5. EXPERIMENTAL

5.1 Self Emulsifying Drug Delivery System - SMEDDS

For the development of SMEDDS formulations, the component selection is an important criterion. The component selection was done on the basis of maximum drug solubility, which was further confirmed by measuring percentage transmittance. The application of a mixture experimental design has been demonstrated to be an efficient and satisfactory method for optimization of the formulation and to acquire the necessary information to understand the relationship between independent variables and dependent variables in a formulation [1]. Experimental mixture design using Design-Expert® software was applied to optimize SMEDDS that contained a minimum amount of surfactant, a maximum amount of oil, and possessed minimum droplet size. The optimization carried out using Design of Experiment (DoE) was compared with Artificial Neural Network (ANN), which is a novel model independent approach for optimization. To apply ANN, MATLAB version R2015b (Mathworks.com, USA) was used. Optimized SMEDDS formulation was characterized for various physicochemical parameters (like droplets size and size distribution, zeta potential, self-emulsification ability, thermodynamic stability study, Cryo-TEM, SANS and stability studies). The release profile of SMEDDS from capsules were evaluated and compared with the release of pure drug. *In vitro* and *ex vivo* drug release studies were performed for formulations and compared with pure drug.

5.2 Development of SMEDDS Formulations

This section describe the development of ILO SMEDDS and VDN SMEDDS. For the development of SMEDDS, general procedure was selection of oil on the basis of solubility. Followed by this, surfactant and co-surfactant selection was done on the basis of emulsification ability.

5.2.1 Solubility Studies

The solubility of ILO and VDN was determined by shake flask method [2]. Briefly, an excess of drug was added to each vial containing 1 g of oil and surfactants. After sealing the vial, the mixture was vortexed to facilitate proper mixing of drug with the selected phase. After that, the vials were shaken for 48 h in shaker (Remi, Mumbai, India) maintained at 37°C. After 48 h, the

vials were centrifuged at 5000 rpm for 10 min. The supernatant was collected and filtered through 0.22 μm nylon membrane filters, diluted with methanol to a suitable concentration range and analyzed by a validated UV method for estimation of ILO and VDN concentration.

5.2.2 Screening of surfactants for emulsifying ability

Surfactant was selected on the basis of emulsification ability [2]. This was calculated on the basis of %transmittance (%T). Briefly, 300 mg of surfactant was added to 300 mg of selected oil phase. The mixture was vortexed for homogenization of oil with surfactant. 50 mg of the isotropic mixture was weighed and diluted with double distilled water up to 50 mL to yield a fine emulsion. The resulting emulsion was checked visually for turbidity and its %T was measured at 638.2 nm against double distilled water as blank using UV-Vis Spectrophotometer (UV 1800, Shimadzu, Japan).

5.2.3 Screening of co-surfactants

The turbidimetric method was used to select the co-surfactant [2]. Surfactant (0.2 g) was mixed with 0.1 g of the co-surfactant. To this, 0.3 g 0f the selected oil phase was added and it was vortexed for mixing of ingredients. From the isotropic mixture, 50 mg was accurately weighed and diluted up to 50 mL using double distilled water to yield fine emulsion. The resulting emulsion was checked visually for turbidity and its %T was determined at 638.2 nm against double distilled water as blank using UV-Vis Spectrophotometer (UV 1800, Shimadzu, Japan).

5.2.4 Pseudo-ternary phase diagram

Pseudo-ternary phase diagrams of oil, surfactant – co-surfactant (Smix) and water were established using water titration method [3]. The mixture of oil and Smix was varied from 1:9 to 9:1 ratio such that for any mixture, total of surfactant, co-surfactant and oil concentrations always added up to 100%. To this, water was added titrimetrically till turbidity was observed. Three pseudo-ternary phase diagrams were established for surfactant to co-surfactant (Smix) ratio of 1:1, 1:2 and 1:3. The mixtures of oil and Smix were vortexed thoroughly to ensure proper mixing of the components. 200 mg of the composition was taken and titrated with water. The concentration of water, at which transition from transparency to turbidity occurred, was

derived from the weight measurements. Phase diagrams were then constructed using CHEMIX software [4,5].

5.3 Optimization of SMEDDS Formulations

5.3.1 Optimization of ILO SMEDDS

5.3.1.1 Optimization by I-optimal design

I-optimal design was used as a statistical tool to quantify the relationship between the critical formulation factors and measured response for optimization. As I-optimal design is a mixture design, the total of all the formulation variables was 100% [6-8]. Based on the screening studies, following parameters were identified as key factors for formulation of self-emulsifying pre-concentrate: (A) Amount of oil (B) Amount of surfactant and (C) Amount of co-surfactant.

A 16-run, three factors, two-level I-optimal mixture design was employed to study the effect of formulation (independent) variables on globule size (dependent variable). Preliminary trials were performed to select the discrete levels of the independent variables. Table 5.1 summarizes the independent and dependent variables evaluated and the goal set for the response.

Table 5. 1 Variables for I-optimal design for optimization of ILO SMEDDS

Independent variables	Low level	High level
Oil-Capmul MCM C8:Oleic acid :: 1:2 (A)	8 mg	14 mg
Surfactant-Cremophor EL (B)	56 mg	65 mg
Co-surfactant-Transcutol HP (C)	25 mg	35 mg

Dependent variables	Goal	Importance
Globule size	Minimize	+++

Based on these input ranges for independent variables and goal for dependent variable, a matrix was created by the Design expert 10.0 stat ease software. Polynomial model was generated for the response i.e. globule size. Model coefficients were generated after conversion of actual component proportion to L-pseudo level using equation 5.1.

$$L\text{-}pseudo = \frac{Component\ weight\ fraction - L_i}{1 - L} \quad \text{.....Equation 5.1}$$

L_i is lower constraint of component weight fraction and L is sum of lower constraint of components' weight fraction [9].

Component weight fraction was calculated as,

$$Component\ weight\ fraction = \frac{Component\ amount}{Sum\ of\ components'amount} \quad \text{.....Equation 5.2}$$

Best fit model was selected based on the R^2 value. After exclusion of statistically insignificant terms by ANOVA, contour plot and Fraction of Design Space (FDS) plots were generated [10].

5.3.1.2 Optimization by Artificial Neural Network

ANN and MATLAB Software

ANN has recently received more attention in the field of formulation development [11,12]. ANN is composed of processing units termed as "neurons" which are interconnected forming a network. Here, we have used multilayer perceptron (MLP) [13]. MLP is organized as set of interconnected input, hidden and output layers [14]. The number of input layers is number of input variables, i.e. 3 in this network. The number of hidden layer is adjusted to decrease the error between experimental target and simulated output values [15]. The number of output layer is number of dependent variables. When the neural network is provided with the input data, the input layer propagates the weighted data to hidden layer. After selection of the bias from this layer, output response is provided by using transfer function. Every time, during learning process, there is adjustment of the interconnection weights between layers [16]. After such training, the network predicts outputs for new set of input data, which is termed as network simulation. This defines generalization ability of the created network. For MLP network formation by feed forward back propagation algorithm, MATLAB R2015b uses 70% of input data for training, 15% data for validation and remaining 15% data for testing purpose [11].

Creating the MLP feed forward back propagation network

This MLP network is created for supervised learning problems, meaning that there is training set of input and output so that the network learns interdependency between them. During this, the weights and biases are adapted to optimal value, which can be evaluated by its performance and mean square error (MSE) values. The activation (transfer) function for MLP was chosen to be logistic sigmoid [15]. The hidden layer contains 10 neurons. Training algorithm was chosen

as Levenberg-Marquardt. To avoid overtraining of the network, 1000 iterations were fixed. Training continued till improvement in MSE value was seen (limit was fixed to 1000 iteration). Training auto-stopped when generalization improvement did not occur, i.e. when MSE value did not decrease.

The input datasets were divided as training (70%), validation (15%) and testing (15%). The training data were presented to network during training and the network adjusted according to its error. The validation data were used to measure network generalization. Based on this, the network stopped training as the generalization stopped improving. Testing data were an independent measure of network performance [11].

Figure 5.1 shows the MLP feedforward back propagation network created using MATLAB 2015b.

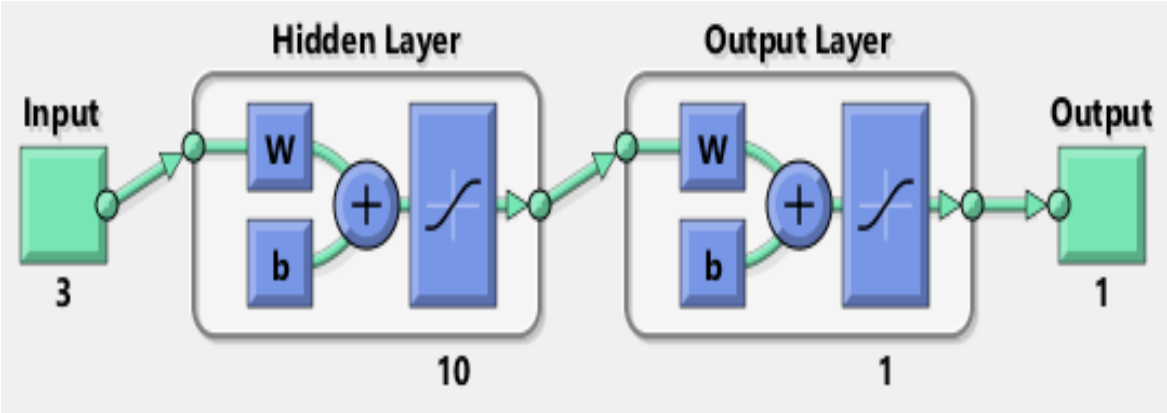

Figure 5.1 MLP feedforward back propagation network

Network training

In this learning and training process, weights and bias of the network changes constantly. This leads to different output due to different weights. Lastly, one can achieve appropriate network after running numbers of iterations that minimize MSE.

The algorithm for the network was feed forward back propagation. During the forward pass, the predicted outputs corresponding to given inputs were evaluated as per equation 5.3.

$$X = f(s) = B\mu(As+a)+b \quad \text{.....Equation 5.3}$$

Where, s is a vector for outputs. A and a are matrix of weights and bias of the first layer respectively. B and b are matrix of weight and bias for the second layer respectively. The function μ denotes elementwise nonlinearity. Reverse transmission was used to transmit error layer by layer. After adjustment of the connection weights among neurons, MSE value was decreased leading to best performance of the network to generate output.

Here, a comparative study was carried out for optimization by DoE based I-optimal design and ANN's predictability. Considering R^2 and MSE values for both the approaches, a conclusion was made for predictability of output for given input variables.

5.3.2 Optimization of VDN SMEDDS by I-optimal design

For optimization by DoE approach, I-optimal design was used to quantify the relationship between the critical formulation factors and responses. Based on the screening studies, following parameters were identified as key factors for formulation of SMEDDS: Amount of oil (X1), amount of surfactant (X2) and amount of cosurfactant (X3). A 16-run, three factors, two-level I-optimal mixture design was employed to study the effect of formulation (independent) variables on globule size and %T (dependent variables). Preliminary trials were performed to select the discrete levels of the independent variables. Table 5.2 summarizes the independent and dependent variables evaluated and the goal set for the response as generated by Design Expert 10.0 Software (Stat-Ease™).

Table 5.2 Variables for I-optimal design for optimization of SMEDDS

Independent variables	Low level	High level
Oil-Capmul MCM C8 (X1)	150 mg	250 mg
Surfactant-Cremophor EL (X2)	300 mg	500 mg
Co-surfactant-PEG 200 (X3)	300 mg	500 mg

Dependent variables	Goal	Importance
Globule size	Minimize	+ + +
%T	Maximize	+ + +

Based on these input ranges for independent variables, a matrix was created by the Design expert 10.0 software. Polynomial model was generated for the response i.e. globule size.
Similarly, SMEDDS were prepared for VDN by replacing Cremophor EL with Tween 20 as surfactant. The two formulations containing either Tween-20 (T-20) or Cremophor-EL (C-EL) were characterized and evaluated for in vivo behavior in animals. Comparing the in vitro and in vivo behavior of these two different surfactants containing SMEDDS, conclusion was made on the fate of VDN after oral delivery. Here, a comparison between two promising surfactants was carried out to understand the role of surfactants for lipid-based drug delivery.

5.4 Characterization of SMEDDS Formulations

For SMEDDS formulation of both the drugs, following parameters were measured.

5.4.1 Globule Size

Briefly, SMEDDS was diluted for 50 times with water. 200 mg of formulation, taken in a beaker was placed on a stirrer and water was added drop wise. The diluted formulation was assessed for self-emulsification efficiency, optical transparency and phase separation. Mean globule size and polydispersity index was measured by Dynamic Light Scattering (DLS) and Small Angle Neutron Scattering (SANS) [17,18].
For DLS principle-based size analysis, Nano ZS90 (Malvern Instruments Ltd., Malvern, UK) equipped with 4.0 mW internal laser was used. The 50 times diluted sample (1 mL) was taken in disposable polystyrene sizing cuvette and intensity-weighted mean diameter measured at 25°C, at a scattering angle of 173°.
The small-angle neutron scattering experiments were performed by SANS diffractometer at Dhruva reactor, Bhabha Atomic Research Centre (BARC), Mumbai, India. The samples were prepared accordingly as prepared for DLS technique in D_2O instead of H_2O [19]. For SANS technique, scattering from each sample was corrected for electronic background, detector deadtime, scattering from the empty cell and sample transmission. The intensity was converted to differential scattering cross-sections in absolute units (cm^{-1}).

5.4.2 Surface Charge

Zeta potential was measured using a Zetasizer (Nano ZS 90, Malvern Instruments Ltd., Malvern, UK. Charge of 50 times diluted SMEDDS sample was measured using the folded capillary cell. The measurement was carried out at 25°C in triplicate using multimodal analysis strategy.

5.4.3 Optical clarity

%Transmittance (%T) was measured at 638.2 nm using UV-Vis Spectrophotometer (UV 1800, Shimadzu, Japan) to determine optical clarity. The SMEDDS were diluted 50 times and checked visually for appearance. Then, %transmittance was measured against double distilled water as the blank [8].

5.4.4 Morphology

Cryo-TEM was used for morphology imaging of oil globules of diluted SMEDDS. SMEDDS sample, 50 times diluted with water, was applied on perforated carbon film supported by copper grid, the surface of which was modified for proper adhesion of sample to the grid surface. After drying by liquid N_2, the grid was transferred to cryo holder already maintained at cryo temperature of -170°C and samples were observed for morphology and size using Tecnai G2 cryo-TEM (FEI Company Ltd., Hillsboro, USA) [20,21].

5.4.5 Thermodynamic stability study

Diluted SMEDDS (50 times diluted with water) were subjected to various thermodynamic studies such as centrifugation, heating – cooling cycle and freeze – thaw cycle to assess their stability. In centrifugation test, the formulation was centrifuged at 2000 rpm for 15 min (PR 24 Centrifuge, Remi Equipment, India) and observed for phase separation, creaming or cracking. The SMEDDS was subjected to six heating-cooling cycles between 4˚C and 40˚C with storage at each temperature for not less than 48h. The formulation was assessed for physical appearance. Three freeze-thaw cycles between -21˚C and +25˚C storage condition (at each temperature for not less than 48h) were performed and observed visually for any phase separation, cracking, creaming and turbidity [22].

5.4.6 Rheology Study

The rheological properties of SMEDDS such as viscosity, Newtonian or Non-Newtonian behavior was determined using RST coaxial cylinder rheometer (Brookfield, USA) at 25°C temperature in triplicate. The instrument was pre-calibrated by silicone oil standards purchased from Brookfield [23].

5.4.7 Cloud Point (Tc) Measurement

The SMEDDS diluted with water at 1:50 ratio was placed in a temperature-controlled water bath and temperature was increased gradually. Tc was determined as the temperature at which there was a sudden appearance of cloudiness. This was checked both visually and by determining % transmittance at 638 nm by using UV–vis spectrophotometer [24].

5.4.8 Dispersibility Study

The effect of dispersibility on SMEDDS was assessed by USP type II (paddle type) dissolution apparatus (DS 8000, M/s LabIndia Instruments, India). 1 mL of SMEDDS was added to 250mL of distilled water at 37.5°C in dissolution vessel. The time required for complete dispersion of the formulation in the aqueous phase to form a microemulsion was recorded as self-emulsification time. Precipitation was evaluated by visual inspection of the resultant emulsion after 24h storage at 37°C. The in vitro dispersibility performance of formulation was visually assessed according to gradation as shown in table 5.3 [25].

Table 5.3 Observation table for Dispersibility Study

Observation	Grade
Rapidly forming (within 1 min) emulsion, having a clear or bluish appearance	A
Rapidly forming, slightly less clear emulsion, having a bluish white appearance	B
Fine milky emulsion that formed within 2 minutes	C
Dull, grayish white emulsion, having slightly oily appearance, that is slow to emulsify (longer than 2 min)	D
Formulation exhibiting either poor or minimal emulsification, with large oil globules present on the surface	E

5.4.9 Drug content

Optimized batch of SMEDDS equivalent to 12mg of ILO and 10 mg of VDN was dispersed into appropriate quantity of methanol and acetonitrile respectively, stirred sufficiently to dissolve the drug, and centrifuged at 3000rpm for 10min. The supernatant was duly diluted and analyzed spectrophotometrically (UV-1800 Shimadzu, Japan) using method described in chapter 4 – analytical techniques, section 4.3.1 for ILO and section 4.4.1 for VDN.

5.5 Drug Release

5.5.1 In vitro drug release study

The dissolution study was performed using USP XXII dissolution apparatus 1 (DS 8000, M/s Lab India Instruments, India), using 900 mL of pH 6.8 phosphate buffer at 37± 0.5°C and 50 rpm [26].

For ILO, SMEDDS formulation and drug each equivalent to 12 mg of ILO were filled into hard gelatin capsules shells. Whereas for VDN, formulation and drug each equivalent to 10 mg VDN were taken in the capsule shell.

An aliquot of 5 mL was withdrawn at each time interval followed by replacement with an equivalent amount of fresh dissolution media to maintain sink condition. The amount of ILO and VDN released was determined using UV spectrophotometer as per method described in analytical techniques (chapter 4 – analytical techniques, section 4.3.1 for ILO and section 4.4.1 for VDN).

5.5.2 Ex vivo drug release study*

Intestinal permeability of ILO or VDN drugs from pure drug suspension and their respective SMEDDS was evaluated using intestinal gut sac technique [27]. Male Sprague-Dawley rats (250 – 300 g) were anaesthetized by intraperitoneal bolus injection of thiopental sodium. A segment of jejunum was excised by making midline incision of 2 – 3 cm in abdominal cavity. The isolated small intestine of rats was then washed thoroughly with PBS to remove the lumen contents. The transport of ILO and VDN in absorptive direction, i.e. from mucosal to serosal direction was studied by using non-everted intestinal sacs immediately after washing. The VDN SMEDDS equivalent to 10 mg VDN and ILO SMEDDS equivalent to 12 mg ILO, were filled

in intestinal tissue which was tied with threads and maintained at 37±0.5°C with continuous aeration. The media was 50 mL of pH 6.8 phosphate buffer. At pre-determined time intervals, 1 mL sample was withdrawn from the receptor compartments. Fresh buffer was replenished to the receptor compartment. Similarly, permeation of equivalent amount of drug suspension in pH 6.8 phosphate buffer was also studied. Samples were analyzed by HPLC (LC20AD system, Shimadzu, Japan) to determine concentration of ILO and VDN (chapter 4 – analytical techniques, section 4.3.2 for ILO and section 4.4.2 for VDN).

*Animal protocol number is MSU/IAEC/2016-17/1627.

Enhancement ratio (Er) i.e. increase in permeability by formulation with reference to drug suspension was calculated using equation 5.4.

$$Er = \frac{Jss\ of\ Formulation}{Jss\ of\ Drug} \quad \ldots\ldots \text{Equation 5.4}$$

Calculation of Jss: The cumulative amount of drug permeated (µg) was plotted as a function of time (t) for each formulation. Drug flux (permeation rate) at steady state (Jss) was calculated by dividing the slope of the linear portion of the graph by the area of the diffusion cell. The diffusion coefficient was calculated by dividing Jss by the initial concentration of drug (C_0) and the mean cumulative values for % drug diffused versus time were plotted against time. The slopes of the graphs were used to calculate the diffusion.

5.6 Stability

As per ICH guideline Q1C, stability study was performed for the developed SMEDDS formulations. The samples were stored at 25±2°C - 60±5% RH (long term storage condition) and 40±2°C - 75±5% RH (accelerated condition) for up to 6 months. Samples were withdrawn at predetermined time intervals after 1, 2, 3 and 6 months. The clarity and globule size after 1:50 times dilution with double distilled water were measured for physical stability. Drug content in SMEDDS was measured after dilution with the solvents as described in chapter 4 of analytical techniques (Section 4.3.1 and 4.4.1).

5.7 NIOSOMES

Ever since the first report on use of self-assembly of non-ionic surfactants into vesicles by researchers in the cosmetic industry in 1970, niosomes have been studied widely as potential drug carriers [28]. Niosomes are formed by self-assembly of non-ionic surfactants in aqueous media resulting in closed bilayer structures.

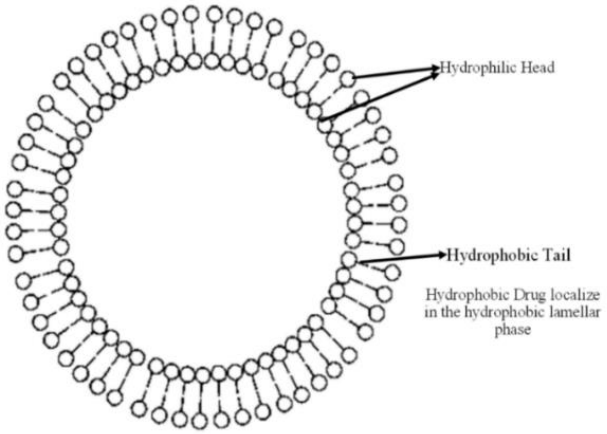

Figure 5.2 Niosomes bilayer structure

For the development of niosomes, selection of the best suited techniques amongst the available different techniques is an important criterion. Alongside, selection of surfactant is also play the major role for drug entrapment.

In this research work, preliminary trials were taken for selection of suitable method to formulate niosomes. Amongst the various methods we had tried ethanol injection, thin film hydration and reverse phase evaporation. As the highest entrapment was obtained using thin film hydration technique, it was used for niosomes preparation. The next major factor affecting entrapment is surfactant type. Amongst the Tween and Span series of surfactants, Span 60 showed highest entrapment. Hence, Span 60 was used as surfactant for niosomes preparation.

In the next step, optimization of process variables and formulation variables were carried out.

5.8 Selection of method for Niosomes formulation development

Here, in this research work we have tried 3 methods for niosomes formulation development.

1. Thin Film Hydration

Span 60 and cholesterol mixture along with 12 mg ILO (drug: lipid mole ratio :: 1:5) was dissolved in solvent mixture of chloroform and methanol (2:1 v/v) in a 100 mL round bottom flask. The flask was connected to vacuum for 30 min at 125 rpm in a thermostatic water bath at a temperature of 50°C to form the dry thin film. The flask was kept under vacuum for 12 h to remove any traces of residual solvent remained in the film. Later on, the film was hydrated with double distilled water (6 mL) at 55°C and 75 rpm for 45 min. Followed by this, size reduction was carried out by ultrasonication. The sonication procedure was 2 cycles of 30 seconds on, 1 min off at 80% amp. To separate unentrapped drug from niosomes suspension, centrifugation was carried out at 3500 rpm for 10 min at room temperature and the supernatant was separated in glass vial. The final dispersion was stored in tight sealed glass bottles. [28].
For VDN niosomes, 10 mg of VDN drug was added. Other parameters were kept same as of ILO Niosomes.

2. Ethanol Injection

Same amount of drug and excipients (Span 60 and Cholesterol) were used for this technique. In this method, accurately weighed quantity of drug, surfactant and cholesterol were dissolved in 1 mL of ethanol. This was injected as rapid injection into 6 mL of aqueous phase (ultra-pure water) which was heated up to 55°C under magnetic stirring. The aqueous phase immediately turned milky due to niosomes formation. The suspension was kept under constant stirring for around 15 minutes. Followed by which ethanol was removed by rotary evaporation under reduced pressure.

3. Reverse Phase Evaporation

In this technique, surfactant, cholesterol and drug were dissolved in chloroform: methanol (2:1 v/v). The organic phase was evaporated under reduced pressure which led to formation of thin film in the round bottom flask. The film was re-dissolved in 6 mL of diethyl ether. To this 3

mL of aqueous phase (ultra-pure water) was added. The mixture was bath sonicated for 2 min with intermittent hand shaking to form w/o emulsion. The resultant opalescent emulsion was rotary evaporated under vacuum to remove organic solvent traces to give the final niosomes aqueous dispersion.

Amongst the tried different methods, method selection was carried out based on the %entrapment efficiency (%EE) and size of niosomes.

5.9 Selection of surfactant

Keeping other variables constant, different surfactants were tried. Span 20, Span 40, Span 60, Span 80, Tween 20, Tween 60 and Tween 80 were used for screening. Based on the %entrapment efficiency and size, surfactant was selected.

5.10 Drug-Excipient interaction studies by FTIR

Fourier transform infrared spectroscopy (FTIR) of pure drugs ILO and VDN and mixture of drugs with the selected excipients (Span 60 and cholesterol) was carried out using FTIR (Bruker, Germany). Thin disk like pellet was prepared with potassium bromide and data were collected using FTIR in the range of 500 to 4000 cm^{-1} to determine compatibility of excipients with drug.

5.11 Optimization

After deciding the method of preparation (thin film hydration) and surfactant selection (Span 60) for niosomes preparation, next step was to identify factors which could affect their vesicular size and %EE. From the multiple factors which may affect quality attributes of niosomes, important factors having significant effect on the desired quality attributes were selected for preliminary investigation. The various factors selected for preliminary investigation included solvent evaporation time, rotation speed for thin film formation, rotation speed for hydration, hydration time, sonication cycle, Span 60: cholesterol ratio and drug: lipid ratio. Amongst these, the amount of drug, Span 60 and cholesterol were found to be most critical, hence, were optimized by mixture design.

5.11.1 Optimization of process variables

Process variables were optimized by One Variable At a Time (OVAT) approach. The various parameters studied for optimization are shown in table 5.4.

Table 5.4 Process variables studied for niosomes formulation development

Variables	Low Range	High Range
Solvent evaporation time	15 min	60 min
Rotation speed for thin film formation	50 rpm	150 rpm
Rotation speed for hydration	25 rpm	100 rpm
Hydration time	30 min	90 min
Sonication cycle	1 cycle	3 cycles

5.11.2 Optimization of formulation variables – ILO Niosomes

A. Optimization of hydration volume

Different batches of niosomes were prepared varying the hydration volume from 3 to 9 mL and optimization was carried out with respect to entrapment efficiency and size. This optimization was carried out during preliminary trials by OVAT approach.

B. Optimization by Combined D-optimal Mixture design

The three factors namely, lipid amount, surfactant amount and drug amount acts as a high-risk factor affecting the quality of the niosomes if not optimized. So, these three factors were optimized by Combined D-optimal Mixture Design. The dependent variables were size and entrapment efficiency. The optimization was done by mathematical equations and desirability function. The effect of these independent variables was studied and analyzed using Design Expert V.10.0. software (Stat-Ease, Inc., USA).

For ILO niosomes, 16-run, three factors, two-level Combined D-optimal mixture design was employed to study the effect of formulation (independent) variables on size and entrapment efficiency (dependent variable). Preliminary trials were performed to select the discrete levels of the independent variables. Table 5.5 summarizes the independent and dependent variables evaluated and the goal set for the response.

Table 5.5 Variables for Combined D-optimal Mixture design for optimization of niosomes

Independent variables	Low level	High level
Span-60 (A)	7 mol	9 mol
Cholesterol (B)	1 mol	3 mol
Drug (C)	1 mol	2 mol

Dependent variables	Goal	Importance
Size	Minimize	+++
% Entrapment Efficiency	Maximize	+++

Here, for the ILO niosomes development, mole ratio was considered instead of weight ratio. So, for conversion of mole (mol) to weight (mg), molecular weight was taken into consideration.

5.11.3 Optimization of formulation variables – VDN Niosomes

For the development of VDN niosomes, we replaced ILO with VDN by keeping all the other parameters same. For VDN niosomes development, 10 mg of VDN drug was added.

5.12 Lyophilization

The optimized batches of nanoparticles were lyophilized using different concentrations of mannitol. It was added to niosomes after separation of unentrapped drug from the niosomes just before the freezing step. The freshly prepared ILO and VDN Niosomes were lyophilized with cryoprotective agent (mannitol) at different ratio (1:1, 1:2, 1:3, 1:4, 1:5 w/w). Briefly, ILO and VDN Niosomes were cooled down to −70°C for 12 h followed by freeze drying in a freeze-drier (Heto Dry Winner, Denmark) under vacuum for 24 h.

5.13 Characterization of Niosomes Formulations

5.13.1 Vesicles Size

For vesicles size determination, sample was diluted 10 times with double distilled filtered water to avoid multiple scattering phenomenon. After placing the sample in disposable polystyrene

sizing cuvette, intensity-weighted mean diameter was measured at 25°C, at a scattering angle of 173° using Malvern Zetasizer. Polydispersity index was also noted to determine their size distribution pattern.

5.13.2 % Entrapment Efficiency (%EE)

Drug entrapment was calculated by determining the unentrapped drug. Briefly, the Niosomal suspension was centrifuged at 3,000 rpm for 5 min to obtain pellet of unentrapped drug. The pellet was then dissolved in methanol and then analyzed by UV spectroscopic method for the respective drug [29] .

The %EE was calculated using the equation 5.5:

$$\%EE = \frac{Total\ Drug - Free\ Drug}{Total\ Drug} * 100 \quad \text{..... Equation 5.5}$$

5.13.3 Bilayer Thickness Measurement

SANS diffractometer was used to determine the bilayer thickness of the niosomes. For this, instead of hydrating the thin film with water (H_2O), D_2O was used as per the principle described in chapter 3 - section 3.3.1.2. After putting the sample in the slit, the intensity of diffraction of neutron beam was converted to differential scattering cross-sections in absolute units (cm^{-1}).

5.13.4 Surface Charge

Zeta potential was measured using a Zetasizer (Nano ZS 90, Malvern Instruments Ltd., Malvern, UK. Zeta potential of ten times diluted sample was measured using the folded capillary cell. The measurement was carried out at 25°C in triplicate using multimodal analysis strategy [30].

5.13.5 Morphology

Cryo-TEM was used for morphology imaging of vesicles of niosomes. Niosomes sample, ten times diluted with water, was applied on perforated carbon film supported by copper grid, the surface of which was modified for proper adhesion of sample to the grid surface. After drying by liquid N_2, the grid was transferred to cryo holder already maintained at cryo temperature of

-170°C and samples were observed for morphology and size using Tecnai G2 cryo-TEM (FEI Company Ltd., Hillsboro, USA).

5.13.6 Drug content

Quantity of Niosomes equivalent to 12mg of ILO and 10 mg of VDN was dispersed into appropriate quantity of methanol, stirred sufficiently to dissolve the drug, and filtered. The filtrate was duly diluted and analyzed spectrophotometrically (UV-1800 Shimadzu, Japan).

5.13.7 Differential Scanning Calorimetry Analysis

Differential Scanning Calorimetry Analysis (DSC) was carried out with Shimadzu DSC 60 (Japan). The samples [(a) Drug (ILO or VDN), (b) cholesterol, (c) Span 60, (d) physical mixture PCT, and (e) ILO niosomes or VDN niosomes] were placed in aluminum pans and sealed. Thermograms were obtained by heating the samples from 20 to 260°C with a scan rate of 10°C/min [31].

5.14 Drug Release

5.14.1 In vitro drug release study

The dissolution study was performed using USP XXII dissolution apparatus 1 (DS 8000, M/s Lab India Instruments, India), using 900 mL of pH 6.8 phosphate buffer at 37± 0.5°C and 50 rpm.

For ILO, Niosomes formulation and drug each equivalent to 12 mg of ILO were filled into hard gelatin capsules shells. Whereas for VDN, formulation and drug each equivalent to 10 mg VDN were taken in capsule shell.

An aliquot of 5 mL was withdrawn at each time interval followed by replacement with an equivalent amount of fresh dissolution media to maintain sink condition. The amount of ILO and VDN released was determined using UV spectrophotometer as per method described in analytical method.

5.14.2 Ex vivo drug release study*

Intestinal permeability of ILO or VDN from pure drug suspension and their respective niosomes was evaluated using intestinal gut sac technique. Male Sprague-Dawley rats (250 – 300 g) were anaesthetized by intraperitoneal bolus injection of thiopental sodium. A segment of jejunum was excised by making midline incision of 2 – 3 cm in abdominal cavity. The isolated small intestine of rats was then washed thoroughly with PBS to remove the lumen contents. The transport of ILO and VDN in absorptive direction, i.e. from mucosal to serosal direction was studied by using non-everted intestinal sacs immediately after washing. The VDN niosomes equivalent to 10 mg VDN and ILO Niosomes equivalent to 12 mg ILO, were filled in intestinal tissue which was tied with threads and maintained at 37±0.5°C with continuous aeration. The media was 50 mL of pH 6.8 phosphate buffer. At pre-determined time intervals, 1 mL sample was withdrawn from the receptor compartments. Fresh buffer was replenished to the receptor compartment. Similarly, permeation of equivalent amount of drug suspension in pH 6.8 phosphate buffer was also studied. Samples were analyzed by HPLC (LC20AD system, Shimadzu, Japan) to determine concentration of ILO and VDN.

*Animal protocol number is MSU/IAEC/2016-17/1627.

Enhancement ratio was calculated for niosomes formulation using equation 5.4.

5.15 Stability

As per ICH guideline Q1C, stability study was performed for the developed Niosomes formulations. The samples were stored at 5±3°C (long term storage condition) and 25±2°C - 60±5% RH (accelerated condition) for up to 6 months. Samples were withdrawn at predetermined time intervals after 1, 2, 3 and 6 months. After the specified time interval, niosomes were analyzed for particle size and drug entrapment efficiency using the methods described in chapter 4 of analytical techniques (Section 4.3.1 and 4.4.1).

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