6.1 \textbf{PART- I: PREPARATION OF SIMVASTATIN NANOPARTICLES}

6.1.1 \textbf{Preformulation Study}

The formulation of the polymeric systems is essential for providing suitable delivery rate of drug. The components of the system have an impact on the rate of drug release and hence affect the bioavailability of the drug to a large extent. It is very important to evaluate the dosage form for its mechanical and physical properties. In assessing the significance of a given property, it is essential to consider not only the end use of the system but also the processing and handling it undergoes before, it is put to the end use.\textsuperscript{114}

6.1.1.1 \textbf{Drug Identification}

Organoleptic property of powder was checked out. Simvastatin samples were identified by melting point, UV spectra and FT-IR spectra.\textsuperscript{115}

6.1.1.2 \textbf{Melting point}

Melting point is one of the key parameter to identify the drug and its crystalline state. Moreover, variation in melting point gives the clue of drug substance purity. Melting point of simvastatin was determined by open capillary tube method. Simvastatin was placed in separate capillary tube that was attached to a thermometer. The whole assembly was kept in oil bath and progress in temperature was monitored. The point at which drug melting starts were noted. The experiment was repeated three times. The mean melting point was considered as the melting point of the drug.

6.1.2 \textbf{UV-spectroscopic studies}

A stock solution of 1 mg/ml of simvastatin was prepared by dissolving 100 mg of drug in small quantity of methanol and sonicated for 10 minutes and diluted with 100 ml of phosphate buffer (pH 6.8) to obtain stock solutions containing 1000 µg/ml in a 100 ml of volumetric flask. Solutions containing 10 µg/ml of simvastatin were scanned in the range of 200-400 nm regions on PC based Jasco V-630 spectrophotometer.

UV-Visible Spectrophotometer (Jasco model V- 530) is a double beam high speed scanning spectrophotometer with a single monochromater with a 1200 grooves/mm concave grating. The software includes fixed wavelength measurement, spectra scan, time scan, trace, quantitative analysis, auto scale, absorbance/ percentage transmittance meter, zoom peak valley picking, repeat scan and derivative for operation. The UV-Visible spectrophotometer specifications given in Table 6.1.1.
Table 6.1.1: UV-Visible spectrophotometer specifications

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Specifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optical system</td>
<td>Single monochromater</td>
</tr>
<tr>
<td>UV-Visible region</td>
<td>1200 lines/mm concave grating, Double beam type</td>
</tr>
<tr>
<td>Resolution</td>
<td>2 nm.</td>
</tr>
<tr>
<td>Light source</td>
<td>Deuterium lamp: 190 to 350 nm. Halogen lamp: 330 to 1100 nm</td>
</tr>
<tr>
<td>Wavelength Range</td>
<td>190-1100 nm</td>
</tr>
<tr>
<td>Wavelength Repeatability</td>
<td>± 0.1 nm.</td>
</tr>
<tr>
<td>Wavelength Accuracy</td>
<td>± 0.3 nm</td>
</tr>
<tr>
<td>Spectral bandwidth</td>
<td>2 nm fixed.</td>
</tr>
<tr>
<td>Photometric mode</td>
<td>Absorbance, % T, %R.</td>
</tr>
<tr>
<td>Photometric Range</td>
<td>2.0 to 3.0 Absorbance (0 to 200 % T).</td>
</tr>
<tr>
<td>Response</td>
<td>Quick, fast / medium / slow</td>
</tr>
<tr>
<td>Wavelength Scanning</td>
<td>40, 100, 200, 400, 1000, 2000, 4000 nm/ min.</td>
</tr>
<tr>
<td>Baseline Stability</td>
<td>± 0.001 Abs. / h.</td>
</tr>
<tr>
<td>Wavelength Moving Speed</td>
<td>8000 nm/ min</td>
</tr>
<tr>
<td>Detector</td>
<td>Silicon Photodiode (S1337).</td>
</tr>
</tbody>
</table>

6.1.2.1 Preparation of standard curve

A stock solution of 1 mg/ml of simvastatin was prepared by dissolving 100 mg of drug in small quantity of methanol and sonicated for a few minutes and diluted with 100 ml of phosphate buffer (pH 6.8). The stock solution was serially diluted to get solutions 10, 20, 30, 40, 50 μg/ml concentrations were prepared. The absorbance of the different diluted solutions was measured using UV-Visible spectrophotometer at \( \lambda_{\text{max}} \) of 238 nm. A calibration curve was plotted by taking concentration of solution in x axis and absorbance in y axis and correlation coefficient ‘r’ was calculated from the equation of straight line i.e \( y = mx + c \)

6.1.3 High performance Liquid Chromatography

6.1.3.1 Selection of Chromatographic Conditions
The selection of HPLC method depends upon the nature of the sample, its molecular weight and solubility. The chromatographic variables such as mobile phase, flow rate and solvent ratios were studied. The resulting chromatograms were recorded and the chromatographic parameters such as capacity factor, asymmetric factor, and peak resolution and column efficiency were calculated. The condition that gave the best resolution, symmetry and capacity factor was selected for estimation. The High Performance Liquid Chromatography specifications and separation conditions given in Table 6.1.2 and 6.1.3.

**Table 6.1.2: High Performance Liquid Chromatography specifications**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Specifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>PU 2080 Pump</td>
<td>Dual piston with gear driven pump</td>
</tr>
<tr>
<td>Flow rate</td>
<td>0.001 to 10 ml/min</td>
</tr>
<tr>
<td>Pressure range</td>
<td>up to 6500 psi</td>
</tr>
<tr>
<td>Rheodyne Injector</td>
<td>20 µl (77251).</td>
</tr>
<tr>
<td>UV 2075 Detector</td>
<td>Jasco intelligent UV-Vis detector model</td>
</tr>
<tr>
<td>Wavelength</td>
<td>190-600 nm.</td>
</tr>
<tr>
<td>Source</td>
<td>Deuterium lamp.</td>
</tr>
<tr>
<td>Stainless steel Column</td>
<td>C\text{18} HIQ-SIL (250 mm X 4.6 mm i.d.), Japan</td>
</tr>
<tr>
<td>Interface</td>
<td>Hercule 2000.</td>
</tr>
<tr>
<td>Software</td>
<td>Borwin® Version 1.5.</td>
</tr>
</tbody>
</table>

**Table 6.1.3: Separation Conditions**

<table>
<thead>
<tr>
<th>Chromatographic Mode</th>
<th>Chromatographic Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard Solution</td>
<td>1000 µg/ml of simvastatin in mobile phase</td>
</tr>
<tr>
<td>Stationary Phase</td>
<td>HIQ Sil C\text{18} column (250 mm x 4.6 mm i.d., 5µm)</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>ACN: Water (80:20 v/v) with 0.1% OPA</td>
</tr>
<tr>
<td>Detection Wavelength</td>
<td>238 nm</td>
</tr>
<tr>
<td>Flow Rate</td>
<td>1 ml/min</td>
</tr>
<tr>
<td>Sample Size</td>
<td>20 µl</td>
</tr>
</tbody>
</table>
6.1.3.2 Optimization of mobile phase strength

For selection of mobile phase, various mobile phase compositions containing acetonitrile: water in different ratios was tried but the resolution was not found to be satisfactory. Mobile phase was selected based on literature reviewed as well as on the basis of best separation of simvastatin, sharp peaks and high number of theoretical plates. The mobile phase included acetonitrile and double distilled water (pH 3) (80:20) with 0.1% O-phosphoric acid.  

6.1.3.3 Optimization of detection wavelength

The sensitivity of HPLC method that uses UV detection depends upon proper selection of detection wavelength. An ideal wavelength is one that gives good response for the analyte to be detected. In the present study drug solution of 10μg/ml of simvastatin prepared in mobile phase. After observing UV spectra of drug, wavelength of 238 nm was selected for further study.

6.1.3.4 Preparation of standard drug solution

Standard stock solution containing simvastatin was prepared by dissolving 50 mg of simvastatin in 20 ml of mobile phase separately. The solution was sonicated for 10 minutes. Then the final volume of the solution was made to 50 ml with mobile phase (1000μg/ml). Accurately measured 0.1, 0.5, 1, 1.5, 2 and 2.5 ml of stock solution were transferred to 10 ml of volumetric flask and the volume was made to 10 ml using mobile phase to obtain of 10, 50, 100, 150, 200 and 250μg/ml. Absorbance of their solution were measured on RP-HPLC (Jasco PU 2080 Pump, UV 2075 Detector) using acetonitrile and double distilled water (pH 3) (80:20) with 0.1% OPA as mobile phase at λ max 238 nm.

6.1.3.5 Linearity study of simvastatin

Standard working solutions of 1000μg/ml of simvastatin, were prepared using mobile phase as a solvent. Required volume of solution from standard working solution was taken to get final dilutions of required strength for calibration curves and made up the volume with mobile phase. The HPLC analysis of all aliquots was carried out and response factor for each analyte was calculated.

6.1.3.6 Recovery Studies

Accuracy of analysis was determined by performing recovery studies by spiking different concentrations of pure drug in the pre-analyzed laboratory sample.

6.1.3.7 System Suitability Parameters

System suitability parameters were analyzed on freshly prepared standard stock solutions of simvastatin. All these analytes were injected into the chromatographic system under the optimized chromatographic conditions. Parameters that were studied to evaluate the suitability of the system were
number of theoretical plates, calibration curve, capacity factor, resolution factor, retention time.

6.1.4 Compatibility Studies

Compatibility of the simvastatin with PLGA used to formulate nanoparticles was established from FTIR spectrum and DSC thermogram analysis. FTIR & DSC spectral analysis of simvastatin and combination of simvastatin and PLGA was carried out to investigate the changes in chemical composition of the drug after combining it with the excipients. Compatibility study was carried out on FTIR (Jasco V-530) and DSC (TA-60, Instruments SDT-2960, USA).

6.1.5 Saturation Solubility Studies

Accurately weighed 10 mg of simvastatin and the nanoparticles equivalent to 10 mg of the drug were separately introduced into 25ml stoppered conical flasks containing 10 ml phosphate buffer (pH 6.8). The sealed flasks were agitated on a rotary shaker for 24 h at 37°C and equilibrated for 2 days. An aliquot was passed through 0.45μm membrane filter and the filtrate was suitably diluted and analyzed for drug content on a UV spectrophotometer at 238 nm wavelength.

6.1.6 Experimental Design

The formulations were fabricated according to a $3^2$ full factorial design, allowing the simultaneous evaluation of two formulation independent variables and their interaction. The experimental designs with corresponding formulations is shown in Table 6.1.4 & 6.1.5. The dependent variables that were selected for study were particle size ($Y_1$) and % drug entrapment ($Y_2$).

Table 6.1.4: Experimental design and Parameters for $3^2$ Full Factorial design batches

<table>
<thead>
<tr>
<th>Batch code</th>
<th>Variables level in coded Form</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$X_1$</td>
</tr>
<tr>
<td>PS1</td>
<td>-1</td>
</tr>
<tr>
<td>PS2</td>
<td>-1</td>
</tr>
<tr>
<td>PS3</td>
<td>-1</td>
</tr>
<tr>
<td>PS4</td>
<td>0</td>
</tr>
<tr>
<td>PS5</td>
<td>0</td>
</tr>
<tr>
<td>PS6</td>
<td>0</td>
</tr>
<tr>
<td>PS7</td>
<td>+1</td>
</tr>
<tr>
<td>PS8</td>
<td>+1</td>
</tr>
<tr>
<td>PS9</td>
<td>+1</td>
</tr>
</tbody>
</table>
Table 6.1.5: Translation of coded levels to actual quantities

<table>
<thead>
<tr>
<th>Coded Levels</th>
<th>+1</th>
<th>0</th>
<th>-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug: Polymer ratios (X₁) (mg)</td>
<td>1:3 (150)</td>
<td>1:2 (100)</td>
<td>1:1 (50)</td>
</tr>
<tr>
<td>PluronicF68 (X₂) %</td>
<td>0.4</td>
<td>0.3</td>
<td>0.2</td>
</tr>
</tbody>
</table>

6.1.6.1 Statistical analysis

The results from factorial design were evaluated using PCP Disso 2000 V3 software. Step wise backward linear regression analysis was used to develop polynomial equations for dependent variables particle size (Y₁) and % drug entrapment (Y₂) equation (6.1).

\[ Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 + \epsilon \]  

Eq...(6.1)

Where, Y is the estimated response of dependent variable, \( \beta_0 \) arithmetic mean response of nine batches, and \( \beta_1 \) estimated coefficient for factor \( X_1 \). The main effects (\( X_1 \) and \( X_2 \)) represent average result of changing one factor at a time from its low to high value. The interaction term (\( X_1 X_2 \)) shows how the response changes, when two factors are simultaneously changed. The polynomial terms (\( X_1^2 \) and \( X_2^2 \)) are included to investigate non-linearity. The term \( \epsilon \) is the random error. The simplified models were then utilized to produce three dimensional response surface plots to analyze the influence of independent variables.

6.1.7 Selection Process for Preparation of Nanoparticles

Nanoprecipitation - solvent displacement method was used for the preparation of nanoparticles. For selection of appropriate process blank nanoparticles were prepared by the nanoprecipitation - solvent displacement method using different concentration (1:1,1:2 and 1:3 ) of PLGA as a polymer and Pluronic F68 as a stabilizer, respectively at stirring speed 1000 to 2000 rpm. The particle size was analysed by Malvern particle size analyzer. The appropriate process for preparation of nanoparticles was selected on basis of particle size analysis data.\(^{119}\)

6.1.8 Preparation of PLGA Nanoparticles

PLGA nanoparticles were prepared by the nanoprecipitation-solvent displacement method. Accurately measured 50 mg of simvastatin was dissolved in sufficient quantity of acetone. Hydrophilic stabilizer pluronic F-68 (0.2%, 0.3% and 0.4 % i.e 100mg, 150mg and 200mg) dissolved in 40ml of water. PLGA was solubilized in 20 ml of acetone at various concentrations (1:1, 1:2 and 1:3. i.e 50mg, 100mg and 150mg). The organic phase was poured into the aqueous solution drop wise, at 1ml/min flow with syringe positioned with the needle directly into stabilizer containing water, which was stirred at 1500 rpm for 2h, thus forming a milky
colloidal suspension. The organic solvent was evaporated using a Rota evaporator. All experiments were performed in triplicates. Nanoparticles were collected by centrifugation at 15,000 rpm for a period of 1h and supernants were discarded. The resultant dispersion was dried using a freeze-drying.¹²⁰⁻¹²¹

### 6.1.9 Freeze Drying of Nanoparticles

Lyophilisation is a promising way to increase physico-chemical stability of nanoparticles over extended period of time. Briefly 5ml of nanoparticles dispersion was filled in 10ml glass vials, covered with special stoppers for lyophilization and placed in a freeze dryer (Martin Christ ALPHA 1-2 LD plus) shown in figure 6.1. For freezing the samples, the vials containing sample were cooled with 0.4°C/min from 20°C to -52°C for 3 h under atmospheric pressure. After 3 h warm-up vacuum was applied for 10 minutes and then main drying was started. In primary drying the pressure was reduced to 0.06 mbar at -20°C and under these conditions samples were dried for 48 h. Secondary drying was performed at pressure 0.002 mbar and sample vials were heated up to 10°C. The condition was maintained for 6 h. Finally sample vials were closed directly in the self stoppering chamber with rubber stoppers. The sealed vials removed from freeze drier and sealed with aluminum crimps using hand operated crippler. Sample vials were stored at 2-8 °C. Free flowing nanoparticles powder was prepared by freeze drying of all batches of nanoparticles dispersion. Different parameters were applied in order to obtain free flowing nanoparticles dispersion. Prior to freeze drying the nanoparticles dispersion was mixed with 5% mannitol, which was used as cryoprotectant. Freeze drying was carried out for 72 h.

![Figure 6.1: Freeze drier](image-url)
6.1.10 Characterization of Nanoparticles

6.1.10.1 Determination of particle size

The particle size and size distribution of the simvastatin loaded PLGA (50:50) nanoparticles was characterized by photon correlation spectroscopy using a Zetasizer 2000 Malvern Instruments, UK. Nanosuspension was diluted with filtered (0.22μm) ultra pure water and analysed using Zeta sizer. This analysis yields the mean diameter (z-average, measuring range: 20–1000nm), which allows sample measurement in the range of 0.020–2000.00μm. The mean diameter for each sample and mean hydrodynamic diameter was generated by cumulative analysis in triplicate.

6.1.10.2 Polydispersity Index

Polydispersity index is the ratio of weight of average molecular mass to the number of average molecular mass. It indicates the distribution of individual molecular masses in a batch of polymers. The PI has a value equal to or greater than 1, but as the polymer chains approach uniform chain length, the PI approaches to 1. Polydispersity was determined using formula (1).\(^\text{(1)}\)

\[
\text{Polydispersity Index} = \frac{D(0.9) - D(0.1)}{D(0.5)} \times 100
\]

Where, \(D (0.9)\) corresponds to particle size immediately above 90% of the sample.

\(D (0.5)\) corresponds to particle size immediately above 50% of the sample.

\(D (0.1)\) corresponds to particle size immediately above 10% of the sample.

6.1.10.3 Determination of entrapment efficiency

The encapsulation efficiency of nanoparticles was determined by first separating the nanoparticles formed from the aqueous medium by centrifugation at 15000 rpm for 1 h. The amount of free simvastatin in the supernatant was measured by UV spectrophotometry at 238 nm (Shimadzu UV-1700) after suitable dilution. The simvastatin entrapped in the nanoparticles was calculated using formula (2).\(^\text{(2)}\)

\[
\% \text{Entrapment Efficiency} = \left(\frac{T_p - T_f}{T_p}\right) \times 100
\]

Where, \(T_p\) is the total simvastatin used to prepare the nanoparticles and \(T_f\) is the free simvastatin in the supernatant. The following calibration curve was used for determination of entrapment efficiency,

\[y = mx + C\]  \hspace{1cm} \text{Eq. (6.2)}
6.1.1.4 Determination of zeta potential

Colloids with high zeta potential (negative or positive) are electrically stabilized, while colloids with low zeta potentials tend to coagulate or flocculate. The zeta potential of the simvastatin loaded PLGA nanoparticles was measured on a zetasizer (Malvern Instruments UK and N5 Beckman submicron zeta analyzer). The measurements were performed using an aqueous dip cell in an automatic mode by placing diluted samples (with ultra-purified water) in the capillary measurement cell and cell position was adjusted. All the samples were measured in water at 25ºC in triplicate.\textsuperscript{124}

6.1.1.5 Percent process yield

Percent process yield was calculated as the weight of the lyophilized nanoparticle from each batch in relation to the sum of starting material multiplied by hundred. The Percent yield was calculated using equation (6.3).

\[
\text{% Yield} = \frac{\text{PracticalYield}}{\text{TheoreticalYield}} \times 100
\]

6.1.1.6 Percent drug content

The lyophilized nanoparticle powder (10mg) was dissolved in 1 ml methanol and volume was made up to mark in 10ml volumetric flask with phosphate buffer pH 6.8. 0.1ml of above solution was further diluted to 10 ml and analyzed by spectrophotometrically at 238nm. The SV contents in nanoparticles (% w/w) were calculated by using eq. (6.2).

6.1.1.7 In vitro drug release study

\textit{In-vitro} drug release studies were performed in USP Type II dissolution apparatus at rotation speed of 50rpm. The simvastatin-loaded PLGA nanoparticles, after separation by centrifugation, were re-dispersed in 5mL phosphate buffer solution pH 6.8, placed in a dialysis membrane bag, tied and immersed in 900ml of phosphate buffer solution in a vessel, and temperature was maintained at 37±0.20°C. The sample weight of formulations equivalent to 10mg of simvastatin was used for dissolution study. Required quantity 5ml of the medium was withdrawn at specific time periods (5, 10, 20, 30, 60 min.) and the same volume of dissolution medium was replaced in the flask to maintain a constant volume. The withdrawn samples were filtered through a filter paper (0.22 μm, Whatman Inc., USA) and 5 ml filtrate was made up to volume with 100ml of Phosphate buffer solution pH 6.8. The samples were analyzed for drug release by measuring the absorbance at 238 nm using UV-visible spectrophotometer and calculated percent cumulative release of simvastatin.\textsuperscript{104}

6.1.1.8 Fourier Transform Infrared Spectroscopy analysis
Infrared spectrum of simvastatin, nanoparticle formulation was determined by using Fourier Transform Infrared Spectrophotometer (FTIR-4100, Shimadzu) using KBr dispersion method. The base line correction was done using dried potassium bromide. The dried mixture of drug and potassium bromide was taken and compressed under 10-ton pressure in a hydraulic press to form a transparent pellet. To evaluate the molecular states of nanoparticles and also for the drug interaction study.

6.1.10.9  Differential scanning calorimetry analysis

DSC is one of the most powerful analytical techniques, which offers the possibility of detecting chemical interaction of simvastatin and PLGA nanoparticles. Temperature and enthalpy were calibrated with the standard materials indium (melting point -56.6°C) and zinc (melting point- 419.5°C) at a heating rate of 5°C/mins. DSC measurements were carried out on a modulated DSC Instrument: SDT Q600 V20.9 Build 20 equipped with a thermal analysis data system (TA instrument). Samples of 2-10 mg were placed in aluminium pans and sealed. The probes were heated from 25 to 250°C at a rate of 10 K/min under nitrogen atmosphere. Thermal data analyses of the DSC thermograms were conducted using STARE software (version 5.21). The % degree of crystallinity of SV, Physical mixture and various batches of NP’s calculated using formula (3).

\[
\text{% of crystallinity} = \frac{\Delta H(\text{sample})}{\Delta H100} \times 100
\]

Where, \(\Delta H\) = Enthalpy of melting formulation, \(\Delta H100\) = Enthalpy of melting of proportional 100% of pure crystalline sample

6.1.10.10  X-ray Diffraction Study

X-ray diffraction analysis was employed to detect the crystallinity of the pure drug and the NPs formulation, which was performed using a Philips PW 3710 x-ray diffractometer (XRD) with a copper target and nickel filter (Philips Electronic Inst, Holland). Powders were mounted on aluminium stages with glass bottoms and smoothed to a level surface. The XRD diffractogram for each sample was obtained from 10 to 80 degrees 2\(\theta\) using a step increment of 0.1- 2\(\theta\) degrees and a dwell time of 1 second at each step. XRD diffraction pattern of simvastatin, Physical mixture and PS6 batch was obtained and peak intensity was calculated using 2-\(\theta\) value by spekwin 32 v 1.71.6.\textsuperscript{125-126}

6.1.10.11  Scanning electron microscopy study

The morphology of nanoparticles was examined by using scanning electron microscopy (SEM, JSM-6360LV scanning microscope Tokyo, Japan). From the structural point of view, the arrangement of components and orientation of molecules within the nanoparticle can determine its behavior and stability. SEM has been used to determine particle size distribution, surface topography, texture and examine the morphology of fractured or sectioned surface. The nanoparticles

---

Formulation and Evaluation of Nanoparticles For Better Drug Bioavailability

Experimental  |  Chapter 6
were mounted on metal stubs using double-sided tape and coated with a 150 Å layer of gold under vacuum. Observation was performed at an accelerating voltage of 5 kV and at a working distance of 10 mm. Stubs were visualized under scanning electron microscope.

**6.1.10.12 Transmission electron microscopy study**

The morphology of nanoparticles was observed by Transmission electron microscopy. Freeze dried powder of nanoparticles was placed on copper grid, which was previously coated with carbon film then sample was dried under lamp for 25-30 minutes. The sample was loaded in instrument (TEM, Tecknai G², 20 U-Twin, FEI, Netherland) operated at 200 kV and image were viewed. The images were recorded with a 1 k CCD camera.

**6.1.11 Preparation of Simvastatin Tablets**

The lyophilized nanoparticles equivalent to 10mg of simvastatin was weighed and mixed with 56mg of Lactose monohydrate, 81mg of hydroxyl propyl methyl cellulose and 10mg of sodium starch glycolate was added and triturated carefully in mortar and pestle. The tablets were prepared by direct compression method using 8mm punches on a rotary tablet machine (Rimek Minipress). Composition for the simvastatin tablet is given in Table 6.1.6.  

<table>
<thead>
<tr>
<th>Tablet Composition</th>
<th>Freeze dried product</th>
<th>Lactose monohydrate</th>
<th>Hydroxyl propyl methyl cellulose</th>
<th>Sodium starch glycolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (mg)</td>
<td>67</td>
<td>56</td>
<td>81</td>
<td>10</td>
</tr>
<tr>
<td>Percentage</td>
<td>31.30</td>
<td>26.16</td>
<td>37.85</td>
<td>4.6</td>
</tr>
</tbody>
</table>

**In-vitro drug release studies**

The in-vitro dissolution study was carried out using USP type II dissolution apparatus at rotation speed of 50 rpm. The study was carried out in 900 ml of phosphate buffer (pH 6.8) for 60 min. The dissolution medium was kept in thermostatically controlled water bath, maintained at 37±0.5°C. At different time intervals, 5ml sample was withdrawn and replaced with fresh medium. Dissolution studies were conducted for pure drug of tablet, marketed tablet and tablets prepared with nanoparticles, and results compared. The drug release was analysed by using UV spectrophotometer at 238 nm wavelength.
6.1.12 In Vivo Evaluation of Simvastatin Nanoparticle Formulation

6.1.12.1 The antihyperlipidemic activity of simvastatin-loaded PLGA Nanoparticles was studied.

6.1.12.1.1 Approval of protocol

The protocol in prescribed Proforma B for animal studies entitled ‘Antihyperlipidemic activity of nanoparticle formulation’ was submitted on August 10, 2010 to IACE of Bharati Vidyapeeth College of Pharmacy, Kolhapur. The protocol was approved by IAEC in presence of CPCSEA nominee with Approval no. BVCPK/ CPESEA/ IAEC/ 01/ 17 dated Jan. 12, 2011 at Bharati Vidyapeeth College of Pharmacy, Kolhapur.

6.1.12.1.2 Selection of Animals

The albino rats of either sex weighing 150 to 250 grams were used for antihyperlipidemic activity of nanoparticles formulation along with pure simvastatin. The albino rats were kept under standard conditions in the animal house of Bharati Vidyapeeth College of Pharmacy, Kolhapur, as per guidelines of CPCSEA.

6.1.12.1.3 Experimental animals

Animals had free access to food and water ad libitum. All experiments were carried out between 10 am and 2 pm. The effect of formulation (test formulation) on plasma lipid profiles was determined by comparison with reference formulation i.e., aqueous suspension containing 1mg/ml simvastatin) and Carboxy methyl cellulose 0.5% w/v as a suspending agent) (equivalent to 10mg/kg per day) in healthy albino rats (Wistar strain). The animals were randomly divided into 4 treatment groups of 3 animals each, viz., control treatment group (CTG), Hyperlipidemic control group (HTG), reference treatment group (RTG) and test treatment group (TTG) and the treatment was given for 21 days. Each treatment group received standard cholesterol diet daily and (2% coconut oil, 2% cholesterol and 1% sodium cholate) orally in the morning throughout 21 days to induce hyperlipidemia except control treatment group. High cholesterol diet was prepared by mixing cholesterol 2%, sodium cholate 1% and coconut oil 2% with standard powdered standard animal food. The diet was placed in the cage carefully for 21 days for Hyperlipidemic control group, reference treatment group and test treatment groups.

Group I: Control treatment group (CTG) (normal pellets diet)

Group II: Hyperlipidemic control (HTG) (normal pellets diet + 2% Cholesterol + 2% coconut oil + 1% sodium cholate)
Group III: Reference treatment group (RTG) Rats treated (with aqueous suspension containing 1mg/ml simvastatin) and Carboxy methyl cellulose 0.5% w/v as a suspending agent) and (normal pellets diet + 2% Cholesterol +2% coconut oil+ 1% sodium cholate).

Group IV: Test treatment group (TTG) Rats treated with simvastatin formulation (equivalent to 10 mg/kg per day) and (normal pellets diet + 2% Cholesterol +2% coconut oil + 1% sodium cholate).

6.1.12.1.4 Collection of Blood samples

Blood samples were collected under light ether anesthesia by retro-orbital puncture at predetermined time intervals, viz., before treatment and after 0, 5, 10, 15, 21 days in anticoagulated (EDTA-treated) polypropylene tube. Plasma was separated by centrifugation at 3000 rpm for 25 min and stored frozen until further use.

6.1.12.1.5 Biochemical analysis

Analysis of lipid profiles for Total Cholesterol, triglycerides, low density lipoproteins and high density lipoproteins levels was carried out. To confirmation induction of hyperlipidemia using respective diagnostic commercial kits (Merck diagnostic kit, Mumbai) and LDL-C, VLDL-C & Al, in plasma was done as per Friedewald estimation using following equations (6.4, 6.5, 6.6) 97

\[
\text{LDL-C} = TC - HDL - \frac{TG}{5} \quad \text{Eq...}(6.4)
\]

\[
\text{VLDL-C} = \frac{TG}{5} \quad \text{Eq...}(6.5)
\]

\[
\text{Atherogenic Index (AI)} = \frac{TC}{HDL - C} \quad \text{Eq...}(6.6)
\]

Briefly, fixed volumes of sample and standard were mixed with the working reagent separately, followed by incubation at 37°C for 10 min. The absorbance of the developed color was read at 505 nm for total Cholesterol and at 546 nm for triglycerides determination.

6.1.12.1.6 Determination of serum total cholesterol

Ready made cholesterol reagent 1, cholesterol reagent 2 and cholesterol standard (200 mg/dl) provided by Merck were used for the determination of serum total cholesterol.
Procedure: In the test tubes, test, standard and blank, add 2.5 mL each cholesterol reagent 1 and in test tube add 0.1 mL of serum, in std. test tube add 0.1 mL of cholesterol standard and in blank tube add 0.1 mL of distilled water. Mix well and cool to room temperature, then add 0.5 mL of cholesterol reagent 2 in test, std. and blank test tubes. Mix thoroughly, keep in a water bath at room temperature for 10 min. Read the absorbance of test and standard against blank at 575 nm using double beam UV visible spectrophotometer. Serum total cholesterol determined by using formula (4).

\[
\text{Serum total cholesterol (mg/dl)} = \frac{O.D_{\text{Test}}}{O.D_{\text{Std}}} \times 200
\]

...(4)

6.1.12.1.7 Determination of serum HDL cholesterol

Ready made cholesterol reagent 1, cholesterol reagent 2 and cholesterol standard (100 mg/dl) provided by Merck were used for the determination of serum total cholesterol. Additional reagents phosphotungstic acid reagent (PTA), magnesium chloride reagent were prepared and used.

Procedure: In a centrifuge tube add 0.5 mL serum, 0.05 mL PTA reagent and 0.02 mL magnesium chloride reagent, mix well, centrifuge at 3000 rpm for 20 min. Separate the supernant and add 0.1 mL in a test tube. In test tubes, test, standard and blank, add 2.5 mL each cholesterol reagent 1, in std. test tube add 0.1 mL of cholesterol standard and in blank tube add 0.1 mL of distilled water. Mix well and keep in water bath at room temperature for min. then add 0.5 mL of cholesterol reagent 2 in test, standard and blank test tubes. Mix thoroughly; keep in the water bath at room temperature. Read the absorbance of test and standard against blank at 520-580 nm using double beam UV visible spectrophotometer. Serum HDL- cholesterol determined by using formula (5).

\[
\text{Serum HDL-cholesterol (mg/dl)} = \frac{O.D_{\text{Test}}}{O.D_{\text{Std}}} \times 100
\]

...(5)

6.1.12.1.8 Determination of serum triglycerides

Ready made cholesterol reagent 1, cholesterol reagent 2 and cholesterol standard (100 mg/dl) provided by Merck were used for the determination of serum total cholesterol. Preparation of working reagent: It is prepared fresh by using two parts of reagent 1 and reagent 2.

Procedure: In the test tubes test, standard and blank, add 1ml each working and in test tube add 0.01 mL of serum, in standard test tube add 0.01 mL of standard and in blank test tube add 0.01 mL of distilled water. Mix well and cool at 37 °C for 1 min. Read absorbance of test and standard against blank at 520 nm using double beam UV visible spectrophotometer. Serum total cholesterol determined by using formula(6).
Experimental | Chapter 6

\[
\text{Serum triglyceride (mg/dl)} = \frac{O.D_{Test}}{O.D_{Std}} \times 100 \quad ...(6)
\]

The results were presented as mean ± standard deviation. Statistical analysis of data was performed by using Student’s unpaired t-test. The differences in lipid profiles were statistically analyzed by using analysis of variance (ANOVA) followed by Dunnets test (significance level \( p < 0.001 \)). All the analysis of data was performed using statistical software package Graph pad prism 5 version.

6.1.12.2 Estimation of Pharmacokinetic parameters of nanoparticle Formulation

Approval of protocol
The protocol no. was same mentioned in part I (6.1.13.)

6.1.12.2.1 Selection of Animals

The albino rabbits of either sex weighing 1.5 to 3.0 kg. were used for estimation of pharmacokinetic parameters of marketed formulation and simvastatin loaded nanoparticles.

6.1.12.2.2 Experimental animals

Nine rabbits were used for the bioavailability study. All the animals were weighed and randomly divided into three groups with three animals in each group. Albino rabbits were grouped as standard I (3), Standard II (3) and Test (3). Simvastatin suspension and simvastatin marketed formulation were administered to standard group. Simvastatin loaded nanoparticles suspension was administered to test group. Animals had free access to food and water ad libitum.

The formulations were provided orally using 23-gauge oral feeding needle. Average weight of tablet of simvastatin providing 10mg dose was found to be 128.15 mg. Hence a fraction of a tablet corresponding to the weight of tablet required to give the desired dose was administered to the rabbits. Simvastatin tablet suspended in purified water and 1 mg equivalent dose was orally administered to RG rabbits. Simvastatin nanoparticles formulation 1 mg equivalent dose was orally administered to TG rabbits. Rabbits were anaesthetized using ether. Blood samples were withdrawn from marginal ear veins of rabbits at 0.5, 1, 2, 4, 8, and 12 h. The plasma was separated and drug content was estimated using HPLC. In this method acetonitrile and double distilled water (pH 3) (80:20) with 0.1% OPA as mobile phase and HPLC (Jasco PU 2080 Pump, UV 2075 Detector).^30

6.1.12.2.3 Processing of Blood samples for HPLC analysis

All frozen plasma samples were thawed at ambient temperature. 200 μl plasma sample was transferred to a 2 ml polypropylene test tube. The tube was vortex
and then liquid extraction was carried out with 1 ml of methyl tert-butyl ether. The tube was vortexing for 30 sec. and centrifuged for 15 min at 4°C at 3000 rpm. The supernatant was separated and transferred to a clean polypropylene test tube and air dried at 40°C. The residue was reconstituted with 100 μl of methanol and filtered through 0.22 μm syringe filter, then 20 μl volume was injected to HPLC. The flow rate was 1 ml/min and UV detection was performed at 238 nm. The retention time and detection of simvastatin was determined. The mean calibration curve was given by equation, \( y = mx + c \), with \( r \) as correlation coefficient, \( y \) represent peak area ratio and \( x \) is the concentration of simvastatin in ng/ml. The data were acquired and calculated on shimadzu controlled using software analyst. Linear regression was used to obtain the best fit of data for the calibration curves.

### 6.1.12.2.4 Estimation of Pharmacokinetic Parameters

A 20 μl of sample solution was injected in the chromatographic system using fixed volume loop injector. The pharmacokinetic parameters for simvastatin pure drug suspension, marketed formulation and nanoparticles dispersion following oral administration were determined from plasma concentration data. The area under the concentration-time curve AUC (0-t) was estimated according to trapezoidal rule. Area under the curve extrapolated to infinity AUC (0-∞) was calculated by formula (7). \(^{131-134}\)

\[
AUC (0 - \infty) = AUC(0-t) + \frac{C_{\text{last}}}{K_e} \quad \text{... (7)}
\]

Where, \( C_{\text{last}} \) and \( K_e \) are the last measurable concentration and the elimination rate constant, respectively.

The Maximum concentration (Cmax), Time required for maximum concentration (Tmax) were determined from plasma concentration time profile. \( K_e \) is the apparent elimination rate constant obtained from the terminal slope of the individual plasma concentration - time curves after logarithmic transformation of the plasma concentration values and application of linear regression. The elimination rate constant was calculated by using formula (8). The terminal half life were calculated from elimination rate constant using formula (9). The Mean residence time was calculated by using formula (10).

\[
K_e = -2.303 \times \text{Slope} \quad \text{... (8)}
\]

\[
t_{1/2} = \frac{0.693}{K_e} \quad \text{... (9)}
\]

\[
MRT = 1.44 \times t_{1/2} \quad \text{... (10)}
\]

The relative bioavailability is determined when there are no i.v. data, by comparing different dosage forms. As with calculation of bioavailability, clearance
is assumed to be constant. The relative bioavailability can be determined from AUC data by using formula (11).

\[
Fr \% = \frac{AUC(0-\infty)_{Formulation}}{AUC(0-\infty)_{Reference}} \times 100
\]  

...(11)

6.1.12.2.5 Statistical analysis

The pharmacokinetic (PK) parameters were performed by non-compartmental analysis. All values are expressed as the mean ± SD. All the analysis of data was performed using statistical software package Graph pad prism 5 version. (GraphPad Software, San Diego, CA, USA), using one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test. Difference between two parameters were considered stastically significant for P<0.001.

6.1.13 Stability Studies

Stability is defined as the ability of a particular drug or dosage form in specific containers to remain with its physical, chemical, therapeutic and toxicological specifications. Stability tests are the series of tests designed to obtained information on the stability of the pharmaceutical product in order to define its shelf life and utilization period under specified packaging and storage conditions. The purpose of stability is to provide information on how the quality of drug product varies with time under the influence of variety of environmental factors such as temperature, humidity and light, to establish a shelf life for the drug product at a recommended storage conditions. Stability studies were carried out according to ICH guidelines Q1A (R2). 135-136

The samples of optimized batch of SV loaded nanoparticles (nanoparticles dispersion and freeze dried nanoparticles) was kept in a refrigerator and programmable environmental chamber for 3 months at 5° ± 3°C and 25° ± 2°C and 60% RH ± 5% RH respectively. The samples were withdrawn at 1, 2 and 3 months from the time of placing samples in chamber, particle size, drug content and % cumulative release was determined.137 The samples of optimized batch of freeze-dried nanoparticles tablet, pure drug tablet and marketed tablet was kept in refrigerator and programmable environmental chamber for 3 months at 5° ± 3°C and 25° ± 2°C and 60% RH ± 5% RH respectively. The samples were withdrawn at 1, 2 and 3 month from time of placing samples in chamber % cumulative release was determined for 60 minutes. 138
6.2  PART-II: PREPARATION OF LOVASTATIN NANOPARTICLES

6.2.1. Drug Identification

Organoleptic property of powder checked out. Lovastatin samples were identified by melting point, UV spectra and FT-IR spectra.\(^{139}\)

6.2.1.2  Melting point

Melting point of lovastatin was determined by open capillary tube method. Detailed procedure given in 6.1.2.

6.2.2  UV-spectroscopic Studies

A stock solution containing 1mg/ml of lovastatin was prepared by dissolving 100 mg of drug in small quantity of methanol and sonicated for 10 minutes and diluted with 100 ml of phosphate buffer (pH 7.4) to obtain stock solutions containing 1000µg/ml in a 100 ml of volumetric flask. Solutions containing 10µg/ml of lovastatin were scanned in the range of 200-400 nm region of PC based Jasco V-63 spectrophotometer.\(^{140}\)

6.2.2.1  Preparation of standard curve

A stock solution containing 1mg/ml of lovastatin was prepared by dissolving 100 mg of drug in small quantity of methanol and sonicated for few minutes and diluted with 100 ml of phosphate buffer (pH 7.4). The stock solution was serially diluted to obtain solution of 5, 10, 15, 20, 25, 30, 35, 40 µg/ml concentrations. The absorbance of the solutions was measured in a UV-Visible spectrophotometer at \(\lambda_{\text{max}}\) 243 nm. A calibration curve was plotted by taking concentration of solution in \(\text{x axis}\) and absorbance in \(\text{y axis}\) and correlation coefficient ‘\(r\)’ was calculated from equation of straight line i.e. \(y = mx + c\).

6.2.3  High Performance Liquid Chromatography

6.2.3.1  Selection of Chromatographic Conditions

The selection of HPLC method depends upon the nature of the sample, its molecular weight and solubility. The chromatographic variables such as mobile phase, flow rate and solvent ratios were studied. The resulting chromatograms were recorded and the chromatographic parameters such as capacity factor, asymmetric factor, and peak resolution and column efficiency were calculated. The condition that gave the best resolution, symmetry and capacity factor was selected for estimation. The High Performance Liquid Chromatography separation conditions given in Table 6.2.1.
Table 6.2.1: Separation conditions

<table>
<thead>
<tr>
<th>Chromatographic Mode</th>
<th>Chromatographic Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard Solution</td>
<td>1000 μg/ml of lovastatin in mobile phase</td>
</tr>
<tr>
<td>Stationary Phase</td>
<td>HIQ Sil C\textsubscript{18} column (250 mm x 4.6 mm i.d., 5µm)</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>ACN: Water (80:20 v/v) with 0.1% OPA</td>
</tr>
<tr>
<td>Detection Wavelength</td>
<td>238 nm</td>
</tr>
<tr>
<td>Flow Rate</td>
<td>1 ml/min</td>
</tr>
<tr>
<td>Sample Size</td>
<td>20 µl</td>
</tr>
</tbody>
</table>

6.2.3.2 Optimization of mobile phase strength

For selection of mobile phase, various mobile phase compositions containing acetonitrile: water in different ratios was tried but the resolution was not found to be satisfactory. Mobile phase was selected based on literature reviewed as well as on the basis of best separation of lovastatin, sharp peaks and high number of theoretical plates. The mobile phase included acetonitrile and double distilled water (pH 3) (80:20) with 0.1% O-phosphoric acid was found to give good resolution, effectively separating lovastatin.

6.2.3.3 Optimization of detection wavelength

The sensitivity of HPLC method that uses UV detection depends upon proper selection of detection wavelength. An ideal wavelength is one that gives good response for the analyte to be detected. In the present study drug solution of 10μg/ml of lovastatin prepared in mobile phase. After observing UV spectra of drug, wavelength of 238 nm was selected for further study.

6.2.3.4 System Suitability Parameters

System suitability parameters were analyzed on freshly prepared standard stock solutions of lovastatin. All these analytes were injected into the chromatographic system under the optimized chromatographic conditions. Parameters that were studied to evaluate the suitability of the system were number of theoretical plates, calibration curve, capacity factor, resolution factor, retention time.

6.2.3.5 Preparation of Standard Drug Solution

Standard stock solution containing lovastatin was prepared by dissolving 50 mg of lovastatin in 20 ml of mobile phase separately. The solution was sonicated for 10 minutes. The final volume of the solution was made to 50 ml with mobile phase 1000μg/ml. Accurately measured 0.05, 0.1, 0.15, 0.2, 0.3 ml of stock solution were transferred to 10ml volumetric flask and volume was made to 10 ml using
mobile phase concentration of 5, 10, 15, 20, and 25\(\mu\)g/ml. Absorbance of their solution were measured using HPLC (Jasco PU 2080 Pump, UV 2075 Detector) using acetonitrile and double distilled water (pH 3) (80:20) with 0.1% O-phosphoric acid as mobile phase at \(\lambda\) max 238 nm.

### 6.2.4 Compatibility Studies

Compatibility of the lovastatin with chitosan used to formulate nanoparticles was established from FTIR spectrum and DSC thermogram analysis. FTIR & DSC spectral analysis of lovastatin and combination of lovastatin and chitosan was carried out to investigate the changes in chemical composition of the drug after combining it with the excipients.

### 6.2.5 Saturation Solubility Studies

Accurately weighed 10mg of lovastatin and the nanoparticles equivalent to 10 mg of the drug were separately introduced into 25ml stoppered conical flasks containing 10ml phosphate buffer (pH 7.4). The sealed flasks were agitated on a rotary shaker for 24 h at 37°C and equilibrated for 2 days. An aliquot was passed through 0.45 µm membrane filter and the filtrate was suitably diluted and analyzed for drug content on a UV spectrophotometer at 243 nm wavelength.

### 6.2.6 Experimental Design

The formulations were fabricated according to a 3\(^2\) full factorial design, allowing the simultaneous evaluation of two formulation variables and their interaction.

**Table 6.2.2: Experimental design and Parameters for 3\(^2\) Full Factorial Design Batches**

<table>
<thead>
<tr>
<th>Batch code</th>
<th>Variables level in coded Form</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Drug:Polymer ratios</td>
</tr>
<tr>
<td></td>
<td>((X_1))</td>
</tr>
<tr>
<td>CL1</td>
<td>-1</td>
</tr>
<tr>
<td>CL2</td>
<td>-1</td>
</tr>
<tr>
<td>CL3</td>
<td>-1</td>
</tr>
<tr>
<td>CL4</td>
<td>0</td>
</tr>
<tr>
<td>CL5</td>
<td>0</td>
</tr>
<tr>
<td>CL6</td>
<td>0</td>
</tr>
<tr>
<td>CL7</td>
<td>+1</td>
</tr>
</tbody>
</table>
Chapter 6

Formulation and Evaluation of Nanoparticles For Better Drug Bioavailability

The experimental designs with corresponding formulations are shown in Table 6.2.2 and 6.2.3. The dependent variables were selected for study particle size \( Y_1 \) and % drug entrapment \( Y_2 \).

### 6.2.6.1 Statistical analysis

The results from factorial design were evaluated using PCP Disso 2000 V3 software. The linear regression analysis was used to develop polynomial equation shown in eq. (6.1), details given in 6.1.6.1.

### 6.2.7 Selection Process for Preparation of Nanoparticles

The objective of the present study was to evaluate the possibility of chitosan nanoparticles as carriers for lovastatin. The challenge was to entrap a hydrophobic molecule into hydrophilic nanoparticles formed by ionotropic pre-gelation of the positively charged polysaccharide chitosan. On the basis of previous work, chitosan nanoparticles were prepared by ionotropic pre-gelation of chitosan polyelectrolyte complexation. Blank nanoparticles were produced using chitosan as a polymer and STPP as a cross linking agent. The particle size was analysed by Malvern particle size analyzer. Based on particle size data obtained, appropriate process for preparation of nanoparticles was selected.

### 6.2.8 Preparation of Chitosan Nanoparticles

Chitosan nanoparticles containing lovastatin were prepared by ionotropic gelation method. Chitosan was dissolved in 1% acetic acid solutions at various concentrations to obtain (0.1%, 0.2% & 0.3% i.e 40mg, 80mg & 120mg) and adjusted the pH 5-6 with 0.1N sodium hydroxide solution, while STPP was dissolved in deionized water at various concentrations to obtain 0.1%, 0.15% and 0.20%. Lovastatin 40 mg was dissolved in ethanol/ water mixture (1:1) to obtain clear solution. Lovastatin solution was added dropwise with syringe needle size 0.45 mm to 40ml chitosan solution. The 20ml of STTP solution was added dropwise 0.75ml/min. under stirring (1000 rpm) at ambient temperature. The formulation was stirred for 30 minutes so as to remove ethanol content. All the

<table>
<thead>
<tr>
<th>Coded Levels</th>
<th>+1</th>
<th>0</th>
<th>-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug: Polymer ratios ( X_1 ) in mg</td>
<td>1:3 (120)</td>
<td>1:2 (80)</td>
<td>1:1 (40)</td>
</tr>
<tr>
<td>STTP ( X_2 ) in %</td>
<td>0.2</td>
<td>0.15</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Table 6.2.3: Translation of coded levels to actual quantities
formulation was sonicated at fixed time for 30 minutes. All experiments were performed in triplicates. Nanoparticles were collected by centrifugation at 15,000 rpm for a period of 1 h and supernant were discarded. The resultant dispersion was dried using a freeze-drying method.141-144

6.2.9 Freeze Drying of Nanoparticles

Briefly, by taking 5ml of nanoparticles dispersion was filled in 10 ml glass vials, covered with special stoppers for lyophilization and placed in a freeze dryer (Martin Christ ALPHA 1-2 LD plus). After freeze drying all sample vials were stored at 2-8 °C, details given in 6.1.9.

6.2.10 Characterization of Nanoparticles

6.2.10.1 Determination of particle size

The particle size and size distribution of the lovastatin loaded chitosan nanoparticles were characterized by photon correlation spectroscopy (PCS) using a Zetasizer 2000 Malvern Instruments, UK and N5 Beckman submicron zeta analyzer at a scattering angle of 90ºC, details given in 6.1.10.1

6.2.10.2 Polydispersity Index

Polydispersity index indicates the distribution of individual molecular masses in a batch of polymers. The PI calculated with particle size data by formula (1), details given in 6.1.10.2.

6.2.10.3 Determination of entrapment efficiency

The encapsulation efficiency of nanoparticles was determined by first separating the nanoparticles formed from the aqueous medium by centrifugation at 15000 rpm for 1h. The amount of free lovastatin in the supernant was measured by UV spectrophotometery at 243 nm (Shimadzu UV-1700,) after suitable dilution. The percentage encapsulation efficiency of nanoparticles was calculated using formula (2), details given in 6.1.10.3.

6.2.10.4 Determination of zeta potential

The zeta potential of the drug-loaded chitosan nanoparticles was measured on a zetasizer (Malvern Instruments UK and N5 Beckman submicron zeta analyzer), details given in 6.1.10.4.

6.2.10.5 Percent process Yield

Percent process yield was calculated as the weight of the lyophilized nanoparticle by equation (6.3), detailed given in 6.1.10.5.
6.2.10.6 Percent drug content

The lyophilized nanoparticle powder was dissolved in 1 ml methanol and volume was made up to mark of 10 ml volumetric flask with phosphate buffer pH 7.4, 0.1ml of above solution was further diluted to 10 ml and analyzed by spectrophotometrically at 243 nm. The calculations were done by using eq. no.(6.2)\textsuperscript{145} details given in 6.1.10.6.

6.2.10.7 In vitro drug release study

In-vitro drug release studies were performed in USP Type II dissolution apparatus at rotation speed of 50 rpm. The lovastatin loaded chitosan nanoparticles, after separation by centrifugation, were re-dispersed in 5mL phosphate buffer solution pH 7.4, placed in a dialysis membrane bag, tied and immersed in 900mL of phosphate buffer solution in a vessel and temperature was maintained at 37±0.20°C. The sample weight of formulations equivalent to 10 mg of lovastatin was used for dissolution study. The required quantity 5ml of the medium was withdrawn at specific time periods (5, 10, 20, 30, 60 min.) and the same volume of dissolution medium was replaced in the flask to maintain a constant volume. The withdrawn samples were filtered through a filter paper (0.22 μm, Whatman Inc., USA) and 5 ml filtrate was made up to volume with 100 ml of Phosphate buffer pH 7.4. The samples were analyzed for drug release by measuring the absorbance at 241 nm using UV-visible spectrophotometer and calculate percent cumulative release of lovastatin.\textsuperscript{97}

6.2.10.8 Fourier Transform Infrared Spectroscopy study

Infrared spectrum of lovastatin, nanoparticle formulation was obtained by using Fourier Transform Infrared Spectrophotometer (FTIR-4100, Shimadzu) using KBr dispersion method, procedure given in 6.1.10.8.

6.2.10.9 Differential scanning calorimetry study

DSC measurements were carried out on a modulated DSC Instrument: SDT Q600 V20.9 Build 20 equipped with a thermal analysis data system (TA instrument). DSC curve of Chitosan, lovastatin, Physical mixture and various batches of NPs using for calculation of degree of crystallinity, melting enthalpy and melting peak.\textsuperscript{146} The procedure is given in 6.1.10.9.

6.2.10.10 X-ray Diffraction Study

X-ray diffraction analysis was conducted using a Philips PW 3710 x-ray diffractometer (XRD) with a copper target and nickel filter (Philips Electronic Inst, Holland). X-ray diffraction pattern of lovastatin, Physical mixture and CL4 batch was obtained and calculate peak intensity using 2 θ value by spekwin 32 v 1.71.6. The procedure is given in 6.1.10.10.
6.2.10.11 Scanning electron microscopy study

The morphology of nanoparticles was examined by using scanning electron microscopy (SEM, JSM-6360LV scanning microscope Tokyo, Japan). The detailed procedure is given in 6.1.10.11.

6.2.10.12 Transmission electron microscopy study

The morphology of nanoparticles was observed by Transmission electron microscopy instrument (TEM, Tecknai G², 20 U-Twin, FEI, Nederland) operated at 200 kV and images were viewed. The images were recorded with a 1 k CCD camera. The detailed procedure is given in 6.1.10.12.

6.2.11 Preparation of Lovastatin Tablets

The lyophilized product equivalent to 10mg of lovastatin was weighed and mixed with 75mg of Lactose monohydrate, 85mg of Hydroxy propyl methyl cellulose and 10mg of sodium starch glycolate was added and triturated carefully in mortar and pestle. The tablet was prepared by direct compression method using 8 mm punches on a rotary tablet machine. Composition of the tablet of lovastatin is given in table 6.2.4.

Table 6.2.4: Composition of 10 mg lovastatin tablet

<table>
<thead>
<tr>
<th>Tablet Composition</th>
<th>Freeze dried product</th>
<th>Lactose monohydrate</th>
<th>Hydroxypropyl methyl cellulose</th>
<th>Sodium starch glycolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight ( mg)</td>
<td>34</td>
<td>75</td>
<td>85</td>
<td>10</td>
</tr>
<tr>
<td>Percentage</td>
<td>16.66</td>
<td>36.76</td>
<td>41.66</td>
<td>4.9</td>
</tr>
</tbody>
</table>

6.2.11.1 In-vitro release studies of tablet

The in-vitro dissolution study was carried out using USP type II dissolution apparatus at rotation speed at 50 rpm. The study was carried out in 900ml of phosphate buffer (pH 7.4) for 60 minute. The dissolution medium was kept in thermostatically controlled water bath, maintained at 37± 0.5°C. At different time intervals, 5ml sample was withdrawn and analyzed spectrophotometrically at 243 nm wavelength for the drug release. At each time of withdrawal dissolution media was replaced with fresh medium. Dissolution studies were conducted for pure drug of tablet and tablets prepared with nanoparticles and results compared.
6.2.12 In vivo evaluation of lovastatin nanoparticle formulation

6.2.12.1 Antihyperlipidemic activity of LV-loaded chitosan nanoparticles was studied

6.2.12.1.1 Approval of protocol is same as mentioned in part-I (6.1.12.1.)
6.2.12.1.2 Selection of Animals: same as given in 6.1.12.1.2
6.2.12.1.3 Experimental animals: same as given in 6.1.12.1.3
6.2.12.1.4 Collection of Blood samples: same as given in 6.1.12.1.4.
6.2.12.1.5 Biochemical analysis: same as given in 6.1.12.1.5
6.2.12.2. Estimation of Pharmacokinetic parameters of nanoparticle formulation
6.2.12.2.1 Approval of protocol no. is same as mentioned in part-I (6.1.12.)
6.2.12.2.2 Selection of Animals: same as given in 6.1.12
6.2.12.2.3 Experimental animals: same as given in 6.1.12

Average weight of tablet of LV providing 10 mg dose was found to be 130.3 mg. Hence a fraction of the tablet corresponding to the weight of the tablet required to give the desired dose was administered to rabbits. Lovastatin tablet suspended in purified water and 1 mg equivalent dose was orally administered to RG rabbits. Lovastatin nanoparticles formulation 1 mg equivalent dose was orally administered to TG rabbits. Rabbits were anaesthetized using ether. Blood samples were withdrawn from marginal ear vein of rabbits at 0.5, 1, 2, 4, 8, and 12h. The plasma was separated and drug content was estimated using HPLC. In this method acetonitrile and double distilled water (pH 3) (80:20) with 0.1% OPA as mobile phase and RP-HPLC (Jasco PU 2080 Pump, UV 2075 Detector).

6.2.12.2.4 Processing of Blood samples for HPLC analysis: same as given in 6.1.12
6.2.12.2.5 Estimation of Pharmacokinetic Parameters: same as given in 6.1.12
6.2.12.2.6 Statistical analysis: same as given in 6.1.13

6.2.13 Stability studies

Stability studies were carried out according to ICH guidelines Q1A (R2). In this study, The samples of optimized batch of LV loaded nanoparticles (nanoparticles dispersion and freeze dried nanoparticles) were kept in a refrigerator and programmable environmental chamber for 3 months at 5° ± 3°C and 25° ± 2°C and 60% RH ± 5% RH respectively. The samples were withdrawn at 1, 2 and 3 month from time of placing samples in to chamber particle size, drug content and % cumulative release was determined. The samples of optimized batch of freeze dried nanoparticles tablet, pure drug tablet and marketed tablet was kept in the refrigerator and programmable environmental chamber for 3 months at 5° ± 3°C and 25° ± 2°C and 60% RH ± 5% RH respectively. The samples were withdrawn at 1, 2 and 3 month from time of placing samples in to chamber % cumulative release was determined for 60 minutes.