a grayish-white appearance.
- **Grade E** - Appearance of large oil globules on the surface of dilution medium with signs of phase separation.
5.6.2 Characterization of CU loaded NE

5.6.2.1 Globule size analysis and Zeta Potential

The information on globule size is particularly important for understanding the behavior of nanoemulsion. Moreover, in addition to composition, the bioacceptability of the delivery system is also influenced by the globule size (Qian et al., 2012). Globule size of the nanoemulsion was determined by photon correlation spectroscopy using Zetasizer Nano ZS90 (Malvern Instruments, Worcestershire, UK) at 633 nm which is based on the principle of dynamic light scattering. Dynamic light scattering (DLS) is a technique for measuring the particle size of colloidal suspensions. In DLS, the sample is illuminated with a laser beam and the intensity of the resulting scattered light produced by the particles fluctuates at a rate that is dependent upon the size of the particles. Analysis of these intensity fluctuations yields the diffusion coefficient of the particles and hence the particle size. The measurements were performed using a He–Ne laser at 633 nm by using Avalanche photo diode detector. Light scattering was monitored at 25 °C at a scattering angle of 90°. The formulation was diluted with distilled water and filtered through 0.22 µm membrane filter in order to eliminate multiscattering phenomena and experimental errors. Droplet size distribution studies were performed at refractive index 1.41, viscosity 5.0 PaS and dielectric constant 79.4.

Zeta potential is the electrical potential which exits at the hydrodynamic plane of shear of a particle. Zeta potential indicates degree of repulsion between adjacent, similarly charged particles in dispersion. For molecules that are small enough a high zeta potential will confer stability that is, the solution or dispersion will resist aggregation (Becher, 1965). Zeta potential controls charge interactions. Zeta potential was measured by applying an electric field across the dispersion. Particles within the dispersion with a zeta potential migrated towards the electrode of opposite charge with a velocity proportional to the magnitude of the zeta potential. The velocity was measured by using M3PALS (Phase Analysis Light Scattering). The magnitude of the zeta potential gives an indication of the potential stability of the colloidal system. A dividing line between stable and unstable aqueous dispersions is generally taken at either +30 or -30mV (Bouchemal et al., 2004).
5.6.2.2 Viscosity determination

Viscosity studies are necessary for nanoemulsion to characterize the system physically and to control its physical stability. Brookfield DV III ultra V6.0 RV cone and plate rheometer (Brookfield Engineering Laboratories, Inc, Middleboro, MA) with spindle # CPE40 at 25 ± 0.5 °C was used for the determination of viscosity of the formulations. The optimized parameters used were: Sample size/wt: 0.5 g, Speed: 30 rpm, Data interval: 1.0, Loop start: 1, Wait time: 30 min, Temperature: 25 ± 0.3 °C, Shear rate: 60 s⁻¹.

5.6.2.3 Refractive index

Refractive index of formulation was determined using an Abbes type of refractrometer (Precision Standard Testing Equipment Corporation, Delhi, India), which was calibrated using castor oil prior to use.

5.6.2.4 Robustness to dilution

The selected CU nanoemulsion (10 mg/mL) were diluted 50, 100, 250 and 1000 times with distilled water and evaluated for changes in transmittance or turbidity.

5.6.2.5 Centrifugation test

The sample was diluted 100 times with distilled water, centrifuged at 5000 rpm on Remi centrifuge (Remi equipments, Mumbai, India) for 10 min and then examined visually for any phase separation.

5.6.2.6 Solubility characteristics of CU in NE

The CU loading potential was evaluated in the selected NE formulations from different combinations. The NEs from different combinations was prepared using different CU concentration (10, 20, 40, 60 100 mg/ml) by optimized procedure. The formation of dispersed CU within the NE was evaluated by measuring transmittance. The yellow sediment (if occur) was evaluated at the bottom of test tubes after centrifugation at 5000 rpm for 10 min. The amount of CU dispersed within the supernatant NE was evaluated using a validated HPLC method. The percentage of CU (CU %) encapsulated within the NE was then calculated using the following equation:

\[ \%CU = \frac{CUs}{CUi} \times 100 \]
where CUs is the CU concentration remained dispersed within the NE at a specific time and 
CUi is the initial CU concentration.

5.6.2.7 Surface morphology by Transmission Electron Microscopy

Transmission electron microscopy (TEM) is the most important technique for the study of microstructures, because it directly produces images at high resolution and it can capture any coexistent structures and microstructure transitions (Ghosh & Murthy, 2006). Morphology and structure of the CU-NE were studied using Morgagni 268D transmission electron microscopy (TEM) (FEI, Netherland) operating at 70 KV and capable of point to point resolution. Combination of bright field imaging at increasing magnification and diffraction modes were used to reveal the form and size of nanoemulsion droplets. In order to perform the TEM observations, a drop of nanoemulsion was applied on carbon coated grid with 2% phosphotungstic acid (PTA) and was left for 30 sec. The dried coated grid was taken on a slide and covered with a cover slip. The slide was observed under the electron microscope.

5.6.2.8 Surface topography by Atomic Force Microscopy

The three dimensional characteristic of globule shape of CU-NE was further characterized by AFM (Nanonics MultiView 2000™, Nanonics Imaging Limited, Israel). A drop of CU-NE was placed on freshly cleaved mica plate. The sample was air dried at room temperature and mounted on the microscope scanner. The shape was observed and imaged in noncontact mode with frequency 312 kHz and scan speed 2 Hz.

5.6.2.9 In vitro release studies

5.6.2.9.1 Dialysis membrane specification

The dialysis membrane used in the study was cellulose membrane (Sigma Aldrich, Bangalore, India). 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.

5.6.2.9.2 Treatment of dialysis bag

Treatment of the dialysis bag was done in accordance with the directions written on the package (Sigma Aldrich) to remove glycerin and sulphur compounds so that pores can be opened.
Chapter 5.6

FORMULATION DEVELOPMENT

I. Removed glycerin by washing in running water for 3-4 h.
II. Removed sulphur compounds by treating it with 0.3% w/v sodium sulphide solution in water at 80 ºC for 1 min.
III. Washed with hot water at 60 ºC for 2 min.
IV. Acidified the procured dialysis bag with 0.2% v/v H₂SO₄ in distilled water.
V. Rinsed it with hot water to remove acid.
VI. 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.

5.6.2.9.3 Procedure for in vitro drug release studies

In vitro release studies were performed using the dialysis bag method. The in vitro release studies were performed to compare the release of CU from CU-NE formulations with CU suspension. CU was mixed with sodium carboxy methyl cellulose solution (2 %w/v) in mortar until the mixture became uniform and was then made up to volume to prepare 10 mg/mL of suspension (CUS). The dialysis bag (MWCO 1200 g/mole, Sigma Aldrich, USA) was treated as per instruction of Sigma Aldrich. One ml of NE formulation (10 mg/ml) and CU suspension (CUS) was filled in treated dialysis bag which was tied using nylon thread. Integrity of bag was assessed visually. The release study was performed in 500 mL of simulated gastric fluid (0.1N HCl) containing 0.1% w/v ascorbic acid using USP apparatus 1 (Basket), at 100 rpm, 37± 0.5 ºC (Hanson Research SR8 plus, California, USA). The dialysis bag was kept inside the basket. 5 ml samples were withdrawn at regular time intervals (10, 20, 30, 60, 120, 180, 240, 300, 360 and 720 min) and aliquot amount of simulated gastric fluid was replaced. The samples were analyzed for the drug content using UV-Vis spectrophotometer method.

5.6.2.10. Ex vivo release studies

All animal experiments were carried out after approval of the protocol by Jamia Hamdard, Institutional Animal Ethics Committee, New Delhi and their guidelines were adhered for the complete study. In order to determine the release using rat intestinal membranes, duodenum was taken, washed with saline to remove excretory product present in the duodenum by flushing.

One ml of NE formulation and suspension (10 mg/ml) was filled in duodenum separately which was tied using nylon thread. The release study was performed in 500 mL of simulated
intestinal fluid (pH 6.8 phosphate buffer) containing 0.1% w/v ascorbic acid using USP apparatus 1 (Basket), at 100 rpm, 37± 0.5 °C (Hanson Research SR8 plus, California, USA). The duodenum was kept inside the basket. 5 ml samples were withdrawn at regular time intervals (10, 20, 30, 60, 120, 180, 240, 300, 360 and 720 min) and aliquot amount of simulated intestinal fluid was replaced in order to maintain sink condition. The samples were analysed for the drug content using UV spectrophotometer method at 424 nm.

5.6.2.11 In vitro lipolysis studies (pH stat method)

The procedure for the dynamic in vitro lipolysis experiment was designed to achieve maximum pseudo-physiological conditions to study the influence of nanoemulsion composition on lipid digestion and CU bioaccessibility, and was a modification of those described previously (Ali et al., 2008).

5.6.2.11.1 Preparation of lipolysis reagents

A digestion buffer (pH 6.8) was prepared by adding the following ingredients to sufficient deionised water to prepare 1L of the buffer; CaCl\textsubscript{2}\cdot2H\textsubscript{2}O (5 mM), NaCl (150 mM), tris-maleate (50 mM), and NaOH (39.75 mM). The pH of the medium was adjusted with 1M NaOH or 1N HCl. In this study, pancreatin with an amylase activity not less than 25 USP, lipase activity not less than 2.0 USP and protease activity not less than 25 USP was used. Fresh pancreatin extract was prepared by adding 1 g of porcine pancreatin powder to 5 ml digestion buffer, stirring for 15 min by homogenizer to produce a 200 mg/ml suspension and incubated for 20 min at 37 °C.

Lipolysis medium was prepared by dissolving 0.26 g of taurocholic acid (5mM) and 0.10 g of L-α-phosphatidylcholine (1.25 mM ) (conditions simulating fasted state GIT) in 100 ml of pH 6.8 digestion buffer at 50 °C with the aid of a magnetic stirrer/hot plate unit (Optics Technology, New Delhi, India). The heat and agitation was maintained for approximately 30 min until L-α-phosphatidylcholine was fully dissolved. The pH of the buffer was re-adjusted to 6.8 using 1.0 M NaOH or 1.0 N HCl.

5.6.2.11.2 In vitro lipolysis experiments using biorelevant media

The experimental medium, which comprised of 35.5 ml of lipolysis medium (pH 6.8) was continuously stirred (100 rpm) and maintained at 37±0.5 °C throughout the experiment. Prior to each experiment, the pH electrode of the pH meter (Mettler, India) was calibrated using
standard buffers of pH values 4 and 10. The tip of the pH electrode was positioned 1 cm below the surface of the biorelevant medium. At the beginning of each experiment, 1 g of the NE formulation was dispersed in the biorelevant medium and stirred for 15 min. The pH of the buffer was re-adjusted to 6.8 using 1.0M NaOH or 1N HCl. About 3.5 ml of the pancreatin extract (200 mg/ml) was inserted into the medium and the enzymatic digestion of the formulation initiated. The pH of the reaction medium 6.8 was maintained with an accuracy of ±0.2 pH units using 0.15M NaOH as the titrant. This is important since during the lipolysis process of triglycerides, free fatty acids are liberated and consequently the pH decreases. The experiment was continued for 30 min, in which time the enzymatic digestion process was completed, as indicated by the completion of the pH titration. The volume of NaOH added to the dispersed nanoemulsion was recorded and was used to calculate the concentration of free fatty acids generated by lipolysis.

Free fatty acids (FFA) are released from the sample when pancreatin lipase in the SIF interacts with triacylglycerols in the digestion vessel. Each triacylglycerol molecule generates two FFAs when fully digested; hence, the fraction of FFA released from a sample can be calculated from knowledge of the total amount of triacylglycerols originally present within the digestion cell and the amount of FFAs released after a particular digestion time. The percentage of FFAs released is calculated from the number of moles of alkali (NaOH) required to neutralize the FFA divided by the number of moles of FFA that could be produced from the triacylglycerols if they were all digested (assuming two FFA produced per triacylglycerol molecule):

\[
\text{% FFA} = \frac{100 \times (V_{\text{NaOH}} \times M_{\text{NaOH}} \times M_{\text{lipid}})}{W_{\text{lipid}} \times 2}
\]

Here \(V_{\text{NaOH}}\) is the volume of sodium hydroxide required to neutralize the FFA produced (L), \(M_{\text{NaOH}}\) is the molarity of the sodium hydroxide solution used (M), \(W_{\text{lipid}}\) is the total mass of triacylglycerol oil initially present in the digestion cell (in g), and \(M_{\text{lipid}}\) is the molecular mass of the triacylglycerol oil (in g mol\(^{-1}\)). The data generated by this method was plotted as a curve between % FFA released versus time.

5.6.2.12 Bioaccessibility determination

The bioaccessibility of CU was evaluated after the samples had passed through the simulated small intestine phase of the \textit{in vitro} gastrointestinal model. To determine the concentration of
CU retained in the aqueous phase of the digestion medium post-lipolysis, 500 µl of the sample was taken out at time interval of 5, 10, 15, 20 and 30 min. At the given time point, samples of the digestion medium were removed and immediately treated with digestion inhibitor (5 µL per 1 mL of digestion medium of 1.0 M 4-bromophenylboronic acid in methanol) to inhibit the digestion (Williams et al., 2012). Samples were then separated by centrifugation using ultracentrifuge (C-24, Remi Instrument Ltd, Mumbai, India) at 10,000 rpm for 60 min at 4°C, into two phases, namely, a dispersed aqueous colloidal phase and sediment phase. After separation, the aqueous fraction was filtered through 0.45 µm membrane filter and analyzed for CU using HPLC. At the end of experiment sediment phase was dissolved in acetonitrile and centrifuged for 10 min at 10,000 rpm. Supernatant was taken out, filtered through 0.45 µm membrane filter and analyzed for CU content by HPLC. The bioaccessibility (BA) of CU was determined using the following equation:

\[ \text{BA(%) = } \frac{\text{CCM}}{\text{CCI}} \times 100 \]

Where, CCM is the CU concentration in micelle/colloidal phase and CCI is the CU initial concentration dissolved in fresh NE.

5.6.2.13 *Ex vivo* everted gut sac studies

An improved everted gut sac model can be used as an *in vitro* tool to study the mechanisms and kinetics of drug absorption. For the study Male Wistar rats (200–250 g), after being fasted for 10–12 h with free access to water, were anesthetized by excessive ether inhalation. Following a midline incision in the abdomen, the small intestine was excised at two positions, at 4 cm distal to the stomach and at the ileocecal junction. The entire length of the small intestine was carefully removed and, before tissue preparation, placed in Krebs Ringer solution (pH 7.4) continuously aerated with the aid of an electrical aerator. Medial jejuna/duodenum segments (~4 cm) were used for the permeation studies. This segment was cut and washed six to eight times with Krebs Ringer solution, ligated with nylon thread at one end, and carefully everted on the glass rod. The everted gut sac, filled with 1 mL of Krebs Ringer solution and ligated, was placed inside the conical flask containing 20 mL of the test solution (10 mg/mL of CU-NE and CUS) continuously bubbled with atmospheric air at 13–19 bubbles per min separately. The gut sac bath was surrounded by an outer water jacket to control the temperature of the bath at 37° ± 5°C. The Krebs Ringer solution outside the sac
was termed mucosal fluid, and the solution inside the gut sac was termed serosal fluid. The amount of CU that permeated across the intestine in serosal fluid was determined using HPLC method after predetermined time period (0.5, 1, 1.5 and 2 h).

Permeability coefficient (Papp) of CU was calculated from mucosal to serosal direction according to the equation:

\[ P_{\text{app}} (\text{cm/sec}) = \frac{dQ/dt}{A \times C_0} \]

Where, \( dQ/dt \) is the rate of drug permeation from the tissue, \( A \) is the cross-sectional area of the tissue, and \( C_0 \) is the initial CU concentration in the donor compartment at \( t = 0 \).
5.7 Pharmacokinetic studies

5.7.1 Animals and dosing

All animal experiments were performed in accordance with committee for the purpose of control and supervision on experiments on animals (CPCSEA) guidelines. The animal experiments were carried out after approval of the protocol by Jamia Hamdard, Institutional Animal Ethics Committee, New Delhi and their guidelines were adhered for the complete study. Wistar rats of either sex (200–250 g) were housed in polypropylene cages and kept on fasting with free access to water for 12 - 18 h before the experimentation. The routine animal handling was performed according to Good Laboratory Practices.

5.7.1.1 Animals Details

a. Species: Albino Wistar
b. Age/weight/Size: 200-250 g
c. Gender: Either sex
d. Total group: Four
e. No of animals in each group: 3/group
f. Total no. of formulations: Four
   Group I - SB1 from LST combination
   Group II - LA1 from LUP combination
   Group III - CC3 from CST combination
   Group IV - CUS (Curcumin suspension)
g. Total time of study and no. of blood samples: 12 h, 8 sample time points, 0.5 ml blood sample withdrawn
h. Dose:
   50 mg/kg (For nanoemulsion)  
   (Seo et al., 2012)
   100 mg/kg (For curcumin suspension)

The animals were divided into four groups, each having three Wistar rats. One group was orally administered CU suspension at a dose of 100 mg/kg, where as other three groups were orally administered NE at a CU dose of 50 mg/kg. The rats were anesthetized using ether, and
blood samples (0.5 mL) were taken from the retro-orbital plexus at 0 (predose), 0.25, 0.5, 1, 2, 4, 8 and 12 h post administration. The samples were collected in microcentrifuge tubes which were first rinsed with EDTA followed by addition of small quantity (4–8 mg) of powdered EDTA to the tube. Blood collected was mixed with anticoagulant properly by shaking and then centrifuged at 10,000 rpm at 4 °C for 10 min and stored at −20 °C until processed and analyzed.

5.7.2 Sample preparation

The plasma samples 100 µL was transferred to a 2 ml eppendorf tube and 50 µL (200 ng/ml) of β-esterdiol (IS) were added to it with thorough mixing. Then 250 µL of acetonitrile was added, vortexed for 1 min and centrifuged at 10,000 g for 10 min. All supernatant was transferred to new 2 ml eppendorf tube and 20 µL of the supernatant was injected directly into the HPLC system for analysis. CU in plasma samples was quantified by a validated HPLC method as described in section 5.4.

5.7.3 Assessment of intestinal transport of curcumin through lymphatic system

The intestinal lymphatic system is also an important route for the absorption of some highly lipophilic drugs. In some cases the intestinal lymphatic absorption plays a significant role in the enhanced bioavailability. Due to the unique anatomy and physiology of the lymphatics, intestinal lymphatic drug transport can provide certain advantages over drug absorption via the portal blood, such as reducing hepatic first pass metabolism and increasing the total systemic bioavailability and maximizing therapeutic benefits. Entry into the lymphatic system is achieved via drug association with intestinal lipoproteins during drug transit across the enterocyte.

The contribution of lymphatic transport in bioavailability enhancement was assessed by blocking the synthesis of chylomicron, a protein which is responsible for the transport of drug to the mesenteric lymph duct. Lymphatic transport is a post-absorption happening and the fraction of drug transported through the mesenteric lymphatic are not subjected to hepatic first pass metabolism. The feasibility of lymphatic transport of CU from NE was assessed using cycloheximide model. Intraperitoneal administration of the protein synthesis inhibitor cycloheximide has been shown to affect the lipid absorption in vivo.

The rats were fasted over night (12–18 h) with free access to water as described previously. The rats were treated with either an intraperitoneal injection of 3 mg/kg cycloheximide in
saline (0.6 mg/ml) or with an equal volume of saline. The animals were divided into four
groups, each having three Wistar rats. One hour after the injection, one group was orally
administered CU suspension at a dose of 100 mg/kg, where as other three groups were orally
administered NE at a CU dose of 50 mg/kg. The blood sample were collected and processed
as described in section 5.7.2.

5.7.3.1 Animals details

   a. Species:          :  Albino Wistar
   b. Age/weight/Size:  :  200- 250 g
   c. Gender:           :  Either sex
   d. Total group       :  Four
   e. No of animals in each group :  3/group
   f. Total no. of formulations :  Four
      Group I - SB1 from LST combination
      Group II - LA1 from LUP combination
      Group III - CC3 from CST combination
      Group IV - CUS (Curcumin suspension)
   g. Total time of study and no. of blood samples :  12 h, 8 sample time points, 0.5 ml blood sample withdrawn
   h. Dose              :  50 mg/kg (For nanoemulsion)
                            (Seo et al., 2012)
                            100 mg/kg (For curcumin suspension)
5.7.4 Pharmacokinetic parameters

Non-compartmental analysis was performed by using Microsoft Excel PK Function. The area under the plasma concentration – time curve (AUC_{0-12h}) was calculated using linear trapezoidal method. The peak plasma concentration (C_{max}) and the time to reach the peak plasma concentration (T_{max}) were observed from the values of experimental data. The elimination rate constant (K_{el}) was determined by regression analysis from the slope of best fit, and half life (t_{1/2}) was computed by the formula

\[ t_{1/2} = \frac{0.693}{K_{el}} \]

Relative bioavailability can be calculated according to Equation

\[ \text{Relative Bioavailability} = \frac{\text{AUC of Test}}{\text{AUC of standard}} \times \frac{\text{Dose of standard}}{\text{Dose of test}} \]
Chapter 5.8

5.8 In vitro cell viability assays against Glioblastoma cells (Cytotoxicity studies)

Brain cancers are the leading cause of cancer-related mortality in children. The nomenclature of these cancers is based on the cell type or part of the brain in which they arise. The most common type of brain cancers arise in the glial cells and hence are called the gliomas. Within the gliomas, astrocytomas (arising from astrocytes) are the most common tumors. Malignancy is graded using a progressive 4 tier scale in which grades I and II are assigned to low grade or benign tumors and grades III and IV are assigned to high grade or malignant tumors (Louis et al., 2007). In order to evaluate the therapeutic effectiveness of CU-NE in human cancer cell line, U 87, a human primary glioblastoma cell line was selected. The cytotoxicity studies were performed with both solutions and the NE formulations at different concentrations of CU.

Glioblastoma multiforme (GBM) is the most frequent primary central nervous system tumor, which represents the second cause of cancer death in adults less than 35 years of age (Allard et al., 2009). Since GBM differs from the other cancers by its diffuse invasion of the surrounding normal tissue, it is impossible to make the complete removal of tumor by the conventional surgical method and tumor recurrence from residual tumors is very possible (Onget al., 2009). Consequently, it is critical to deliver the therapeutic agent effectively to the tumor as well as to infiltrate cells that are not located in the tumor bed for GBM treatment.

5.8.1 Cytotoxicity studies with U-87 cell line

5.8.1.1 Cell culture

The U-87 glioma cell line was purchased from American Type Culture Collection and cultured in Eagle’s minimum essential medium (MEM) containing 10% (v/v) fetal bovine serum (FBS), 2 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin, at 37 °C under a humidified atmosphere containing 5% CO₂.

5.8.1.2 Cell viability assay

Solutions of CU were prepared by dissolving the CU in DMSO and then adding them to culture medium to obtain the desired concentrations of CU (5, 10, 25, 50 and 100 µM) and maintaining the DMSO at 0.1% concentration in the well. Similarly NEs were also prepared at the above mentioned concentrations of CU in minimum essential medium (MEM). The
U87 cells were allowed to adhere on the surface of 96-well microplates at a density of $5 \times 10^3$ cells per well. After 48 h, when the cells in the wells were approximately confluent, the culture media was replaced with the solutions and nanoemulsions of CU separately. After dosing, the U87 cells were incubated at 37°C for 48 hours. Cells treated with MEM containing 0.1% DMSO and also cells treated with MEM containing blank nanoemulsion formulation alone was used as negative controls in determining the cytotoxicity of solutions and nanoemulsion formulations respectively.

For the cytotoxicity study, the MTT reagent was prepared at a concentration of 5 mg/mL by dissolving the yellow dye (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole dye) in MEM. Following a 2-day (48 h) incubation period, the media from each well was replaced with 50 µL of freshly prepared MTT reagent into each well and again incubated for 2 h in the CO₂ chamber at 37°C. Then 150 µL DMSO was added into each well to dissolve the insoluble purple-colored formazan crystals formed in the mitochondria of living cells. MTT gets reduced to purple formazan in living cells by mitochondrial reductase. This reduction takes place only when reductase enzymes are active, and therefore this conversion is used as a measure of viable (living) cells. The absorbance in each well was measured using a UV/Visible/fluorescence microplate reader at 570 nm. The percentage cell viability was calculated at each concentration of CU by dividing the absorbance of the treated cells over the control cells and multiplying it by one hundred. Then IC50 was determined for the solutions and NE on U87 cells. Results were expressed as % cell viability versus dose.
5.9 Stability studies

5.9.1 Accelerated stability studies as per ICH guidelines

The purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product varies with time, under the influence of a variety of environmental factors, such as temperature, humidity and light, as well as to establish a re-test period for the drug substance or expiration date for the drug product, and recommend storage conditions. NE was assessed for any change during storage at accelerated condition as per ICH guidelines Q1A (R2) (2003). NE was subdivided into 5 ml glass vials, sealed and were stored upright. Physical and chemical stability of NE were evaluated for six months by storing them at 40 ± 2 °C/75 ± 5% RH. Samples were withdrawn at specified time intervals (0, 1, 2, 3 and 6 months) and assessed for remained CU content and globule size. The NE was also observed for physical evaluation such as turbidity (after 100 times dilution), phase separation and drug precipitation. Analysis with HPLC method was carried out at each time interval by diluting the NE with mobile phase to 5 µg/mL. The amount of drug degraded and the amount remaining at each time interval were calculated.

5.9.2 Stability studies of CU loaded NE formulation using Arrhenius equation (Estimation of residual drug content)

According to ICH guideline forced decomposition studies (stress testing) at temperatures in 10 °C increments above the accelerated temperatures should be carried out on the drug substance so as to establish the inherent stability characteristics and degradation pathways to support the suitability of the proposed analytical procedures (Bakshi and Singh, 2002).

The selection of higher temperatures and humidity was designed to accelerate reaction rates significantly, so that even relatively slow reactions become evident in a short period of time. For the determination of shelf life by conventional method, CU loaded NE formulations (NE-SB1, NE-LA1 and NE-CC3) were kept at 40 °C, 50 °C and 60 °C for 30 days. Samples were withdrawn after specified time intervals (0, 7, 15, and 30 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 0.45 µm filter before being injected into the HPLC system.
The order of degradation was determined by the graphical method. Degradation rate constant (K) was determined at each temperature. The Arrhenius plot was constructed between logK and 1/T to determine the shelf-life of optimized NE formulation, where T is the absolute temperature in degrees Kelvin. The value of K at 25 °C (K_{25}) was obtained by extrapolation of the plot and shelf-life was then calculated by substituting K_{25} in the following equation:

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

where \( T_{0.9} \) is the time required for 10% drug degradation is referred to as shelf-life.