Formulation and Evaluation of Nanosuspension Formulation for Drug Delivery of Simvastatin

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ABSTRACT
Poor water solubility and slow dissolution rate are issues for the majority of upcoming and existing biologically active compounds. Simvastatin is poorly water-soluble drug and its bioavailability is very low from its crystalline form. The purpose of the present investigation was to increase the solubility and dissolution rate of simvastatin by the preparation of nanosuspension by Emulsification Solvent Diffusion Method at laboratory scale. Prepared nanosuspension was evaluated for its particle size and in vitro dissolution study and characterized by zeta potential, differential scanning calorimetry (DSC) and X-Ray diffractometry (XRD), Motic digital microscopy, entrapment efficiency, total drug content, saturated solubility study and in vivo study. A 2³ factorial design was employed to study the effect of independent variables, amount of SLS (X1), amount of PVPK-30 (X2) and Poloxamer-188 (X3) and dependent variables are Total drug content and polydispersity index. The obtained results showed that particle size (nm) and rate of dissolution has been improved when nanosuspension prepared with the higher concentration of PVPK-30 and Poloxamer-188 and lower concentration of SLS. The particle size and zeta potential of optimized formulation was found to be 258.3 nm and 23.43. The rate of dissolution of the optimized nanosuspension was enhanced (90.02% in 60min), relative to plain simvastatin (21% in 60 min), mainly due to the formation of nanosized particles. These results indicate the suitability of 2³ factorial design for preparation of simvastatin loaded nanosuspension significantly improved in vitro dissolution rate, and thus possibly enhance fast onset of therapeutic drug effect. In vivo study shows increase in bioavailability in nanosuspension formulation than the plain simvastatin drug.

KEYWORDS: Simvastatin; Nanosuspension; Emulsification Solvent Diffusion; Dissolution; Particle Size; Factorial design.

Introduction
It is estimated that more than 1/3 of the compounds being developed by the pharmaceutical industry are poorly water soluble. An important property of a drug substance is solubility, especially aqueous system solubility (Katteboinaa et al., 2009). The solubility/dissolution behavior of a drug is key factor to its oral bioavailability. The bioavailability of these drugs is limited by their low dissolution rates. An improvement of oral bioavailability of poor water-soluble drugs remains one of the most challenging tasks of drug development. To overcome poor solubility, many approaches have been studied. They are generally salt formation, use of surfactant, use of prodrugs and micronization. In micronization, the particle size of a drug powder is reduced to a micron scale size (typically 2-10 micron), which increases the specific surface area and dissolution rates. However, many new drugs are so poorly soluble that micronization is not sufficient, which motivated the development of nanoscale systems. By decreasing the particle size from a micron to a nanometer scale, there is a significant increase in the surface area and related dissolution rate. Nanosuspensions are sub-micron colloidal dispersions of pure drug particles in an outer liquid phase. Nanoparticle engineering enables poorly soluble drugs to be formulated as nanosuspensions alone, or with a combination of pharmaceutical excipients.

Nanosuspension engineering processes currently used are precipitation (Patel et al., 2011), high pressure Homogenization (Kumar et al., 2011) and pearl milling (Wagh et al., 2011), either in water or in mixtures of water and water-miscible liquids or non-aqueous media. (Nagare et al., 2012), precipitation method presents numerous advantages, in that it is a straightforward technique, rapid and easy to perform. In this method, the drug is dissolved in an organic solvent such as acetone, acetonitrile, methanol or ethyl acetate. The organic solvent is evaporated either by reducing the pressure or

ABBREVIATIONS: PCS - Photon Correlation Spectroscopy; PI - Polydispersity Index; ZP - Zeta Potential; SDP - Spray Drying Process; DSC - Differential Scanning Calorimetry; XRD - X-ray Diffraction; DLS - Dynamic Light Scattering; FT-IR - Fourier Transform Infrared Spectroscopy; PD - Plain Drug Suspension; SIM - Simvastatin; SNS - Simvastatin Nanosuspension; SLS - Sodium Lauryl Sulphate; PVP K-30-Polyvinyl Pyrolidine k-30; Polo-188 - Poloxamer-188
by continuous stirring. Particle size was found to be influenced by the type of stabilizer, concentrations of stabilizer, and homogenizer speed. In order to produce small particle size, often a high-speed homogenization or ultrasonication may be employed. The super-saturation is further accentuated by evaporation of drug solvent. This yields to the precipitation of the drug. High shear force prevents nucleus growth and Oswald's ripening.

Simvastatin (SIM) is a lipid lowering agent derived synthetically from a fermentation product of Aspergillus terreus. After oral ingestion, SS, an inactive lactone, is hydrolyzed to the corresponding β-hydroxyacid form. This is a principal metabolite and an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme-A (HMG Co-A) reductase, the enzyme that catalyses an early and rate-limiting step in the biosynthesis of cholesterol (Patil et al., 2011). Simvastatin is a white, crystalline, non-hygroscopic powder, insoluble in water and 0.1N HCl (30 µg/ml and 60 µg/ml, respectively). It is generally considered that compounds with very low aqueous solubility will show dissolution rate-limited absorption. Improvement of aqueous solubility in such case is a valuable goal to improve therapeutic efficacy. The dissolution rate is a function of the solubility and the surface area of the drug, thus, dissolution rate will increase if the solubility of the drug is increased, and it will also increase with an increase in the surface area of the drug. In this present study, nanoprecipitation technique is used where a drug solution in a water miscible organic solvent is mixed with an aqueous solution containing a surfactant(s). Upon mixing, the supersaturated solution leads to nucleation and growth of drug particles, which may be stabilized by surfactants.

The aim of this work is to optimize and characterize the formulation prepared by Emulsification Solvent-Diffusion method for the preparation of nanosuspensions in order to identify formulation parameters. A 2^3 factorial design was applied to investigate the combined effect of 3 formulation variables i.e. amount of SLS (X1), PVPK-30 (X2) and Poloxamer-188 (X3). The Total Drug Content (% Y1), and Polydispersity Index (Y2) were taken as responses. Characterization of optimized nanoparticles was carried out by X-Ray Diffractometry (XRD), Differential Scanning Calorimetry (DSC), Photon Correlation Spectroscopy, Motic Digital Microscopy and Fourier Transform Infrared Spectroscopy (FT-IR). Dissolution study of nanosuspension formulations was performed in distilled water, pH 1.2 and 7.4 buffer and was compared to the plain simvastatin drug. Pharmaco-dynamic study was performed.

**Materials and Methods**

**Chemicals and drug**

Simvastatin was obtained from Dr. Reddy Laboratories, Ltd., Hyderabad, India, Poloxamer-188 was obtained from Alkem Pharmaceutical Ltd., Mumbai, India and polyvinyl pyrrolidone (PVPK-30) was obtained from Research lab Fine Chem. Industries, Mumbai, India, sodium lauryl sulphate (SLS) was obtained from Loba Chemie Pvt. Ltd., Mumbai, India, Ethyl Acetate was obtained from Analab Fine Chemicals, Mumbai, India Bidistilled water was prepared in laboratory for study. All materials used in study conformed to USP-24 standards.

**Melting point**

Melting point was measured with the use of Thiele’s Tube apparatus by using paraffin oil, thermometer, thread and burner. The capillary is tight with thermometer and placed in Thiele’s tube containing paraffin oil, the tube is heated by using burner. The range of temperature when drug just start melting and till it completely melts was noted.

**Calibration plot in methanol**

Accurately weighed 20 mg of Simvastatin was transferred in 25 ml of volumetric flask, dissolved in methanol and volume was made with methanol to obtain stock solution (1000 µg/ml). From this stock pipette out 10 ml solution and diluted up to 100 ml by using methanol to obtain stock solution (100 µg/ml). This stock was suitably diluted with methanol to obtain concentration in the range of 2-16 µg/ml and absorbance was recorded at 238 nm using UV-Spectrophotometer. The linear correlation was obtained between absorbance and concentration. The data was statistically treated using the least square regression method.

**Calibration plot in water**

Accurately weighed 20 mg of Simvastatin was transferred in 25 ml of volumetric flask, dissolved in methanol and volume was made with distilled water to obtain stock solution (1000 µg/ml). From this stock pipette out 10 ml solution and diluted up to 100 ml by using methanol to obtain stock solution (100 µg/ml). This stock was suitably diluted with distilled water to obtain concentration in the range of 2-20 µg/ml and absorbance was recorded at 238 nm using UV-Spectro-photometer. The linear correlation was obtained between absorbance and concentration. The data was statistically treated using the least square regression method.

**Calibration plot in pH 7.4 phosphate buffer**

Accurately weighed 20 mg of Simvastatin was transferred in 25 ml of volumetric flask, dissolved in methanol and volume was made with pH 7.4 Phosphate buffer to obtain stock solution (1000 µg/ml). From this stock pipette out 10 ml solution and diluted up to 100 ml by using methanol to obtain stock solution (100 µg/ml). This stock was suitably diluted with pH 7.4 Phosphate buffer to obtain concentration in the range of 2-16 µg/ml and absorbance was recorded at 238 nm using UV-Spectrophotometer. The linear correlation was obtained between absorbance and concentration. The data was statistically treated using the least square regression method.

**Calibration plot in pH 1.2 acidic buffer**

Accurately weighed 20 mg of Simvastatin was transferred in 25 ml of volumetric flask, dissolved in methanol and volume was made with pH 1.2 Acidic
buffer to obtain stock solution (1000 µg/ml). From this stock solution, 10 ml solution and diluted up to 100 ml by using methanol to obtain stock solution (100 µg/ml). This stock was suitably diluted with pH 1.2 Acidic buffer to obtain concentration in the range of 2-18 µg/ml and absorbance was recorded at 238 nm using UV-Spectrophotometer. The linear correlation was obtained between absorbance and concentration. The data was statistically treated using the least square regression method.

**FTIR spectroscopic analysis**

Fourier–transform infrared (FT-IR) spectra of moisture free powdered samples of SS, SLS, PVPK-30, Poloxamer-188 and physical mixture were obtained using a spectrophotometer (FTIR-Shimadzu, India) by potassium bromide (KBr) pellet method. The scanning range was 750 - 4000 cm⁻¹ and the resolution was 1 cm⁻¹.

**Preparation of simvastatin nanosuspensions by emulsification solvent diffusion**

Nanosuspensions were prepared by the solvent evaporation technique. Simvastatin (20 mg) was dissolved in an 10 ml Ethyl Acetate (organic phase) at room temperature. This was poured into 40 ml of water containing different amount of SLS, PVPK-30 and Poloxamer-188 maintained at room temperature continuously stirring at 7500 rpm for 6-7 min by using Ultra Turrax stirrer. The addition of organic solvent was done with the help of syringe positioned with a needle directly into the aqueous surfactant (at a rate of 0.5 ml/min). The solution was stirred for 5-6 min at 20,000-25,000 rpm (T-10 basic Ultra Turrax) and then was subjected to the sonication for 10 min (By using probe sonicator). The suspension was then diluted with 60 ml of double distilled water and then stirred for 1 hr to induce diffusion of organic solvent into the continuous phase. The prepared nanosuspension was left stirring at room temperature to evaporate the organic solvent.

**Particle size, zeta potential and its morphology**

Particle size and zeta potential was determined by photon correlation spectroscopy (PCS) using a Beckman Coulter. This analysis yields the mean diameter. All the data presented are the mean values of three independent samples produced under identical production conditions. Particle morphology was examined by Motic Digital Microscopy.

**Entrapment efficiency**

The method is suitable for determining entrapment efficiency of nanosuspension when fairly high concentration of free drug is present in the supernatant after centrifugation. 10 ml portion of the freshly prepared cooled nanosuspension was centrifuged at 10,000 rpm for 10 minutes using microcentrifuge. The supernatant was removed and the amount of unincorporated drug was measured by taking the absorbance of supernatant solution at 238 nm by using UV spectrophotometer. Entrapment Efficiency was calculated by using formula

\[
\text{Entrapment Efficiency} = \frac{(W_{\text{initial drug}} - W_{\text{free drug}})}{W_{\text{initial drug}}} \times 100
\]

**Total drug content**

An aliquot (0.5 ml) of the prepared nanosuspension was evaporated to dryness. The residue was dissolved in methanol and filtered with a 0.45 µm filter. Total drug content was determined by UV spectrophotometer at λmax 238 nm.

**Formula:**

Total Drug Content = (Total volume of nanosuspension \times amount of drug in aliquot) / Volume of aliquot

**In vitro dissolution profile**

In vitro dissolution study of plain simvastatin drug, SNS (Simvastatin Nanosuspension), Physical Mixture and Spray dried powder of Optimized Nanosuspension was carried out using USP Dissolution apparatus type II. Dissolution studies were carried out using different dissolution medium and condition given in Table 1. 10 ml of samples were withdrawn at regular time interval of 10, 20, 30, 40, 50 and 60 minutes, samples were replaced by equivalent volume of fresh dissolution media. The samples were analyzed using UV-Spectrophotometer at 238 nm, for determining of drug release.

**Spray drying of Nanosuspension**

In general, spray drying was employed to obtain nano size powder. An optimized batch of aqueous nanosuspension was transferred into nano size powder by a Lab spray dryer LU-222 advanced (twin cyclone) labultima. Spray-dried powder were directly collected after the process. In this process, the spray dryer was set to the conditions given in Table 2.

**Determination of saturated solubility**

Excess amount of plain simvastatin drug and spray dried powder of optimized nanosuspension batch are added to 40 ml of distilled water in 250 ml volumetric flask and then kept on rotary shaker for 48 hours at room
temperature. After equilibrium (48 hr) the suspensions were filtered through membrane filter paper (0.45 µm) and analyzed by UV-Spectrophotometer at 238 nm.

**Differential Scanning Calorimetry (DSC) analysis**

DSC scans of the prepared spray dried powdered drug sample, pure drug and physical mixture were recorded using DSC-Shimadzu 60 with TDA trend line software. All samples were weighed (8-10 mg) and heated at a scanning rate of 10 °C/min under dry nitrogen flow (100 ml/min) between 50 and 300 °C. Aluminum pans and lids were used for all samples. Pure water and indium were used to calibrate the DSC temperature scale and enthalpy response.

**X-Ray diffractometer**

Plain Simvastatin drug and Spray dried powder of optimized batch of nanosuspension were subjected to XRD studies using an automatic X-Ray diffractometer model PW 1710.

**In Vivo Study**

**Materials and Methods**

**Animals:** Healthy albino rats of both sexes weighing from 100-250 gm were purchased from local supplier of Pune University. The animals were provided standard diet and water ad libitum. During the overall period, a hygienic condition with no physical stress was provided to study subjects.

**Drug and chemicals:** Simvastatin was provided by Dr. Reddy’s Pharmaceutical (Pvt.) Ltd., India and used as Standard at a dose of 20 mg/kg. Triton (X-100). Non-ionic detergent triton X-100 (Iso octyl polyoxy ethylene phenol, formaldehyde) was obtained from the Analab Chemicals, Mumbai (India) and experimentally used as hyperlipemia inducing agent. Freshly prepared doses of Triton intraperitoneally in a dose of 400 mg/kg were used in this study. Carboxy Methyl Cellulose (CMC) and its 0.5% concentration in distilled water were used as vehicle for injecting triton (i.p) and administrating the doses of Simvastatin and Simvastatin nanocrystal in experimental test rats.

**Experimental Procedures:** An overnight fasted twenty four experimental rats were divided into four groups viz., Normal, control, triton-induced hyperlipidemic standard (plain simvastatin drug) and test (Simvastatin Nanosuspension powder) group. Each group individually contained six (06) rats. The Normal group was treated orally with distilled water. The control group was treated with 400 mg/kg Triton-X 100, Standard group was treated with simvastatin (20 mg/kg) after 72 hr of i.p. triton treatment. Test group rats were treated orally with Simvastatin Nanosuspension powder (equivalent to 20 mg/kg of simvastatin drug) after the 72 hr of i.p. dose of triton. After 24 hours of respective treatments, collected blood from retro orbital of each group to separate serum to analyze biochemical parameters by using Erba Chem 5 X (Biochemical Analyzer).

**Biochemical analysis:** Lipid profile includes serum Total Cholesterol (TC), Triglyceride (TG), High Density Lipoprotein cholesterol (HDL-c), LDL-c; VLDL was determined by the commercially available Kits (pathoynme diagnostics).

LDL-c was calculated by formula given in reagent kit (pathoynme diagnostics) as:

\[
LDL-c = Total\ cholesterol -(HDLcholesterol-VLDL)
\]

VLDL-c was calculated by formula given in reagent kit (pathoynme diagnostics) as:

\[
VLDL = Triglyceride/5
\]

**Statistical analysis:** Results are expressed as mean ± SEM. Comparison between the groups was made by one way analysis of variance (ANOVA) followed by Tukey’s test.

**Stability study:** For the stability study of optimized batch nanosuspension were stored at 40 °C ± 2 °C and 75% ± 5% RH for 3 months to access their stability. The protocol of stability studies were compliance with WHO guidelines for stability testing. After 30 days, samples were withdrawn and rested for Entrainment efficiency and Total drug content.

**Optimization of formulation using 2³ factorial design:**

2³ factorial design is one of the tools to study the effect of different variables on the quality determinant parameters of any formulation. Based on the principle of design of experiments, this design was employed to investigate the effect of three independent factors. A 2³ factorial design for three factors at two levels each was selected to optimize the varied response variables. The three factors, amount of amount of SLS (X1), PVP K-30 (X2) and Poloxamer-188 (X3) were varied and the factor levels were suitably coded. The Total Drug Content (%), and Polydispersity Index (Y2) were taken as responses variables. In this design, 3 factors are evaluated, each at 2 levels. Experimental trials were performed at all (B1-B8) possible combinations. All other formulation variables and processing variables were kept invariant throughout the study.

**TABLE 3**

<table>
<thead>
<tr>
<th>Variable Level</th>
<th>-1 (Low)</th>
<th>+1 (High)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLS (mg) (X1)</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>PVP K-30 (mg) (X2)</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>Polo-188 (mg) (X3)</td>
<td>15</td>
<td>30</td>
</tr>
</tbody>
</table>

SLS-sodium lauryl sulphate, PVPK-30-polyvinyl pyrolidine k-30, Polo-188-poloxamer-188

**TABLE 4**

Formulation of Simvastatin Nanosuspension using 2³ factorial design

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>B1</th>
<th>B2</th>
<th>B3</th>
<th>B4</th>
<th>B5</th>
<th>B6</th>
<th>B7</th>
<th>B8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simvastatin (mg)</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>SLS (mg)</td>
<td>4</td>
<td>8</td>
<td>4</td>
<td>8</td>
<td>4</td>
<td>8</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>PVP K-30 (mg)</td>
<td>25</td>
<td>25</td>
<td>50</td>
<td>50</td>
<td>25</td>
<td>25</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Polo.-188 (mg)</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Ethyl Acetate (ml)</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Distilled Water (ml)</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
</tbody>
</table>

SLS-sodium lauryl sulphate, PVP K-30-polyvinyl pyrolidine k-30, Polo-188-poloxamer-188.
Results and Discussion

Melting point

Melting point was measured with the use of Thiele’s Tube apparatus by using paraffin oil, thermometer, thread and burner. The melting point of plain simvastatin drug was found to be 139 °C.

Calibration curves in different solvents

**Calibration plot in methanol:** Simvastatin in methanol showed absorption maximum at 238 nm and this wavelength was chosen as the analytical wavelength. Beer’s law was obeyed between 2 and 16 μg/ml. Regression analysis was performed on the experimental data. Regression equation for standard curve was $y = 0.068x$. Correlation coefficient for developed method was found to be 0.985 signifying that a linear relationship existed between absorbance and concentration of the drug. The interference studies with formulation excipients studies were carried out and no difference in absorbance was observed at 238 nm. The calibration plot in methanol was shown in Fig.1.(A)

**Calibration plot in water:** Simvastatin in water showed absorption maximum at 238 nm and this wavelength was chosen as the analytical wavelength. Beer’s law was obeyed between 2 and 20 μg/ml. Regression analysis was performed on the experimental data. Regression equation for standard curve was $y = 0.054x$. Correlation coefficient for developed method was found to be 0.999 signifying that a linear relationship existed between absorbance and concentration of the drug. The calibration plot in water was shown in Fig.1.(B)

**Calibration plot in pH 7.4 phosphate buffer:** A characteristic spectrum was obtained for Simvastatin in pH 7.4 Phosphate buffer when scanned in the ultraviolet range between 200 and 400 nm. The scan showed absorption analytical methods maximum at 238 nm and this wavelength was chosen as the analytical wavelength. Beer’s law was obeyed between 2 and 16 μg/ml. Regression analysis was performed on the experimental data. Regression equation for standard curve was $y = 0.066x$. Correlation coefficient for developed method was found to be 0.998 signifying that a linear relationship existed between absorbance and concentration of the drug. The calibration plot in pH 7.4 phosphate buffer was shown in Fig.1.(C)

**Calibration plot in pH 1.2 acidic buffer:** A characteristic spectrum was obtained for Simvastatin in pH 1.2 Acidic buffer when scanned in the ultraviolet range between 200 and 400 nm. The scan showed absorption Analytical Methods maximum at 238 nm and this wavelength was chosen as the analytical wavelength. Beer’s law was obeyed between 2 and 18 μg/ml. Regression analysis was performed on the experimental data. Regression equation for standard curve was $y = 0.058$. Correlation coefficient for developed method was found to be 0.996 signifying that a linear relationship existed between absorbance and concentration of the drug. The calibration plot in pH 1.2 acidic buffer was shown in Fig.1.(D)

**Fig. 1.** Calibration plot in (A) methanol (B) Water (C) pH 7.4 phosphate buffer (D) pH 1.2 acidic buffer.
Fourier transforms infrared spectroscopy (FT-IR)

Fourier transform infrared spectroscopy (FT-IR) has been used to assess the interaction between carrier and guest molecules in the solid state. The FT-IR spectra of Simvastatin drug, SLS, PVPK-30, Poloxamer-188 and Physical mixture was shown in Fig. 2.

Motic digital microscopy

Pure drug and optimized nanoparticles surface appearance and shape were analyzed by Motic digital microscopy. Optimized nanosuspension showed uniform shapes with particle size (0.2 µm) shown in Fig. 3.

Particle size

The optimized batch-7(b7) had a Z-average particle size of 258.3nm with 0.381 poly-dispersity index which indicate the particles are in uniform distribution and it is shown in Fig. 4.
Fig. 2. FTIR spectra of (A) Simvastatin (B) Sodium Lauryl Sulphate (C) Poly-Vinyl Pyrolidine K-30 (D) Poloxamer-188 (E) Physical mixture.

Fig. 3. Motic digital microscopy image of Batch-7 Simvastatin Nanosuspension.

Fig. 4. Particle size distribution of Batch-7 Simvastatin nanosuspension.

Zeta potential

It is generally acknowledged that a zeta potential of approximately ± 20 mV is required. Zeta potential analysis was performed to get information about the surface properties of the nanocrystal. The zeta potential of the prepared Simvastatin nanosuspension was + 23.43 mV and it is shown in Fig. 5.

Entrapment efficiency

Entrapment efficiency of all SNS formulation was found to be greater than 70% except batch-1 and 2,
indicating suitability of these methods for particle size reduction. And the optimized batch-7 shows 92.78% Entrapment efficiency shown in Fig. 6.

![Mobility Distribution](image)

**Table 5**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Powder</th>
<th>Absorbance</th>
<th>Saturated Solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pure Drug</td>
<td>0.194</td>
<td>3.037 µg/ml</td>
</tr>
<tr>
<td>2</td>
<td>Nanosuspension powder</td>
<td>0.8989</td>
<td>129.42 µg/ml (after dilution)</td>
</tr>
</tbody>
</table>

**Spray drying of Nanosuspension**

In general, spray drying was employed to obtain nano size powder. Anaqueous nanosuspension was transferred into nano size powder by a Lab spray dryer LU-222 advanced (twin cyclone) labultima. The formed Spray dried powder of Nanosuspension shown in Fig. 8.

**In vitro drug release study**

The release rate profiles were drawn as the percentage simvastatin dissolved from the nano-suspension and pure drug versus time. Dissolution studies of pure simvastatin and all other prepared nano-suspension (B1-B8) were carried out in pH 7.4. From this data, it was evident that onset of dissolution of pure simvastatin was very low. Dissolution of simvastatin nanosuspension was affected by different surfactant concentrations and organic solvent. It can be observed that, 90.02% of the simvastatin nanosuspension was dissolved in 60 min; while in the same period, 21% of the plain simvastatin was dissolved. The dissolution rate of simvastatin nanoparticles is 4 times that of plain drugs. According to Noyes–Whitney equation, the dissolution rate is directly proportional to its surface area exposed to the dissolution medium. The increase dissolution for drug nanoparticles could thus be mainly ascribed to their greater surface area in comparison with raw drug (Fig. 10).
In vitro dissolution profile of plain drug and SNS batches in pH 7.4 phosphate buffer.

The in vitro dissolution studies showed increase in drug release as compared to plain drug suspension at pH 1.2. Plain drug suspension showed only 16.06% drug dissolution in 80 min while in all nanosuspensions Batch-7 shows 88.98% drug dissolution in 80 min. Improved drug dissolution which could be attributed to enhanced solubility of Simvastatin and dissolution rate which in turn can be due to low droplet size and surface properties of the nanosuspension (Fig. 11).

In vitro dissolution profile (In Distilled Water)

The in vitro dissolution profiles of Batch-7 nanosuspension powder, physice mixture and plain drug suspension in distilled water. Plain drug suspension showed only 13.85% drug dissolution and Physice Mixture of Simvastatin, SLS, PVP K-30, Poloxamer-188 shows 17.08% dissolution, while nanosuspension Batch-7 powder shows 81.83% drug dissolution in 60 min. Improved drug dissolution which could be attributed to enhanced solubility of Simvastatin and dissolution rate which in turn can be due to low droplet size and surface properties of the nanosuspension (Fig. 12).

Differential scanning calorimetry

DSC was also performed for Simvastatin, SNS and SCF formulation in order to further confirm the physical state. In this case, DSC scan of bulk Simvastatin sample showed a single sharp endothermic peak at 144 °C ascribed to the melting of the drug. In case of SNS the crystalline structure had been reduced substantially or lost after nanosizing as reflected by disappearance of melting endothermic peak. Incase of physical mixture the peak at 114 °C. Thus DSC study proved that crystallanity of Simvastatin was significantly reduced due to the size reduction in the crystals. DSC graphs are shown in Fig. 13.
X-Ray diffractometry

In order to investigate the physical nature of the encapsulated drug, the powder X-ray diffraction technique was used. Crystalline state is another factor influencing the dissolution and stability behavior of a compound. The crystalline state of the samples was evaluated to prove the effect of stirring. Experimental Simvastatin Nanosuspensions on the physical state of Simvastatin. Upon X-ray examinations; it was observed that the specific peaks for Simvastatin at specified 2θ value in X-RD graph at 10, 15, 17, 20 were not observed for SNS. This suggested that the crystallinity of Simvastatin was not preserved in the SNS formulation, indicating that the crystalline state of Simvastatin was altered following stirring. The absence of major peaks of Simvastatin in case of SNS confirmed formation of amorphous product which might leads to enhanced solubility of the drug in case of SNS. Nanoparticles reduced its crystallinity. This is evident from the disappearance of most peaks in the nanoparticles compared to the drug. X-Ray diffractometry graphs are shown in Fig. 14.
**In vivo study**

Injection of Triton X-100 (400 mg/kg) has successfully induced hyperlipidemia in rats by increasing the serum TC, TG, LDL-c and VLDL-c levels. Treatment with standard drug (Simvastatin) altered this elevation to different degrees. In animals that were treated with Triton X-100, the total lipid level was increased in serum, while the increase was prevented in animals that received standard drug. Oral administration of the nanosized simvastatin powder equivalent to 20 mg/kg had shown significant decrease \((p < 0.001)\) in serum TC level up to 78.28 ± 1.088 mg/dL when compared with Standard group (Plain drug) that showed TC 101.07 ± 2.307 mg/dL in rats treated only with triton (400 mg/kg) intraperitoneally, decrease in serum TG level significantly up to 191.56 ± 2.103 mg/dL when compared with Standard group (Plain drug) having value 225.44 ± 6.049 mg/dL \((p < 0.001)\). Same dose decreased serum LDL-c level in test group 67.50 ± 1.008 mg/dL as compared to Standard group (Plain drug). The LDL-c levels found in Standard group 85.86 ± 1.383 mg/dL. Similarly, serum VLDL-c level 32.43 ± 1.205 mg/dL in test group and in standard group it was found to be 43.191 ± 1.654 mg/dL. The HDL-c levels found in control, test and standard were 35.08 ± 1.023 mg/dL, 51.91 ± 2.586 mg/dL and 46.74 ± 3.239 mg/dL respectively. The change in lipid levels in test groups were comparable with group of Simvastatin treated rats. The Simvastatin Nanosuspension Powder demonstrated anti-hyperlipidemic activity. The Simvastatin Nanosuspension reduced the elevated total cholesterol, LDL, VLDL and triglyceride level more significantly \((p < 0.001)\) and increased HDL level than Plain Simvastatin drug. From the above value it was found that the simvastatin nanosuspension powder shows greater decrease in lipid profile than the plaine drug (Fig. 15).

### TABLE 6

Biochemical analysis of blood sample after induction of hyperlipidemia.

<table>
<thead>
<tr>
<th>Bio-analytical Test</th>
<th>Normal Group Mean ± SEM</th>
<th>Control Group Mean ± SEM</th>
<th>SNS Powder Group Mean ± SEM</th>
<th>Plaine drug Group Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC</td>
<td>75.10 ± 3.2</td>
<td>113.43 ± 3.115***</td>
<td>114.83 ± 3.234***</td>
<td>113.47 ± 2.822***</td>
</tr>
<tr>
<td>TG</td>
<td>130.20 ± 1.095</td>
<td>301.38 ± 8.155***</td>
<td>300.48 ± 6.370***</td>
<td>289.01 ± 9.147***</td>
</tr>
<tr>
<td>LDL</td>
<td>82.84 ± 2.315</td>
<td>133.26 ± 1.744***</td>
<td>133.76 ± 2.438***</td>
<td>133.18 ± 2.479***</td>
</tr>
<tr>
<td>VLDL</td>
<td>28.66 ± 1.918</td>
<td>62.32 ± 2.462***</td>
<td>64.25 ± 1.968***</td>
<td>62.57 ± 3.070***</td>
</tr>
<tr>
<td>HDL</td>
<td>34.39 ± 1.415</td>
<td>34.24 ± 1.828</td>
<td>34.84 ± 2.088</td>
<td>38.15 ± 1.559</td>
</tr>
</tbody>
</table>

TC-Total Cholesterol, TG-Triglyceride, LDL-Low Density Lipoprotein, VLDL-Very Low Density Lipoprotein, HDL-High Density Lipoprotein, SNS-Simvastatin Nanosuspension, Plaine drug (Simvastatin), *Normal vs,*** = Extremely Significant \((P < 0.001)\)

### TABLE 7

Biochemical analysis of blood sample after drug and SNS powder dosing (After 24 hr)/Pharmacodynamic study after 24 hr. (After Dosing).

<table>
<thead>
<tr>
<th>Test</th>
<th>Normal Group Mean ± SEM</th>
<th>Control Group Mean ± SEM</th>
<th>SNS Powder Group Mean ± SEM</th>
<th>Plaine drug Group Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC</td>
<td>78.00 ± 1.020</td>
<td>112.17 ± 2.801***</td>
<td>78.28 ± 1.088***</td>
<td>101.07 ± 2.307**##@ @@@</td>
</tr>
<tr>
<td>TG</td>
<td>134.99 ± 1.773</td>
<td>282.27 ± 8.279***</td>
<td>191.56 ± 2.103***</td>
<td>225.44 ± 6.049###@@@</td>
</tr>
<tr>
<td>LDL</td>
<td>65.61 ± 1.344</td>
<td>134.71 ± 1.492***</td>
<td>67.50 ± 1.006##</td>
<td>85.86 ± 1.383###@@@</td>
</tr>
<tr>
<td>VLDL</td>
<td>25.78 ± 1.100</td>
<td>55.59 ± 1.479***</td>
<td>32.43 ± 1.205###</td>
<td>43.191 ± 1.654###@@@</td>
</tr>
<tr>
<td>HDL</td>
<td>34.47 ± 1.085</td>
<td>35.08 ± 1.023</td>
<td>51.91 ± 2.586###</td>
<td>46.74 ± 3.239##</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM. Comparison between the groups was made by one way analysis of variance (ANOVA) followed by Tukey’s test TC:Total Cholesterol, TG:Triglyceride, LDL:Low Density Lipoprotein, VLDL: Very Low Density Lipoprotein, HDL:High Density Lipoprotein, SNS-Simvastatin Nanosuspension, Plaine drug (Simvastatin), *Normal vs,*** = Extremely Significant \((P<0.001)\), ** = Highly Significant \((P < 0.01)\), * = Non Significant \((P>0.05)\), # = Control vs, ## = Extremely Significant \((P < 0.001)\), # = Highly Significant \((P < 0.01)\), @ = Non Significant \((P > 0.05)\), @ = SNS Powder vs Plaine drug, @@ = Extremely Significant \((P < 0.001)\), @@ = Highly Significant \((P < 0.01)\), @ = Non Significant \((P > 0.05)\)
Stability study

The stability studies were carried out on optimized formulations. The samples were stored at 40 °C ± 2 °C and 75% ± 5% RH for 3 months to access their stability. After 1, 2, 3 months samples were withdrawn and rested for Entrapment efficiency and Total drug content. The optimized formulation did not show any significant difference in both parameters. It indicates that this formulation was able to retain its stability up to 3 months.

TABLE 8
Stability data of optimized formulation of nanosuspension.

<table>
<thead>
<tr>
<th>Time period</th>
<th>Entrapment efficiency</th>
<th>Total drug content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>92.78%</td>
<td>95.74%</td>
</tr>
<tr>
<td>After storage</td>
<td>92.53%</td>
<td>95.49%</td>
</tr>
<tr>
<td>1 month</td>
<td>91.87%</td>
<td>94.91%</td>
</tr>
<tr>
<td>2 month</td>
<td>91.30%</td>
<td>93.65%</td>
</tr>
<tr>
<td>3 month</td>
<td>91.30%</td>
<td>93.65%</td>
</tr>
</tbody>
</table>

Experimental data analysis

A three factor, two level full factorial design was adopted for optimization employing the amount of SLS, amount of PVPK-30 and Poloxamer-188 as independent variables. Total drug content(%) and Polydispersity Index as dependent variables.

Experimental trials were performed at all 8 possible combinations. Y1 is indicating Total drug content(%) whereas Y2 is Polydispersity Index. X1= amount of SLS, X2 = amount of PVPK-30 and X3 = amount of Poloxamer-188. Each batch contains 20 mg of simvastatin.

In order to investigate the factors systematically, a 2³ factorial design was employed. A statistical model incorporating interactive and polynomial terms was used to evaluate the responses. Total Drug Content, the results of multiple linear regression analysis showed that the coefficients b1 bear a negative sign and coefficients b2 and b3 bear positive sign (R² = 0.9395). 3-D Response Surface Plot of Total Drug Content was shown in Fig.16(A).

Final Equation in Terms of Actual Factors:

$$TDC = + 93.18 - 1.62*A + 0.29*B + 0.59*C$$

It can be concluded from the equation (2) that when the concentration of X2 and X3 was increase with decrease in the concentration of X1 then desired Total Drug Content could be obtained and its controlling the stabilization to the nanoparticles for coalescence and high Total Drug Content was observed in the formulation Batch-7.

Final Equation in Terms of Coded Factors:

$$PI = + 0.46 - 0.054*A - 0.079*B - 0.041*C$$

The coefficients b1, b2, and b3 were found to be significant at P < 0.05. Concerning Polydispersity Index, the results showed that the coefficients b1, b2 and b3 bear a negative sign (R² = 0.9061) 3-D Response Surface Plot of Polydispersity Index was shown in Fig.16(B).

**Table 9**
Design matrix of independent and dependent variable as per 2³ factorial design

<table>
<thead>
<tr>
<th>Run Sample</th>
<th>X1 (SLS)</th>
<th>X2 (PVPK-30)</th>
<th>X3 (Polo-188)</th>
<th>Y1 (TDC%)</th>
<th>Y2 (PI)</th>
<th>*% E.E</th>
<th>*MPD (nm)</th>
<th>*Particle size (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>4</td>
<td>25</td>
<td>15</td>
<td>94.11%</td>
<td>0.689</td>
<td>58.33%</td>
<td>1916.7</td>
<td>0.3-0.5</td>
</tr>
<tr>
<td>B2</td>
<td>8</td>
<td>25</td>
<td>15</td>
<td>90.01%</td>
<td>0.496</td>
<td>67.67%</td>
<td>1117.4</td>
<td>0.5-0.6</td>
</tr>
<tr>
<td>B3</td>
<td>4</td>
<td>50</td>
<td>15</td>
<td>94.45%</td>
<td>0.431</td>
<td>70.83%</td>
<td>653.8</td>
<td>0.2-0.3</td>
</tr>
<tr>
<td>B4</td>
<td>8</td>
<td>50</td>
<td>15</td>
<td>91.77%</td>
<td>0.414</td>
<td>74.62%</td>
<td>903.9</td>
<td>0.3-0.5</td>
</tr>
</tbody>
</table>

Table 9 Contd…
TABLE 10
ANOVA for Response Surface Linear Model: Response-1 (Total Drug Content).

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>df</th>
<th>Mean squares</th>
<th>F value</th>
<th>P-value</th>
<th>Probe &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>24.39</td>
<td>3</td>
<td>8.13</td>
<td>20.69</td>
<td>0.0067</td>
<td>significant</td>
</tr>
<tr>
<td>A-SLS</td>
<td>20.93</td>
<td>1</td>
<td>20.93</td>
<td>53.27</td>
<td>0.0019</td>
<td></td>
</tr>
<tr>
<td>B-PVPK-30</td>
<td>0.67</td>
<td>1</td>
<td>0.67</td>
<td>1.71</td>
<td>0.2608</td>
<td></td>
</tr>
<tr>
<td>C-Polo-188</td>
<td>2.78</td>
<td>1</td>
<td>2.78</td>
<td>7.09</td>
<td>0.0563</td>
<td></td>
</tr>
<tr>
<td>Residual</td>
<td>1.57</td>
<td>4</td>
<td>0.39</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cor Total</td>
<td>25.96</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Std.Dev.  0.63  R-Squared  0.9395
Mean  93.17  Adj R-Squared  0.8941
C.V%  0.67  Pred R-Squared  0.7578
PRESS 6.29  Adeq Precisor  11.270

TABLE 11
ANOVA for Response Surface Linear Model: Response-2 (Polydispersity Index)

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>df</th>
<th>Mean squares</th>
<th>F value</th>
<th>P-value</th>
<th>Probe &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>0.086</td>
<td>3</td>
<td>0.029</td>
<td>12.86</td>
<td>0.0160</td>
<td>Significant</td>
</tr>
<tr>
<td>A-SLS</td>
<td>0.023</td>
<td>1</td>
<td>0.023</td>
<td>10.33</td>
<td>0.0325</td>
<td></td>
</tr>
<tr>
<td>B-PVPK-30</td>
<td>0.050</td>
<td>1</td>
<td>0.050</td>
<td>22.17</td>
<td>0.0092</td>
<td></td>
</tr>
<tr>
<td>C-Polo-188</td>
<td>0.014</td>
<td>1</td>
<td>0.014</td>
<td>6.08</td>
<td>0.0692</td>
<td></td>
</tr>
<tr>
<td>Residual</td>
<td>8.95E-003</td>
<td>4</td>
<td>2.238E-003</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cor Total</td>
<td>0.095</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Std.Dev.  0.047  R-Squared  0.9061
Mean  0.46  Adj R-Squared  0.8356
C.V%  10.26  Pred R-Squared  0.8243
PRESS 0.056  Adeq Precisor  10.389

Fig.16 (A) 3-D Response Surface Plot of Total drug content (B) 3-D Response Surface Plot of Polydispersity Index.
Conclusions

Emulsification Solvent Diffusion method was employed to producing nanosuspension of simvastatin, a poorly water-soluble drug, for the improvement of solubility and dissolution velocity. In this process, the particle size of simvastatin can be obtained in the nano-size ranges, by adjusting the operation parameters, such as the stabilizer concentration. The best nanosuspension of simvastatin can be obtained by 4 mg SLS, 50 mg PVPK-30, and 30 mg Poloxamer-188 using Emulsi-fication solvent diffusion technique at laboratory scale. The dissolution of nanosized simvastatin is significantly enhanced compare with the pure simvastatin suspension. From the In-vivo study value it was found that the simvastatin nanosuspension powder shows greater decrease in lipid profile than the plain drug. These results show that the bioavailability of simvastatin nanosuspension is greater than the Plain Simvastatin drug due to the decrease in particle size. Emulsification solvent diffusion is a simple and effective approach to produce submicron particles of poorly water-soluble drugs.

References


Shid et al: Formulation and Evaluation of Nanosuspension Formulation for Drug Delivery of Simvastatin


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