Fabrication of lipidic nanocarriers of loratadine for facilitated intestinal permeation using multivariate design approach

Samridhi Verma, Sandeep Kumar Singh, and Priya Ranjan Prasad Verma

Abstract
In this investigation, multivariate design approach was employed to develop self-nanoemulsifying drug delivery system (SNEDDS) of loratadine and to exploit its potential for intestinal permeability. Drug solubility was determined in various vehicles and existence of self-nanoemulsifying region was evaluated by phase diagram studies. The influence of formulation variables $X_1$ (Capmul MCM C8) and $X_2$ (Solutol HS15) on SNEDDS was assessed for mean globule sizes in different media ($Y_1- Y_3$), emulsification time ($Y_4$) and drug-release parameters ($Y_5-Y_6$) to improve quality attributes of SNEDDS. Significant models were generated, statistically analyzed by analysis of variance and validated using the residual and leverage plots. The interaction, contour and response plots explicitly demonstrated the influence of one factor on the other and displayed trend of factor-effect on responses. The pH-independent optimized formulation was obtained with appreciable global desirability (0.9266). The strenuous act of determining emulsification time is innovatively replaced by the use of oil-soluble dye to produce visibly distinct globules that otherwise may be deceiving. TEM images displayed non-aggregated state of spherical globules (size < 25 nm) and also revealed the structural transitions occurring during emulsification. Optimized formulation exhibited non-Newtonian flow justified by the model-fit and also presented the stability to dilution effects and thermodynamic stress testing. The ex vivo permeation study using confocal laser scanning microscopy indicate strong potential of rhodamine 123-loaded loratadine-SNEDDS to inhibit P-gp efflux and facilitate intestinal permeation. To conclude, the effectiveness of design yields a stable optimized SNEDDS with enhanced permeation potential, which is expected to improve oral bioavailability of loratadine.

Keywords
Confocal laser scanning microscopy, dye colored emulsification, ex vivo intestinal permeation, multivariate design, self-nanoemulsifying drug delivery system, transmission electron microscopy

Introduction
Drug discovery process involving combinatorial chemistry and high throughput screening leads to the discovery of large number of pharmacologically active compounds, of which, just a handful is ultimately available as medicines due to drug delivery issues. With the advancement in technology and rapid growth in research and development, an uphill in number of drugs being modified at formulation stage, is observed which has already reached or likely to reach the final clinical stage. Nanotechnology aided drug delivery has taken a new dimension over the past decade and is increasingly enhancing the solubility and bioavailability of pre-existing lipophilic drugs as well as the therapeutically active new chemical entities (NCE). Pharmaceutical nanocarriers in various forms, due to their high surface area to volume ratio and their site-specific delivery are providing enhanced drug solubility, permeability and GI-stability of such entities. Lipid-based drug delivery systems (LBDDS) developed with the aid of nanotechnology are extensively being investigated for the drug delivery of challenging drugs. One such lipidic nanocarrier system comprising the potentials of lipidic formulations and nanocarriers is the self-nano emulsifying drug delivery systems (SNEDDS) which has proved its ability to deliver such drugs through its commercialized products including Sandimmune® and Neoral® (cyclosporine A), Fortovase® (saquinavir) and Norvir® (ritonavir). SNEDDS are isotropic mixtures of oils, surfactants and cosolvents which upon agitation and aqueous dilution in gastrointestinal (GI) fluids result in nanosized droplets forming nanoemulsions. An overwhelming research thrust in this domain is seen in the past couple of years by virtue of their enhanced drug solubilization capability and intraluminal solubility, attenuation of enterocyte-based efflux, selective lymphatic uptake and bypassing the first-pass drug metabolism.

The formulation by design (FbD) incorporating the multivariate design of experiments (DOE) approach involves the selection and testing of important variables affecting the system function at all possible combinations for determination of statistically significant factors, followed by optimization for the identification of optimal factor settings, in order to hit specific targets. The DOE tool provides us with the two-way ANOVA along with the graphical outcomes. Response surface methodology (RSM) is used to obtain the optimal levels of the most influencing factors derived through the contour plots and response surface plots of the responses and optimized using desirability function.
Allergic rhinitis resulting from an IgE-mediated inflammation of the nasal mucosa, currently affects 10–30% of the population with prevalence rates increasing worldwide. The World Allergy Organization (WAO) and the organization of Allergic Rhinitis and its Impact on Asthma (ARIA), in collaboration with World Health Organization (WHO) have reported allergic rhinitis as a major systemic allergic disease that has high comorbidity with rhinitis among asthmatic patients and therefore should be properly evaluated and treated\textsuperscript{18}. Loratadine (LTD), an orally effective, long-acting and non-sedating second generation H1-antihistaminic agent is widely prescribed for allergic rhinitis and chronic urticaria. It is almost completely absorbed with peak plasma levels achieved in 1–1.5 h. However, its absolute oral bioavailability is highly variable (10–40%) due to its ionizable weakly basic nature, pH-dependent solubility and high pre-systemic first pass metabolism (85%). With better prospects of SNEDDS as solubility and bioavailability enhancer, LTD belonging to BCS class-II with low oral dose (10 mg) was chosen as the model drug\textsuperscript{19,20}. Apart from the various advantages rendered by the SNEDD formulation, following added advantages can be hypothesized: (a) The developed formulation is expected to improve bioavailability and efficacy owing to nanometric size and by circumventing the first pass metabolism through lymphatic absorption, (b) LTD in addition to their antihistaminic properties has also been reported to exert in vitro growth-inhibitory effects on neoplastic mast cells\textsuperscript{21}. Hence, it can hypothesize that the antineoplastic activity of LTD can also be augmented by delivering through nanoparticles based drug delivery system such as SNEDDS formulations. It is widely accepted fact that the nanoparticles could target cancerous tumor cells more effectively. Furthermore, it has also been reported that LTD displayed the lowest incidence of heart rhythm disorders and cardiac deaths per million amongst other non-sedating antihistaminics\textsuperscript{22}. The goal of this research is to formulate, evaluate and investigate the potential of LTD-loaded SNEDDS. DOE approach is hereby utilized with the aid of mathematical equations, graphical outcomes and statistical analysis, in order to comprehend the impact of process variables and their interactions influencing the quality attributes. Moreover, the theoretical concept of the dye test is hereby applied to achieve accuracy in judgment of emulsification time of SNEDDS, not reported previously in such systems. Also, the lesser explored morphological changes occurring during emulsification is studied by transmission electron microscopy (TEM) to provide an insight into the likely in vivo behavior of these systems. The optimized SNEDDS was also characterized for morphology, size distribution and rheology. Furthermore, in order to evaluate stability potential, optimized product was subjected to dilution test and thermo-dynamic stress test by freeze–thaw and centrifugation. Finally, the developed LTD-SNEDDS were evaluated for their effectiveness and potential to permeate the intestinal epithelial membrane using the fluorescence detection technique through confocal laser scanning microscopy (CLSM), which is increasingly exploited for the studying interaction of pharmaceutical formulations with biological barriers in particular.

Materials and methods

Materials

Loratadine (LTD) was a generously gifted by Cipla (Mumbai, India). Capmul\textsuperscript{®} MCM C8 (CMC8), capmul MCM L, capnex 200P, captex 355 and caprol PGE860 were gifted by Abitec Corporation (Janesville, WI). Solutol\textsuperscript{®} HS15 (SHS15), cremophor EL, cremophor RH40 and lutilot E300 were gifts from BASF (Ludwigshafen, Germany). Pecol, labrasol, laurolglycol, capryol PGMC and plurol oleque were gifts from Gattefosse (Saint-Priest, Cedex, France). Miglyol 829 and lipoxol were gifted by Sasol (Witten, Germany). HPLC grade methanol, acetonitrile, orthophosphoric acid, potassium dihydrogen orthophosphate and Rhodamine 123 were purchased from Sigma Aldrich Ltd. (Mumbai, India). All other chemicals were of AnalR grade.

Quantification of LTD by HPLC analysis

The quantitative analyses of LTD were determined by high-performance liquid chromatography (HPLC) method using a quaternary gradient HPLC Knauer (Germany) with Smartline manager-5000 degasser and Smartline pump-1000. The method was adopted from the report by Frizon et al. with modification\textsuperscript{23}. LTD was chromatographed on ThermoScientific\textsuperscript{®} ODS C18 RP column (150 × 4.6 mm, 5 μm) in isocratic mode, at 30 °C. The optimized degassed mobile phase was a 35:45:20 (v/v/v) mixture of acetonitrile, methanol and a phosphate buffer solution (0.01 M, pH 7.2 ± 0.1, adjusted with dilute orthophosphoric acid). The flow rate was 1.0 mL/min with injection volume of 20 μL and the analyte was monitored at 254 nm (UV detector). The reliability of the analysis method was determined by obtaining linearity and accuracy.

Phase solubility studies

Phase solubility studies were carried out to obtain the maximum solubility of the drug in various excipients in a manner performed by us and other researchers previously\textsuperscript{24–25}. Saturated solution of drug is obtained when equilibrium is established between undissolved and dissolved solute in a dissolution process. To obtain the saturation solubility of LTD in 17 different vehicles (oils, surfactants and co-surfactants), 3 mL of each excipient was taken in screw-capped vials. An excess amount of LTD was introduced into each excipient, followed by vortexing for 5–10 min (Cyclo, Remi Equipments Pvt. Ltd., Mumbai, India). The samples were equilibrated in a water bath shaker (Remi Equipments Pvt. Ltd., Mumbai, India) at 50 °C for 48 h to facilitate solubilization. After equilibrium had been attained, the samples were filtered using Sartorius filter paper (5–8 μm) to remove the undissolved drug. The filtrates were suitably diluted with methanol and the amount of LTD was quantified by the peak area obtained from HPLC. All measurements were done in triplicate.

Ternary phase diagram studies

Self-emulsifying formulations constituting oil, surfactant(s)/cosurfactant form/o/w emulsion upon dilution by the aqueous medium or GI fluid. Such systems are described by ternary/pseudo-ternary phase diagrams where a constant ratio of two components is used and the other(s) is varied\textsuperscript{26}. Varying proportions of oil and water were vortexed for 5 min followed by triturating with the surfactant solution. The mixtures were continuously stirred on magnetic stirrer. The minimum amount of surfactant consumed to make the system clear was noted. The fraction of each component was then plotted on ternary graph using Origin 9.0 software (Microcal Software Inc., Northampton, MA).

FbD: 3\textsuperscript{2}-full factorial design

Formulation by design (FbD) was carried out by the DOE approach of a two-factor, three-level full factorial design (3\textsuperscript{2}-FFD) with nine randomized experimental runs using Design-Expert\textsuperscript{®} software version 7.0.0 (Stat-Ease, Inc., Minneapolis, MN). An elaborate study of significant formulation variables (factors) affecting the quality attributes or responses (optical clarity, globule size, emulsification time and drug release) were explored.
using FFD. The independent factors and the dependent variables (responses) used in this statistical design are listed in Table 1. Data was modeled to generate mathematical relationships between them, which was of the form: \( Y = b_0 + b_1X_1 + b_2X_2 + b_3X_1X_2 + b_4X_1^2 + b_5X_2^2 + E \), where \( Y \) is the measured response, \( b_0 \) is the intercept, \( b_1-b_5 \) are the regression coefficients, \( X_1 \) and \( X_2 \) are the factors and \( E \) is the error term. The model was statistically analyzed by two-way analysis of variance (ANOVA), lack-of-fit and multiple correlation coefficients (R²) tests. Interaction plots were used to demonstrate the effect of one factor influencing the effect of another factor onto any response. Graphical depiction with the help of 2D contour plots and 3D response surface plots allows us to understand the behavior of the system by demonstrating the effects of the independent factors. The diagnostic residual plots and the influence plot were also used to diagnose the statistical properties of the model and also find the statistical outliers.

**Optimization using desirability function**

The multivariate DOE involving a large number of responses utilizes desirability function tool for the optimization purpose. Desirability function, developed by Derringer and Suich in 1980, aims to find the optimum conditions which comply with the desired criteria of the response variables.27 The optimal point was achieved by transforming the estimated ‘\( m \)’ response variables to an individual desirability value ‘\( d_i \)’ using the fitted models and desired criteria of each response. This value is restricted to the [0,1] interval where ‘0’ corresponds to an undesirable response while ‘1’ represents the most desirable response. In order to extract the best joint responses, the individual ‘\( d_i \)’ are combined into an Overall or Global Desirability (D) which is the geometric average of the ‘\( m \)’ individual desirabilities, calculated by the software as described in Equation (1).29

\[
D = \sqrt[2]{d_1d_2, \ldots, d_m} \quad (1)
\]

**Preparation of liquid-SNEDDS of LTD**

Nine liquid LTD-SNEDDS (F1–F9) constituting varying proportions of oil (CMC8), surfactant (SHS15) were formulated as per 3²-FFD (Table 1). The precisely weighed excipients were mixed in the desired ratios in a beaker and gently heated at 50 °C with continuous stirring to obtain an isotropic concoclusion. Accurately weighed LTD (10 mg) was introduced to each of these (F1–F9), followed by application of gentle heat to solubilize the drug completely. These isotropic mixtures were stored in ambient conditions until analyzed for the said responses.

**Evaluation and characterization**

**Optical clarity**

The absorbances of aqueous dispersion (100 mL) of F1–F9 formulations (100 mg) were obtained spectrophotometrically at 400 nm (UV–Vis Spectrophotometer, 1800, Shimadzu, Japan) at 2 and 24 h, in order to categorize the formulations based on their visual clarity and also to study their precipitation behavior 24 h post-dispersion.30 The correlation between the absorbance and mean globule size was later determined. Moreover, the effect of pH on the absorbance and mean globule size were observed for all the nine formulations. Furthermore, in post-optimization studies, the optimum formulation was evaluated for the % transmittance calculated from the absorbance values, before and after subjecting it to thermodynamic stress testing and dilution effect.

**Globule size determination**

Dynamic light scattering (DLS), also known as photon correlation spectroscopy is an essential technology in formulation development for monitoring the colloidal state of particles in the sub-micron range. The globule sizes (Z-Avg) of SNEDDS (F1–F9) and optimized SNEDDS were determined by DLS, with a Zetasizer Nano ZS software version 6.0 (Malvern Instruments, Worcestershire, UK) wherein the sample was illuminated with a He–Ne Red laser beam and the intensity of the resulting scattered light produced by the particles fluctuates at a rate that is dependent on the size of particles. These formulations (100 mg) were dispersed in 100 mL of different media, namely Millipore Water (Molsheim, France), 0.1 N HCl and phosphate buffer (pH 6.4) to simulate the in vivo behavior and inspect the dispersion in gastric and intestinal pH. This was followed by gentle agitation to ensure complete dispersion. The formed nanoemulsions were then filtered through Millipore filter (8 μm) and the filtrate was taken for analysis. Even though the presence of dust particle is less catastrophic in the backscattered configuration of the instrument, filtration is still performed. The globule size and poly dispersity index (PDI) were analyzed at a wavelength of 633 nm at 25 °C after 2 and 24 h post-dispersion. All the analyses were carried out in triplicate.

**Zeta potential measurement**

Zeta potential of the optimized formulation was measured on Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) after aqueous dispersion of the SNEDDS pre-concentrate in the similar manner as in globule size determination. The measurement was made at 2 and 24 h post-dispersion to obtain the surface charge which is directly linked to nanoemulsion stability. The surface charge is reflected by their zeta potential, the higher values of which would present strong repellent forces among globules thereby preventing aggregation.30 All studies were repeated in triplicate.

**Assessment of self-emulsification**

Self-emulsification behavior was evaluated as a function of emulsification time upon dilution in the aqueous media. The emulsification time of all nine SNEDDS formulations (F1–F9) and the optimized SNEDDS were carried out by the method described by other scientists previously.32–34 One milliliter of each pre-concentrate was added into 500 mL of Millipore water in USP dissolution apparatus 2 (TDT-08L, Electrolab, Mumbai, India) maintained at 37 ± 0.5 °C and rotated at 50 rpm. However, since it is visually strenuous to note the exact time at which the formulations completely emulsify, an oil-soluble dye (Sudan III) which is insoluble in aqueous medium was added to the pre-concentrate prior to incorporation in the media. The time at which the distinct globules got completely emulsified (as observed by visibly uniform coloration of the medium) was noted down. The timely snapshots of the emulsification process of all the formulations were taken to avoid delusive or erroneous results. Reproducibility was attained by triplicate measurements.

**In vitro drug release studies**

The in vitro drug release profiles of the drug loaded SNEDDS (F1–F9) and optimized SNEDDS was investigated using USP dissolution apparatus 2. Each formulation equivalent to 10 mg of LTD was filled in hard gelatin capsules prior to release studies. With the aid of sinkers, the filled capsules were immersed in 900 mL of 0.1 N HCl maintained at 37 ± 0.5 °C and rotated at 50 rpm (United States Pharmacopeia, 2009). Aliquots (5 mL) were withdrawn at pre-determined intervals up to 120 min and replaced.
Table 1. $3^2$ Full factorial design: independent ($X$) and dependent variables ($Y$) chosen and the outcomes of observed and predicted responses.

| Independent variables | Levels | Low | Middle | High |
|-----------------------|--------|-----|--------|------|
|                       | Coded  | Actual (mg) | Coded | Actual (mg) | Coded | Actual (mg) |
| X1: Capmul MCM C8     |         | 80 0 190 | +1 300 |      |        |
| X2: Solutol HS 15     |         | 125 0 162 | +1 200 |      |        |

| Constraints | Low | High | Goal |
|-------------|-----|------|------|
| Y1: Mean globule size, Millipore water (nm) | 19.56 | 57.31 | Minimize |
| Y2: Mean globule size, 0.1 N HCl (nm) | 16.83 | 51.21 | Minimize |
| Y3: Mean globule size, PB 6.8 (nm) | 20.7 | 55.74 | Minimize |
| Y4: Emulsification time (Min) | 1.00 | 2.00 | In range |
| Y5: Percent DE (%) | 62.96 | 78.45 | Maximize |
| Y6: T 65% (Min) | 4.34 | 24.89 | Minimize |

| Runs | X1 (mg) | X2 (mg) | Y1 (nm) | Y2 (nm) | Y3 (nm) | Y4 (Min) | Y5 (%) | Y6 (Min) |
|------|---------|---------|---------|---------|---------|----------|--------|---------|
| F1   | 80      | 125     | 24.37 ± 0.31 | 21.76 ± 0.42 | 26.25 ± 0.03 | 2.41 ± 0.27 | 70.00     | 4.91 ± 0.03 |
| F2   | 80      | 162     | 20.01 ± 0.64 | 19.24 ± 0.83 | 23.22 ± 0.61 | 1.33 ± 0.17 | 76.46     | 4.34 ± 0.05 |
| F3   | 80      | 200     | 19.56 ± 0.38 | 16.83 ± 0.02 | 20.70 ± 0.09 | 1.00 ± 0.13 | 78.46     | 4.66 ± 0.03 |
| F4   | 190     | 125     | 37.52 ± 0.18 | 35.69 ± 0.73 | 39.73 ± 0.75 | 9.11 ± 0.35 | 65.59     | 15.83 ± 0.08 |
| F5   | 190     | 162     | 33.63 ± 0.47 | 30.08 ± 1.49 | 33.81 ± 0.09 | 5.30 ± 0.13 | 67.90     | 7.73 ± 0.05 |
| F6   | 190     | 200     | 28.98 ± 1.4  | 27.60 ± 0.52 | 30.67 ± 0.28 | 2.31 ± 0.05 | 69.02     | 10.81     |
| F7   | 300     | 125     | 57.31 ± 0.60 | 51.21 ± 1.61 | 55.74 ± 0.43 | 16.00 ± 0.22 | 62.96     | 24.89 ± 0.06 |
| F8   | 300     | 162     | 41.57 ± 0.07 | 40.53 ± 1.71 | 41.99 ± 0.42 | 12.29 ± 0.21 | 65.92     | 18.34 ± 0.10 |
| F9   | 300     | 200     | 37.93 ± 0.74 | 35.67 ± 0.66 | 38.44 ± 0.50 | 9.00 ± 0.17 | 67.28     | 10.02 ± 0.08 |

Values in the parentheses indicate the predicted responses obtained upon model fitting. Standard deviation of the values are shown as ±SD are within ±5%; $n = 3$. DE: Dissolution Efficiency; T65%: Time taken for 65% drug release.
with equal volume of fresh media each time, to maintain sink conditions. The cumulative amount of LTD released was analyzed by the in-house validated HPLC method described previously. The dissolution experiments were performed in triplicate, and data were expressed as mean value ± SD.

**Rheological study of SNEDDS**

Rheological evaluations of optimized SNEDDS were performed using a rotational viscometer (Bohlin Model Viscos 88, Bohlin Instruments, UK) equipped with cone-plate geometry (5.2° cone angle, 30 mm diameter and 1 mm gap at 25 ± 0.5°C). Samples were loaded on the plate and shear stress (σ) was measured over shear rates (γ) ranging from 10 to 250 s⁻¹ with linearly increasing and decreasing scale using the Bohlin software. The flow behavior was described using rheological models [Equation (2)] to fit the decreasing curve and determination coefficient (r²) was used to choose the most appropriate model²⁵.

\[
\text{Newtonian: } \sigma = \eta \gamma; \\
\text{Power Law: } \sigma = \eta \gamma^n; \\
\text{Bingham: } \sigma = \sigma_0 + K\gamma; \\
\text{Herschel–Bulkley: } \sigma = \sigma_0 + K\gamma^n,
\]

where \( \sigma \) = shear stress (Pa), \( \eta \) = viscosity (Pa s), \( \gamma \) = shear rate (s⁻¹), \( n \) = flow behavior index, \( \sigma_0 \) = yield stress (Pa) and \( K \) = consistency coefficient (Pa sⁿ).

**Transmission electron microscopy (TEM)**

The globule shape and size of the optimized LTD-loaded SNEDDS were investigated by TEM (Tecnai 2, 120 KV, FEI Company, Eindhoven, the Netherlands). The aqueous dispersion for analysis was obtained by dispersing the formulation (100 mg) in Millipore water (100 mL) and vortexing for 15 min. A drop of nanoemulsion was placed over Cu-grid coated with carbon film in 1% (w/v) phosphotungstic acid. The nanoemulsion was then negatively stained using 1% (v/v) phosphotungstic acid. The grid was air-dried at ambient temperature before loading in the microscope³⁶,³⁷.

**Stability study: dilution, thermodynamic, centrifugation test**

The stability and phase integrity of the optimized SNEDDS were evaluated by assessment of the effect of dilution, temperature variation (three freeze–thaw cycles) and centrifugation on precipitation behavior, percentage transmittance and globule size at 2 and 24 h. These studies were performed as reported previously with slight modifications³⁸–⁴⁰. Optimized SNEDDS (100 mg) were taken and diluted \( \times 100, \times 250, \times 500 \) and \( \times 1000 \) times with aqueous media to investigate the robustness to dilution. The optimized liquid SNEDDS were also subjected to three freeze-thaw cycles of \(-4 \text{ to } 40°C\) with storage at each temperature for 24 h. For centrifugation stress test, the pre-concentrate was centrifuged at 15,000 rpm for 20 min using a table-top Ultracentrifuge (Optima Max XP, Beckman Coulter, Fullerton, CA). Study was performed in duplicate for a reproducibility check.

**Ex vivo intestinal permeation by confocal laser scanning microscopy**

The transport of LTD-loaded SNEDDS across the epithelial mucosa of rat ileum was ex vivo as described by Negi et al.⁴¹. The animal experiments were performed on Male Sprague–Dawley rats (body weight 180–200 g) approved by the institutional animal ethical committee (No. BIT/PH/IAEC/12/2014) of Department of Pharmaceutical Sciences and Technology, Birla Institute of Technology, Mesra, Ranchi. Rats were housed under standard conditions and acclimatized for 5 days prior to the study and were provided food and water ad libitum. Briefly, after the rats were sacrificed, 5 cm longitudinal sections of the ileum were excised, washed with 0.9% sodium chloride solution and tied at one end. Different test samples (Rhodamine 123 solution and Rhodamine loaded LTD-SNEDDS) were introduced from the other end and suspended in aerated Tyrode solution for 60 min at room temperature. After 60 min, the tissues were cut open and fixed onto different slides. The drug transport across the intestine into the epithelial cells were then visualized by detecting the degree of fluorescence by confocal laser scanning microscopy (CLSM) at excitation and emission wavelengths of 540 and 600 nm, respectively.

**Results and discussion**

**Quantification by HPLC**

The HPLC method developed for quantitative in vitro analyses of LTD showed the retention time at 3.45 min in methanol and 3.61 in 0.1 N HCl. The calibration curves tested in the range of 4.0–24.0 μg/mL showed excellent linearity in methanol \( R^2 = 0.9999 \) as well as in 0.1 N HCl \( R^2 = 0.9986 \). To validate the HPLC method and to verify the linearity range, the calibration curve was contemplated by analyzing different concentrations within the same range. The corresponding peak area was fitted into the calibration equation in order to obtain calculated concentration.

**Phase solubility**

Components of SNEDDS were selected to maximize the solubility of LTD in order to obtain a stable and spontaneous nanoemulsion. The saturation solubility of LTD in each component viz. oils, surfactants and co-surfactants is presented in Figure 1. The medium chain glycerides especially the mixed-glyceride ones due to their high solvent capacity, their ability to promote emulsification and being unsusceptible to oxidation, offer popular choice for use in lipidic formulations⁴². LTD exhibited poor solubility in medium chain tri-glycerides (capteks 355, capteks 200P, miglyol 829) but in medium chain monoglycerides and di-glycerides (capmul MCM C8 and capmul MCM L), the drug was found to have excellent solubility. Capmul MCM C8 (CMC8) showing maximum drug solubility \( (267.37 \pm 0.84 \text{ mg/gm}) \) was chosen for further studies as lipid loading capacity is an essential factor for self-emulsification and for the enhancement in intestinal absorption through lymphatic route. Amongst various surfactants tested, labrasol demonstrated highest solubility \( (150.69 \pm 2.42 \text{ mg/gm}) \). However, when the mixture of selected oil and labrasol was subjected to aqueous dispersion, the emulsification appeared to be improper as evident from the largely dispersed oil droplets. Similar result with labrasol was also observed by Tran et al.’s (2013) work⁴³. Therefore, SHS15 was selected as the second best surfactant since its solubilizing capacity of 130.13 ± 1.58 mg/gm was very close to that of labrasol. Water-soluble non-ionic surfactants (like SHS15) tend to enhance drug absorption by increasing the dissolution rate and by maintaining the drug’s solubilized state for prolonged period of time, and also being lesser toxic, they provide better in vivo nanoemulsion stability⁴⁴. Moreover, due to their high HLB values (14–16), they easily form aqueous micellar solutions at low concentrations above their critical micellar concentration⁴²,⁴⁵. Thus, the selection of surfactants based on their toxicity, HLB, hydrophilicity etc. becomes a crucial factor.

**Ternary phase diagram studies**

Selection of excipients was followed by some preliminary trials and thereafter phase diagram studies were performed. As shown
in Figure 2, the non-shaded monophasic formulations were secluded from the shaded biphasic ones for further studies. This study benefited in exclusion of a large number of inappropriate combinations of formulation components and provided the basis for selection of low and high levels of each component required for DOE and optimization studies.

3²-Full factorial statistical analyses

The specific purpose of experimentation through the three-level full factorial design is to provide means whereby the factors having an influence on the process or quality may be evaluated simultaneously with their interaction and significance being assessed. A total of nine randomized experimental runs, as shown in Table 1, performed by varying the combinations of low (−1), intermediate (0) and high (+1) levels of each factor were evaluated for optical clarity at 2 h, globule sizes in different media at 2 h (Y1−Y3) and 24 h, emulsification time (Y4), percent dissolution efficiency (%DE; Y5) and time taken for 65% drug release (T65; Y6).

Mathematical model: screening, generation and evaluation

The model summary statistics of all responses (Y1−Y6) are presented in the Table 2. Selection of the model and their subsequent analysis of variance is primarily based on the significance (p < 0.05) level and the model being not aliased. The p values of the best-fit model for Y1−Y6 were 0.0333, 0.0107, 0.0345, 0.0002, 0.0019 and 0.0055, respectively, which were well below 0.05. The secondary statistical parameters which are of equal importance in modelling, curve fitting and equation predictability include $R^2$ (signifying variation around mean), adjusted $R^2$ ($R^2_a$ representing proportion of data variability) and predicted $R^2$ or goodness of prediction ($Q^2$ representing the proportion of variability in predicted data) which are calculated as per Equation (3)

$$R^2_a = 1 - (n - 1) \frac{\text{MSE}}{\text{SST}}$$

$$Q^2 = 1 - \frac{\text{PRESS}}{\text{SST}}$$

where $n =$ number of experimental runs, MSE = mean square for error, SST = total sum of squares and PRESS = prediction residual sum of squares. In our study, these parameters were close to unity indicating goodness of model fit, explanation of variation and response prediction (Table 2). Apart from these, PRESS and $F$ value are equally important statistical parameters. Smaller deviations between actual and predicted residuals will render a low PRESS value and a high prediction variation. The $F$ value calculated by the ratio of mean sum of squares and MSE, indicates how the different means are relative to variability within the sample. Its larger values indicate greater real effects of variable and $F$-observed should be greater than $F$-critical at $p = 0.05$ level of significance.

The general mathematical model: $Y = b_0 + b_1X_1 + b_2X_2 + b_3X_1X_2 + b_4X_1^2 + b_5X_2^2 + E$ generated upon model fitting represents $Y$ as the measured response, $b_0$ as the overall mean effect, $b_1$ and $b_2$ as the effect of the respective $X_1$ and $X_2$ factors with $b_3$ as their interaction effect, $b_4$ and $b_5$ as the second degree polynomial coefficient and $E$ as the random error. The values of these coefficient estimates and standardized main effects (SMEs) of $X_1$ and $X_2$ on the responses ($Y_1−Y_6$) along with their corresponding summary of ANOVA are tabulated in Supplemental Table S1. The SMEs were calculated by dividing the main effects by their
standard errors. The positive and negative signs associated with these coefficients represent the synergistic and antagonistic effect of the factors on a particular response. As evident from the Supplemental Table S1, $X_1$ (CMC8) affects the globule size ($Y_1-Y_3$) and emulsification time ($Y_4$) significantly ($p \leq 0.0001$) but affects the drug release profile ($Y_5-Y_6$) less significantly ($0.0001 < p < 0.05$). For an explicit understanding of factor effects, the next in the sequence is the study of interaction plots, two-dimensional contour plots and three-dimensional response surface plots which displays a quick visual guide to observe the trend in the responses with increasing or decreasing factor ($X_1, X_3$) amounts.

**Model validation**

Generation of significant model with evaluation of statistical parameters alone may not explain the variation in data completely. Consequently, it is essential to evaluate residual plots as a requirement for validity of ANOVA. One such plot, i.e. plot of residual versus predicted response values, is hereby used to access homogeneity of variance. The internally studentized residual compares each residual to all the residuals including itself, and is calculated as the quotient obtained from the ratio of residual and the estimated standard deviation of that residual. Furthermore, if there is no outlier, then each leverage point should be less than twice of Average Leverage ($2 \times AL$).

### Evaluation of developed LTD-loaded SNEDDS

#### Optical clarity

Nanoemulsions formed upon aqueous dilution of self-emulsifying formulation represents a system of single optically isotropic and thermodynamically stable solution, which is qualitatively and quantitatively assessed by visual observation and optical clarity evaluation, respectively. Higher transmittance (lower absorbance) is obtained with optically clear solutions due to the low scattering of the incident radiation. This was particularly seen with $F_1-F_3$ formulations having lower $X_1$ (oil) contents (Figure 3A). Moreover, optical clarity (absorbance) and mean globule size ($Z$-Avg) were found to be very well correlated with $R^2$ of 0.9592 when the formulations were dispersed in Millipore water (Figure 3B). Thus, spectrophotometric absorbance of nanoemulsions was used as a simple direct technique to distinguish between formulations with larger globule size and those with smaller ones, rather than using the more expensive and sophisticated globule size measuring equipment, specifically in the pre-development stages.

#### Effect of pH

The phase behavior of the self-emulsifying systems is reported to be considerably affected by the pH of the aqueous phase. Also, the solubility of pure LTD is largely affected by pH of the medium. LTD being a weak base ($pK_a = 4.85-6.0$) is ionized at lower pH values and is more soluble, while at pH ~ 7 it is in unionized state with low solubility but capable of being absorbed. For these reasons, evaluation of the effect of pH on the developed LTD-loaded SNEDDS is mandatory. The effect of pH on globule size was least with $F_8$ formulation showing a change in $Z$-avg from

| Responses | $p$ Value | $R^2$ | Adjusted $R^2$ ($R^2_a$) | Predicted $R^2$ ($Q^2$) | PRESS |
|-----------|-----------|-------|--------------------------|--------------------------|-------|
| $Y_1$     | 0.0004    | 0.9265| 0.9020                   | 0.7857                   | 246.01|
| Linear    | 0.0333    | 0.9728| 0.9564                   | 0.8660                   | 153.85|
| 2FI       | 0.4491    | 0.8940| 0.9574                   | 0.8406                   | 183.00|
| Cubic     | 0.6823    | 0.9926| 0.9405                   | -0.3535                  | 1555.71|
| $Y_2$     | <0.0001   | 0.9622| 0.9495                   | 0.8871                   | 110.59|
| Linear    | 0.0107    | 0.9909| 0.9854                   | 0.9642                   | 35.03 |
| 2FI       | 0.3283    | 0.9957| 0.9884                   | 0.9471                   | 51.84 |
| Cubic     | 0.0267    | 1.0000| 1.0000                   | 0.9994                   | 0.55  |
| $Y_3$     | 0.0002    | 0.9419| 0.9225                   | 0.8330                   | 158.81|
| Linear    | 0.0345    | 0.9782| 0.9650                   | 0.9063                   | 89.15 |
| 2FI       | 0.3555    | 0.9890| 0.9708                   | 0.8700                   | 123.60|
| Cubic     | 0.2658    | 0.9992| 0.9938                   | 0.8588                   | 134.27|
| $Y_4$     | 0.0002    | 0.9371| 0.9161                   | 0.8265                   | 1.435E+005|
| Linear    | 0.0591    | 0.9712| 0.9539                   | 0.8697                   | 1.078E+005|
| 2FI       | 0.2007    | 0.9901| 0.9737                   | 0.8797                   | 99464.42|
| Cubic     | 0.0516    | 1.0000| 0.9998                   | 0.9952                   | 3969.00|
| $Y_5$     | 0.0019    | 0.8769| 0.8359                   | 0.7313                   | 55.63 |
| Linear    | 0.3620    | 0.8975| 0.8360                   | 0.7330                   | 55.27 |
| 2FI       | 0.0812    | 0.9808| 0.9488                   | 0.7743                   | 46.72 |
| Cubic     | 0.3067    | 0.9982| 0.9855                   | 0.6706                   | 68.20 |
| $Y_6$     | 0.0038    | 0.8443| 0.7924                   | 0.5459                   | 191.86|
| Linear    | 0.0055    | 0.9710| 0.9535                   | 0.9408                   | 25.01 |
| 2FI       | 0.4907    | 0.9819| 0.9518                   | 0.8486                   | 63.97 |
| Cubic     | 0.8957    | 0.9855| 0.8840                   | -1.6423                  | 1116.40|

Values given in Italics present the data for the best-fit model. PRESS: prediction residual sum of squares.
40.53 nm (pH 1.2) to 41.99 nm (pH 6.8) with a difference of 1.46 nm (Table 1 and Figure 3A). F$_7$ formulation was found to be most affected by change in pH with increase in Z-avg from 51.21 nm (pH 1.2) to 57.31 nm (pH 7.0) with a difference of 6.1 nm (Table 1 and Figure 3A). However, this marginal change in $F_7$ was insignificant since the solutions were still optically clear even up to 24 h post-dilution with no signs of precipitation, exhibited by low absorbance values indicating high percentage transmittance (Figure 3A). Thus, the developed self-emulsifying formulations of LTD are made independent of pH, thereby preventing the probable insolubility in terms of precipitation behavior justified by the optical clarity and mean globule size.

**Evaluation of responses (Y$_1$–Y$_6$)**

The globule sizes (Y$_1$–Y$_3$) of the nine formulations when dispersed in different aqueous media ranged between 16.83 and 57.31 nm with eight nanoemulsion formulations lying below 50 nm. However, emulsification time (T$_2$) varied from as low as 60 s ($F_3$) to as high as 16 min ($F_7$) due to varied ratios of oil and surfactants. The drug release (Y$_3$–Y$_6$) from the liquid SNEDDS was characterized in terms of cumulative percentage drug released over a specified time wherein it was observed that %DE for all formulations varied over a narrow range of 62.96–72.46% but with >50% of the drug being released within 15 min. Since manual study of all responses simultaneously is a tedious job with possible inaccuracy, statistical evaluation using the Design-Expert software version 7.0.0 was undertaken which involves a sequential process of (1) estimating the factor effects, their signs and significance, (2) generation of mathematical model fitting a polynomial function, (3) statistical evaluation using two-way ANOVA, (4) segregating the insignificant terms and variables and finally, (5) check the model adequacy using model diagnostic plots.

**Impact of formulation components on globule size (Y$_1$–Y$_3$) and size distribution.** Globule size and size distribution are the prime factors which define the efficiency of a nanosized self-emulsifying formulation as it directly influences the drug release profile and are, therefore deemed important for oral bioavailability of the drug. Due to nanosize, the carrier-mediated systems gain better access to biological membranes, since larger particles tends to get cleared very rapidly by the clearance mechanisms like mono-nuclear phagocytic system (MPS). Also, sizes <100 nm is preferred for the nanoparticle transport via lymph to the blood from interstitium. In an attempt to simulate in vivo behavior of SNEDDS, effect of different dilution media on Z-avg was evaluated by DLS that measures the hydrodynamic radius and refers to the way a particle diffuses within a fluid. The aqueous dispersion of $F_1$–$F_9$ formulations in Millipore water (Y$_1$), 0.1 N HCl (Y$_2$) and phosphate buffer 6.8 (Y$_3$) resulted in clear and transparent nanoemulsions ranging from 19.56 to 57.31 nm, 16.83 to 51.21 nm and 20.70 to 55.74 nm, respectively, with smaller sizes being observed at lower pH (Table 1). The responses Y$_1$–Y$_3$ followed the 2-factor interaction (2FI) model, the significance of which is defined by extremely low p values (<0.001) and R$^2$ and R$^2$ being close to unity. This model has added advantage of including the 2FI terms to the mean and block with linear terms already present. The observed values were found to be in proximity to the predicted ones as shown in Table 1. The low PRESS values and high F values (Y$_1$–Y$_3$)>>F critical (F$_{3,8}$ = 5.41, p = 0.05) of the best-fit model, adds to its significance (Table 2 and Supplemental Table S1). SMEs of Y$_1$–Y$_3$ present high overall main effects (Y$_2$ dominating) and its factor-effects ($b_1$–$b_2$) present the quantitative effect onto the responses (Supplemental Table S1).

The red and black lines in interaction plots represent the high and low levels of X$_2$ respectively whereas the red and the blue lines in contour and response surface plots represent the regions with high and low values of the responses respectively (Figure 4). Slopes of low and high values of X$_2$ as shown in interaction plot (Figure 4A) are not same, which indicates that the effect of X$_1$ (CMC8) on Y$_1$ depends on the settings of X$_2$ (SHS15) parameter. This plot reveals that at high settings of X$_2$, X$_1$ has a lesser effect on Y$_1$ (i.e. low globule size) and hence desirable. The contour plot and response surface plot for Y$_1$ between X$_1$ and X$_2$ indicates an increment in globule size with the increase in X$_1$ content (Figure 4B and C). At low level of X$_2$, the Z-avg increased from 24.37 to 57.31 nm when X$_1$ was increased from 80 to 300 mg. This rise of ~30 nm globule size (highest increment achieved) was observed in all the three media, at low levels of X$_1$ (Table 1). The synergistic effect of X$_1$ (CMC8) on globule size leading to coarse and large sized globules is plausibly due to the increase in surface tension at the oil–water interface caused by increase in hydrophobic lipid content of CMC8. At high level of X$_1$, ~20 nm decrease was observed in responses Y$_1$–Y$_3$ from 57.31 to 37.93 nm due to an increase in the X$_2$ (surfactant) content. The lowest Z-avg was obtained at high levels of surfactant (X$_2$) but in the presence
of low levels of oil ($X_1$). The surfactant $X_2$ (SHS15) successfully counteracted the increase in surface tension caused by CMC8 by localizing its molecules at the oil–water interface and lowering the surface tension that lessens the free energy required for finer emulsion. This in turn decreased the globule size significantly and provided nanoemulsion stability. Such effects of surfactant have also been reported by us and other researchers in past\textsuperscript{10,51,52}. In addition, as a matter of fact, minimum droplet size
is only achieved at particular lipid and surfactant compositions since mean droplet size are affected by both the oil phase properties (viscosity, interfacial tension etc) and surfactant (solubility, partitioning etc) properties\textsuperscript{53}. Moreover, the nanoe-mulsion when observed after 24 h, revealed an insignificant rise in globule sizes but with no signs of precipitation or agglomeration indicating the physical stability of the system.

The residual analysis being necessary to confirm the ANOVA assumptions were investigated through internally studentized diagnostic plot of residual versus predicted for response \(Y_1\) as shown in Supplemental Figure S1(A). This graphic representation with no regular pattern verifies that the assumptions are undisputedly being met. The leverage plot shows that there is no outlier lying outside the upper red line. Also, with \(2 \times \text{AL} (0.888)\) being close to but less than 1.0, the validation is still agreeable (Supplemental Figure S1B).

However, \(Z\text{-Avg}\) does not provide a description of size distribution and the latter is derived by fitting correlogram to a multi-exponential algorithm. The correlation curves obtained from DLS (Figure 5) depicts clearly that the smaller and faster moving globules (red lines) decays to the baseline within 100 µs, while the larger globules (green lines) requires nearly 1000 µs to decay to baseline. The corresponding size distribution curve is shown in the inset of Figure 5. This width of size distribution is represented by PDI that is closer to unity for highly polydisperse sample. The \(F_1\)–\(F_9\) nanoemulsions presented PDI ranging from 0.075 to 0.686.

**Impact of formulation components on emulsification time (\(Y_4\)).** Emulsification studies demonstrated that the ability of varied amounts of oil and surfactant to emulsify LTD-loaded SNEDDS. This low energy emulsification process determines the spontaneity of self-emulsifying formulations when subjected to aqueous dilution under mild agitation. The surfactant being used to emulsify the oil spontaneously to form SNEDDS are preferred to be non-ionic (being less toxic), water-soluble, and having HLB of 12–18. Labrasol which presented maximum LTD solubility amongst surfactants and also met the above criteria, produced turbid and coarse emulsion with CMC8 in preliminary studies, even in the absence of the drug. Therefore, SHS15 shown to have similar drug solubility, having HLB 14–16 was chosen for development of SNEDDS. In addition, SHS15, a P-gp inhibitor reduces the P-gp drug-efflux transportation and its 30% content of free polyethylene glycol (PEG) is known to increase the stability of colloidal dispersion thereby enhancing the blood circulation time by reduced macrophage uptake\textsuperscript{54,55}. Emulsification time studies carried out with addition of oil-soluble dye (Sudan III) into the pre-concentrate, resulted in formation of visibly discrete red colored-oil globules post-dispersion, which otherwise resulted in colorless globules whose emulsification time was misjudged in colorless aqueous medium. The time taken by \(F_1\)–\(F_9\) formulations for complete emulsification ranged from 1.0 to 16.0 min (Table 1). \(F_7\) containing the highest amount of oil \((X_1)\) with the lowest amount of surfactant \((X_2)\) took maximum time for emulsification and also produced nanoemulsion with largest globule size of 51.21–57.31 nm in different media \((Y_1\)–\(Y_3)\). In contrast, \(F_3\) consisting of low levels of \(X_1\) and high levels of \(X_2\) presented minimum globule size and emulsification time. The photographs of formulation undergoing emulsification presenting the initially large globule size to the smaller globule size are displayed in Figure 6(A–C). These photographs of dye colored oil globules undergoing emulsification, authenticates the accuracy and reproducibility of results that otherwise might be deceiving.

The interaction plot affirms absence of any interaction which implies that the effect of \(X_1\) is same, regardless of the level of \(X_2\) (Figure 4D). It is explicit from the plot that at high levels of \(X_2\) (red line) and low levels of \(X_1\), spontaneous emulsification is achieved (Figure 4D). Emulsification occurs spontaneously with SNEDDS due to low surface energy required to form nanoemulsion, for which undoubtedly there should not be any resistance by
interfacial structures towards surface shearing\textsuperscript{8}. However, an increase in $X_1$ content (at fixed levels of $X_2$) leads to gradual increase in emulsification time plausibly due to hindrance towards surface shearing. Conversely, lowest level of $X_1$ with highest levels of $X_2$ resulted in smallest globule size ($Z$-avg) due to decrease in surface tension that undeniably favors faster emulsification (Table 1). A thorough study of response surface plots (Figure 4E and F) reveals that with increasing amounts of surfactant ($X_2$), there is linear decrease in emulsification time ($Y_4$) having $p$ value of 0.0019 with $R^2$ and $R^2_a$ being well correlated (Table 2). Even though the PRESS value best-fit model was high and SME was low (12.61) as compared to other responses, the $F$ value-observed (44.67) was $>>F$ critical value ($F_{2,6} = 5.14$, $p = 0.05$), signifying model adequacy (Table 2 and Supplemental Table S1). This spontaneity in emulsification might be attributed to the increased aqueous penetration of SHS15 ($X_2$) into the oil globules leading to interfacial disruption and release of nanosized droplets into bulk aqueous phase. Such potential of SHS15, also being reported by Heshmati et al., requires molecular investigation wherein apart from the high HLB value of this micelle-forming agent, its lower lipophilicity also favors smaller globule size and its hydrophilicity (30% PEG) probably contributing to emulsification\textsuperscript{38}.

The diagnostic plot shown (Supplemental Figure S1C) for response $Y_4$ although shows randomly distributed points, is not a perfectly scattered one. Even though all the points lie within 2.0, the validation is further looked for “unusualness” of the observation indicating problems with the model, if any. This being done by study of leverage plot, which displays all points lying within the limits and $2 \times AL = 0.666$, which is quite less than 1.0 implying no matter of concern regarding the model (Supplemental Figure S1D).

**Impact of formulation components on drug release profile ($Y_5$–$Y_6$).** Drug release from nanoemulsions involves interfacial transport followed by diffusive and convective transport, across the layer of surfactant coating the oil globules and through the surrounding aqueous medium, respectively. The slower dissolution rate exhibited by the poorly soluble drugs leads to erratic and incomplete absorption and therefore presents a major challenge in the drug development process. The dissolution study is optimized using two parameters, namely $Y_5$: % dissolution efficiency (%DE) and $Y_6$: the time taken for 65% release ($T_{65}$). The $F_1$–$F_9$ LTD-loaded SNEDDS showed varied but reproducible drug release owing to differences in the composition ratios. $F_3$ formulation showed highest DE of 78.46% with $T_{65}$ of 4.66 min (Table 1). This increased dissolution at low levels of $X_1$ and high levels of $X_2$ is attributed to the small globule size ($Y_1$–$Y_3$) of 16.83–20.70 nm in different media (Table 1) with low PDI which eventually increases surface area, resulting in rapid drug release. Comparable release profile was observed with $F_2$ having low level of $X_1$ and middle level of $X_2$ but with a relatively higher globule size, though not significant. The delay in $T_{65}$ in some formulations might be closely related to the increased emulsification time of the same.

The interaction plot of $Y_5$ illustrates that the effect of $X_1$ on %DE is not influenced by $X_2$ and that the components are not interdependent on each other for increased drug release, though their presence in the formulation is a necessity (Figure 4G). In contrast, the slope of interaction plot of $Y_6$ indicates that at low and high levels of $X_2$ (SHS15), the increasing amounts of $X_1$ (CMC8) results in sharp and gradual increase in $T_{65}$, respectively (Figure 4I). The contour and response surface plots of $Y_5$ and $Y_6$, as shown in Figure 4 appears to be very much different to each other, with $Y_5$ decreasing linearly ($p = 0.0019$) and sharply with
increase in oil content ($X_1$) and $Y_6$ increasing with increase in oil content ($X_3$; Supplemental Table S1). A gradual and not steep increase in $Y_5$ is observed with an increase in surfactant content ($X_2$) as displayed in Figure 4 (H and I). The improved dissolution may also be contributed to spontaneous emulsification and high hydrophilic–lipophilic balance (HLB) of the surfactant, apart being influenced by smaller globule size. The high HLB of SHS15 (14–16) resulting in increased hydrophilicity that causes increased drug dissolution and diffusion in the dissolution medium.\(^{56,57}\) The predicted values of $Y_5$ and $Y_6$ were found to be in proximity to the experimental ones (Table 1). The PRESS value of $Y_5$ and $Y_6$ corresponds to 55.63 and 25.01 are lowest among PRESS values of all responses implying high significance of prediction variation value (Table 2). The SMEs of $Y_5$-$Y_6$, as shown in Supplemental Table S1, displays that the main effect of $Y_5$ has greater model significance than $Y_6$. Moreover, the $F$ values of $Y_5$ (21.37) and $Y_6$ (55.72) were still much greater than their $F$ critical values of $F_{2,6} = 5.14$ and $F_{3,5} = 5.41$, respectively (Supplemental Table S1).

The residual analysis clearly indicates perfectly and randomly scattered points of responses $Y_5$-$Y_6$ on the internally studentized residual plot (Supplemental Figure S1E and G) with no specific pattern being observed, thereby confirming the constant variance assumption. Moreover, as calculated from leverage points, $2 \times AL$ for $Y_5$ and $Y_6$ were 0.666 and 0.888, respectively, both being $<1.0$ and all the leverage points on leverage plot (Supplemental Figure S1F and H) were $<1$ and also close to each other ($Y_5$ dominating over $Y_6$). Thus, the undesirable high leverage points are not present which otherwise would negatively influence the selected model and lead to unexpected errors. The model validation is therefore achieved, confirming the predictions by the same.

**Optimization using desirability function**

The comprehensive study of the influences of independent variables onto the dependent variables is followed by the optimization studies which identifies and determines the conditions of the significant factors required to produce a robust product with desired high quality characteristics. For this purpose, desirability function, a numerical optimization technique was employed wherein every response is assigned a goal to either minimize, maximize or to fall in-range, with the effect under a set of constraints. Thereafter, all the responses ($Y_1$-$Y_5$) are simultaneously optimized and the most desirable optimum point in the design space fulfilling the set goals is identified by first transforming each response into individual desirability scales ($d_i$) followed by its conversion into Global desirability function as per Equation (1). Among all the responses, $Y_1$-$Y_3$ and $Y_6$ was set to be minimized, $Y_4$ was set in-range and $Y_5$ was set to maximize with same weightage and importance being given to all. Figure 7A–C shows the global desirability bar graph consisting of individual desirabilities ($d_i$), the 2D-contour and 3D-response surface plots for the desirability function holding the variables $X_1$ (CMC8) and $X_2$ (SHS15). The optimized formulation was achieved at lower levels of $X_1$ and medium levels of $X_2$, the optimized amounts of which are 80.0 and 163.95 mg, respectively, having an overall desirability ($D$) of 0.9266, satisfying the maximum requirements of all responses. The predicted values of this combination of factors and its optimized levels, for responses $Y_1$, $Y_2$, $Y_3$, $Y_4$, $Y_5$ and $Y_6$ were 21.22 nm, 19.28 nm, 23.41 nm, 60 s, 74.18% and 4.24 min, respectively (Table 3).

**Evaluation and validation of optimized formulation**

The validity of optimized parameters was confirmed by the evaluation of the same for the aforementioned responses. The predicted values of optimized formulation, its observed values and % bias for $Y_1$-$Y_6$ are tabulated in Table 3. It can be easily deduced that the experimental (observed) values are in close agreement to the predicted ones with smaller percent bias. Moreover, the observed values fell within the two-sided 95% prediction interval range presented by the Design-Expert software during optimization of each response, indicating the accuracy in prediction of 95% population mean to fall in the specified range. The globule size in $Y_1$, $Y_2$, $Y_3$ were 23.33, 19.16 and 23.94 nm, respectively (Table 3). The effect of pH on nanoemulsion is of paramount importance in formulation design. In our study, the pH-dependent pure LTD, when formulated in SNEDDS is found to be independent of pH as visualized from the absence of precipitation and insignificant effect on globule size in 0.1 N HCl ($Y_2$, pH 2), phosphate buffer ($Y_1$, pH 6.4) or Millipore water ($Y_1$, pH 7). Optimized formulation (OF) completely emulsified in 1.11 min and released 65% of the drug in 4.79 min with DE of 73.84% in 120 min (Table 3). Figure 8(A) shows the plot of percentage dissolution efficiency versus time. The efficiency of drug release from SNEDDS was achieved due to the small globule sizes (19.16 nm in 0.1 N HCl) resulting in larger surface area and also due to faster emulsification of the larger oil globules into finer ones (1.11 min). The optimized LTD-SNEDDS formulation was further evaluated for its rheological behavior, surface charge, surface morphology by TEM and stability studies as an adjunct to the characterization of a developed robust formulation. The aqueous dispersion of optimized formulation was negatively charged at their surface as reflected by their zeta potential of $-11.4 \pm 1.63$ and $-20.9 \pm 0.21$ mV at 2 and 24 h, respectively. The higher zeta potential $>\pm 30$ mV prevents aggregation due to strong repulsion between droplets\(^{31}\). The change in zeta potential values, if any were also observed in stress-induced stability studies of the same. However, the charge alone cannot predict the stability of SNEDDS and therefore the non-aggregated state of the individual globules evident from TEM images affirm the stability of formulation. Moreover, the stability was also assessed by stability studies as discussed in later section.

**Rheological studies**

The rheological study of the undiluted LTD-SNEDDS pre-concentrate revealed that when fitted on the power law model, $r^2$ of 0.977 was obtained and the power law index ($n$) was 3.728 which being $>1$ indicated shear thickening nature of the system. Power law is good for describing material flow under a small range of shear rates. However, the model fit of the obtained data showed best fit in the Herschel–Bulkley model ($r^2 = 0.9834$) which describes the non-Newtonian behavior after yielding and is basically a power law model with a yield stress term. The viscosity being a crucial parameter determines the ability of formulation to be filled in hard or soft-gelatin capsules. Moreover, the SNEDDS comprising of CMC8 a medium-chain glyceride being sufficiently dense results in an average viscosity of 34.94 cp over the increasing and decreasing shear rate as presented in Figure 8(B). The down-curve is relatively displaced from the up-curve (arrowed) indicating the non-Newtonian nature of the fluid\(^{58}\). This sufficiently viscous formulation is expected to minimize the weight variation, present low rate of capsule-leakage and prevent the risk of splashing during liquid capsule-filling which is unlikely in case of viscosities similar to that of water (1.0 cp)\(^{59}\).

**Transmission electron microscopy**

Electron microscopy, an important asset in pharmaceutical technology is the most popular method to characterize the nanoemulsions for their morphology and particle size estimation with TEM being most frequently employed to serve the purpose.
This single particle-based imaging technique represents a complementary tool to the routine particle size analyzer like DLS that provides population-based results of hydrodynamic radius of dispersed oil droplets. Negative staining method was applied to improve contrast of nanoemulsion. Keeping in mind, the theoretical knowledge of the self-emulsification processes involving erosion of fine cloud of small globules from the surface of large droplets, a significant effort was made by us to provide an insight into the self-emulsification process with the help of TEM analysis, which is likely to predict the in vivo behavior of liquid SNEDD pre-concentrate when subjected to aqueous medium. The representative TEM images from different areas of the same copper grid depicting various stages of self-emulsification process are depicted in Figure 9. Aqueous dispersion of SNEDDS depicted non-spherical worm/hook-shaped globules at initial stages of emulsification (Figure 9A). As, visualized in TEM images, this was followed by the break-up of those non-spherical globules (indicated by arrows at the break-up points) and the larger globules (132 nm) breaking-up into smaller globules (46 and 20 nm) at the middle stages of emulsification (Figure 9B). This process indicates a degree of possible stratification of globule sizes when presented in vivo, and apparently involves the intermediate mesophase-like structures being formed before reaching the final stage of nanosized globules as shown in Figure 9(C). Some mesophasic structures were also observed by Singh et al. and Mercuri et al. The entire process of structural transitions occurring at initial, middle and final stages of emulsification are enclosed within rectangular boxes for an apt visualization (Figure 9A–C). The results obtained from TEM studies were further construed diagrammatically for an explicit understanding of the emulsification process taking place at different stages (Figure 9D). The arrows indicate the points of break-up of globules. The spherical shape of globule with size around 20 nm was concordant with the results obtained by DLS.
technique which was in accordance with the findings of Malvern Zetasizer. A smaller yet significant similar depiction of self emulsification was shown diagrammatically by Chang et al., wherein they illustrated the movement of hydrophilic surfactant from the oil phase to the aqueous phase, upon pouring the organic phase (oil and surfactant) into an aqueous phase which lead to low energy spontaneous formation of fine oil droplets. The rapid and spontaneous stratification of globule sizes involving structural changes occur during emulsification could therefore be well related to the ease of water penetration into various phases formed on the droplet surface existing in and around the indicated break-up points (Figure 10A and B) leading to interface disruption and formation of fine droplets (Figure 10C), thereby supporting the speculations by other researchers.

Stability studies: dilution, thermodynamic and centrifugation

The self-nanoemulsifying formulations, due to their nanosized globules offer greater surface area and surface energy that may lead to inherent physical and chemical instability when presented in vitro or in vivo in their dispersed colloidal state. Since the globule sizes are found to be <25 nm (optimized formulation), these nanoparticles may exhibit higher surface interactions and lead to precipitation, aggregation or change in globule size under different conditions of dilution, temperature variation or centrifugal force. The effect of dilution and thermodynamic

Table 3. Optimized formulation: validation and stability studies.

| Responses | Globule size (nm) | Emulsification time (min) | DE (%) | $T_{65\%}$ (min) |
|-----------|-------------------|--------------------------|--------|-----------------|
| Predicted | $Y_1$             | $Y_2$                    | $Y_3$  | $Y_4$           | $Y_5$  | $Y_6$  |
| 21.22     | 19.28             | 23.41                    | 1.00   | 74.18           | 4.24   |
| Observed  | 23.33 (0.25)      | 19.16 (0.37)             | 23.94 (0.77) | 1.11 (0.02) | 73.84 | 4.79 (0.02) |
| Residuals | -2.11             | 0.12                     | -0.53  | -0.11           | 0.34   | -0.55  |
| %Bias*    | -9.04             | 0.63                     | -2.21  | -9.91           | 0.46   | -11.48 |

%Transmittance | Globule Size (nm) | PDI |
|----------------|-------------------|-----|
| 24 h           | 2 h               | 2 h |
| 23.33 (0.25)   | 19.16 (0.37)      | 23.89 (0.77) | 1.11 (0.02) | 73.84 | 4.79 (0.02) |

*Bias (%) = [(Predicted value – Observed value)/Observed value] × 100.
Values in parentheses indicate standard deviations, all being within ± 5%.

Figure 8. (A) Drug release profile of optimized formulation (OF) of SNEDDS showing plot of per cent dissolution efficiency versus time. (B) Rheological study of Optimized formulation: Plot of the effects of shear rate on shear stress and viscosity.
stress-testing were observed for the changes in precipitation behavior, % transmittance, globule size and PDI at 2 and 24 h post-dilution. This stability study is performed for at least 6 h post-dilution supposing the gastric retention time of 2 h38. However, the formulation was found to be stable up to 24 h with the absence of any sign of visual precipitation or aggregation, thereby maintaining the phase integrity. The percentage transmittance revealed greater than 90% transmission of light in dilutions equal to or greater than 250 times and also post-stress testing. However, the light seems to be transmitted less at C100 dilution, probably due to the closeness of the dispersed particles. The analysis by DLS revealed that there is no effect of dilution on globule sizes at 2 h with size around 18 nm upon dilution. Insignificant changes at 24 h (<2.5 nm difference) shown in the Table 3, might be the result of instrumental variation. Robustness to dilution and stress-testing without any phase separation indicates the existence of stable interfacial film around the oil globules which avoids coalescence of globules. The presence of such films has been supported by others as well64. However, the stress-induced samples reported relatively larger globule size of 27.19 nm but <30 nm. It is explicit from the PDI data that the formulation presented monomodal distribution of particles with all values <0.7. The zeta potential of the diluted optimized formulation after subjection to stress-testing, were −14.4 ± 1.77 and −12.0 ± 0.14 mV at 2 and 24 h, respectively. Relatively insignificant change in zeta potential of observed in pre-treated and post-treated formulation. Stability of the formulation under these conditions predicts and ensures its shelf-life stability.

**Ex vivo intestinal permeation by confocal laser scanning microscopy**

The transport of dye loaded LTD-SNEDDS and dye solution across the intestinal epithelium was investigated using *ex vivo* in ligated ileum loops using Rhodamine 123 (R123) dye as a biological tracer and was confirmed using CLSM.

Figure 10(A) shows a diagrammatic representation of the passage of drug-loaded formulation in the GIT undergoing structural changes upon emulsification (shown by arrows), followed by intestinal permeation after transit through stomach (shown by arrow). The process of structural changes upon emulsification was deduced from TEM studies as discussed earlier. The encircled portion of the intestinal cross-section (Figure 10A) was observed under CLSM after 60 min of permeation study as displayed in Figure 10(B and C). The insets of Figure 10(B and C) show the CLSM images of R123 solution after 60 min of the study. High uptake of R123-loaded LTD-SNEDDS by the mucosal membrane and epithelium was observed (Figure 10B and C) as compared to plain R123 solution (insets of Figure 10B and C), exhibited by the strong and intense green intracellular fluorescence (shown by arrows). R123 being widely accepted as a marker for P-gp-mediated efflux in intestinal epithelium, indirectly helps in the investigation of P-gp inhibition activity of the developed drug-loaded formulation. Internalization of R123 (P-gp substrate) into the cells (strong green fluorescence) demonstrates high potential of LTD-SNEDDS to inhibit the P-gp-mediated efflux and promote the permeation across the
intestinal membrane and thereafter gaining entry into the enterocytes (Figure 10B and C). The P-gp efflux of R123 was quite evident by the low uptake of the R123 aqueous solution (0.05% w/v) by the epithelial cells, displayed by the extremely weak fluorescence observed, indicating insignificant permeation of the plain dye solution alone (insets of Figure 10B and C). These results suggest the P-gp inhibitory activity of the excipients in the SNEDDS decreasing the efflux of drug. Researchers have reported that surfactants with HLB in the range of 10–17 improve the intestinal permeability and absorption of drugs by disturbing the cell membrane and also by inhibiting the efflux transporter. Thus, in our case, inhibitory activity may probably be due to SHS15 (HLB 14–16) present in LTD-SNEDDS. The efficient uptake of the developed LTD-SNEDDS by the biological membranes may also be attributed to the PEG content (about 30%) of SHS15 which has been incorporated as surfactant in the formulation. This enhanced permeation due to the PEG content is possibly because it dissolves in both the polar and non-polar solvents and also has high solubility in cell membranes. The hydrophilicity of PEG promotes the initial penetration of the

Figure 10. Diagrammatic representation of the GI-transit of drug-loaded formulation and subsequent intestinal penetration (A); the enhanced permeation of the R123-loaded LTD-SNEDDS (indicated by arrows) into the epithelial cells as compared to R123 solution (insets) (B and C); and the illustrative diagram showing the various routes of drug transport (D).
formulation through the aqueous mucus layer. Thus, the surface property of SHS15 is of great significance in absorption because mucous membrane of GIT represents a significant barrier to the diffusion of high molecular weight compounds or hydrophobic molecules. Furthermore, the size of the nanoparticles could also be a reason for enhanced permeation since the particles in the range of 50 nm are said to be rapidly taken up by different cell types as compared to those having particle sizes around 200 nm.

In addition, the likely fate of the drug-loaded formulation in the intestinal lumen while crossing the intestinal membrane has been diagrammatically represented in Figure 10(D). This figure provides an explicit understanding of the structural forms of micelles which passes through the various possible routes viz. (1) paracellular, (2) transcellular, (3) active transport, (4) opening of tight junctions and (5) uptake by chylomicrons (Figure 10D). The efficient passage of the formulations through the enterocyte thus directly influences the absorption, distribution and excretion processes. The nanocarrier systems following paracellular and transcellular route, release their payload upon reaching the systemic circulation. Furthermore, the particles can reach lymphoid system via active transport or cellular uptake. The particles transported by cellular uptake seem to have shown greater potential owing to their intimate contact with the absorption membrane leading to steeper concentration gradient across the mucosa, which acts as driving force for drug uptake. The validity of these routes has been well documented by other researchers in the past.

Conclusion

This investigation successfully demonstrated the use of 3²-FFD combined with a desirability function for the optimization of LTD-loaded SNEDDS (F1–F9) and also exhibited the potential of LTD-SNEDDS to permeate across intestinal epithelium using CLSM. Higher drug solubilities in CMC8 (X₁) and SHS15 (X₂) presented better emulsifying properties. A comprehensive study of the effects of formulation composition (factors) on the quality attributes of the SNEDDS (responses) were performed and elaborated with the help of interaction, contour and response surface plots. The SMEs and ANOVA quantified the influence of factors on the responses and also identified the ones affecting the performance significantly. The validation of the model fits were subjected for pharmacokinetic evaluation and subsequent scale-up. As encouraging results are obtained in this study, further studies are ongoing to confirm the potential of SNEDDS for oral delivery of poorly water soluble drugs like LTD.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article. Samridhi gratefully acknowledges the financial support in the form of INSPIRE-JRF (IF120784) provided by the Department of Science and Technology, Government of India (Ref. No. DST/INSPIRE fellowship/2012 dated 25 February 2013).

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Supplementary material available online
Supplementary Figure S1 (A–H) and Table S1.