ORIGINAL ARTICLE

Effect of Formulation Variables on Physicochemical Properties of Cholecalciferol Non-aqueous Nanoemulsion

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Abstract

Aim: The study was to design a stable non-aqueous nanoemulsion (NANE) using cosmetically approved ingredients as a vehicle for the water sensitive active ingredients. **Materials and Methods:** NANE was designed to increase the dermal penetration and permeation and study solubility and dermal bioavailability of cholecalciferol. For better compliance incorporated the NANEs in cosmetics or personal care products. A non-aqueous system was obtained with glycerin and mineral oil stabilized by glycerol monosterate. It was observed that emulsification behavior is completely unpredictable and conventional theories of emulsification and HLB system cannot be applied here. An optimized non-aqueous cream was obtained through implementation of Box-Behnken experimental design. Cholecalciferol was used as model drug which converts into dehydro cholecalciferol in the presence of water. **Result and Discussion:** NANE was evaluated by pH, rheology, spreadability, drug content, globule size analysis, zeta potential, and stability. The stability studies (agitation, centrifugation, freeze thaw cycle, accelerated stability) were carried out at 5°C, 25°C and 40°C. Cream was stable at 5°C and 25°C. *In vitro* drug release shows slow permeation rate but increased retention of cholecalciferol in skin. A comparative study with aqueous formulation shows that non-aqueous cream offers a good stability for cholecalciferol. **Conclusion:** Results proved that NANE can be used as vehicle for the poorly water-soluble drug, suspension vehicles, and oleogels.

Key words: Box-Behnken design, cholecalciferol, non-aqueous nanoemulsion, stability

INTRODUCTION

The nanoemulsions can be used to deliver drugs via several routes, and their composition and structure enables them to incorporate greater amount of drug than other drug delivery systems.[1] Nanoemulsions are comparatively thermodynamically stable systems and gained the wide acceptance because of their enhanced drug solubilization, thermodynamic stability, and ease of manufacture.[2] The non-aqueous nanoemulsion (NANE) useful for drug delivery and principally overcomes the problem of slow and incomplete dissolution of poorly water-soluble drugs with water unstable and/or unsavory drug.[3-6]

Emulsion is one of the most convenient and advantageous formulation in which one of the liquid phases is water; however, emulsion can be formulated without an aqueous phase to produce anhydrous, non-aqueous or oil-in-oil emulsions (OOE)/microemulsion, nanoemulsion.[6] Such systems which can replace conventional emulsions where the presence of water to be avoided.[7-14] Such systems can reduce the inherent limitations and facilitates the formation of solubilized phases from which absorption may occur. Unfortunately, the major difficulty in formulating NANE arises from the lack of appropriate data on surfactant action in relevant non-aqueous media, or indeed, the dearth of suitable surfactant designed for such specialized system.[15] Oil-in-polyhydroxylic solvent nanoemulsion of water unstable cholecalciferol was designed and developed using mineral oil, glycerin, and glycerol monosterate to improve stability and elegancy of NANE formulation.[16-19]

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Vitamin D3 exerts most of its effects either directly via its receptor (the vitamin D receptor, known as VDR) acting in the nucleus and promoting protein synthesis, or by a "non-genomic" action which may still be through the VDR localized not in the nucleus but in cell membrane caveolae.[20]

While classically the VDR was thought to exert its action solely in the nucleus by mediating genomic transcription, it was later shown to translocate from the nucleus toward the cytoplasmic membrane when activated by hormonally active vitamin D3, suggesting that VDR may play a role in both the genomic and non-genomic actions of vitamin D. Additional evidence for the dual role of the VDR comes from another study in spermatids, which noted that activation of the VDR induced changes in the cell that were abolished by VDR inhibitors yet were not genomic in nature. At the same time, it seems that there are additional membrane-bound non-VDR receptors for vitamin D which may also play a role in the non-genomic actions of vitamin D, such as the $1,25(OH)_2D_3$ membrane-associated, rapid-response steroid-binding protein, which has no sequence similarity to the classical VDR. VDR acts on keratinocytes normalize the proliferation.[21-23]

For cosmetic use, it is best stabilized in an anhydrous lipid or silicone base. Cholecalciferol can be solubilized into non-aqueous polar solvents such as glycerin, polyethylene glycol, and propylene glycol to form an anhydrous emulsion.[24-27] It can be further converted into cream, lotion or gel to improve its stability, and elegance. The anhydrous composition is exceptionally pleasing and cosmetically appealing when topically applied to the skin surface and is surprisingly percutaneously absorbent and maintains the working ingredient stable for extended periods. As a result, lower concentrations of working ingredient are utilized, thus avoiding irritation sometimes associated with active ingredient.[28]

The use of a Box-Behnken experimental design is required to map the optimal composition range for excipients; this technique is mainly used to map the optimum nanoemulsion.[26,27] On the other hand, from the pharmacological point of view, surfactants with low critical micelle concentration value have more stable micelles.[29] NANEs are characterized using dynamic light scattering, polarized light microscopy, electrical conductivity, and rheology.[8,30,31] Dynamic light scattering is used to measure nanoscale particles of liquid mediums such as nanoemulsions.

In the present work, the formulation of NANE using silicone oil and castor oil (CO) with cyclomethicone is discussed and use of BBD explored to map the optimal composition range for three excipients and can be used to show the influence of changes in the volume fractions of the different phases on the phase behavior of the system. Therefore, with an aim to focus on the development of drug delivery systems for enhancing the stability of formulation which contains water sensitive materials (active ingredients); exploring the possibilities of anhydrous vehicles for controlled drug delivery and to achieve stability of non-aqueous emulsions by finding a suitable surfactant whose two structural parts were selectively soluble in either of the immiscible phases.[4]

The precise finding a suitable solvent system for the formulation of NANE, consisting of SO and CO with cyclomethicone, at the critical volume fraction and a constant molar ratio was determined.

MATERIALS AND METHODS

Materials

Analytical grade materials were used for the study. Cholecalciferol collected as a gift sample, from Shreeji Pharma, India. CO, SO, olive, arachis oil, soybean oil, cottonseed oil, and sesame oil; liquid paraffin, oleic acid, Tween 20, Tween 80, Span 20, Span 80, Span 85, Triton X100, and Silicone Emulsifier; Transcutol P, Labrasol, Labrafil, Captex 355, Acconon, Capmul MCM, Cremophore RH40, Maisine, Sodium Lauryl Sulfate; Methanol, purchased from Research Lab Fine Chem Industries, Mumbai, India. Silicone Emulsifier was received as a gift sample from Supreme Silicone, Pune, India.

A preformulation study was performed through its identity, purity, and physicochemical nature; by way of certain tests *viz*.; infrared spectrum, UV spectra, melting point, solubility, drug excipient compatibility, and few physicochemical tests.

Primary screening of materials for OOE

Anhydrous emulsion prepared using mortar and pestle, ultrasonicator, homogenizer, and hand stirring methods. Individual surfactant and its combination were tried for screening a stable OOE. Both oil phases were screened based on immiscibility of oil phases. The surfactant selection was based on miscibility in continuous phases.[30]

Formulation of OOE

A wide range of oils and surfactants were screened for the formulation of OOE. CO-SO emulsion was formulated with Triton X100 as surfactant using ultraturrex homogenizer (5000-6000 rpm).[8,31] The formulations were prepared using different phase volume ratio, silicone surfactant concentration, and hand stirring. From the preliminary studies among the few surfactant silicon surfactant shows below 3% concentration at 1:9, 2:8 and 3:7 ratio gives good stability against phase separation. Based preliminary results formulations were exposed to the Box-Behnken experimental design.[32]

Box-Behnken experimental design

The objective functions for this study were selected as maximizing the stability while controlling the viscosity as responses depending on three independent variables stirring time, surfactant concentration and phase volume ratio at three different levels. Hence, the Box-Behnken statistical design with 3 factors, 3 levels and 15 runs was selected to statistically optimize the formulation parameters and evaluate the main, interaction and quadratic effects of the formulation ingredients on the stability and viscosity of OOE. 3-factor, 3-level design was used to explore the quadratic response surfaces and for constructing polynomial models thus helping in optimizing a process using a small number of experimental runs.[33]

Statistical analysis of the Box-Behnken design batches was performed by multiple regression analysis using Reliasoft DOE. The contribution of each factor with different levels to the response was evaluated with two-way analysis of variance (ANOVA). The models were evaluated in terms of statistically significant coefficients and R^2 values. The experimental design consists of a set of points lying at the midpoint of each edge and the replicated center point of the multidimensional cube [Table 1].

Characterization of oil-in-oil nanoemulsion (OONE)

Measurement of pH

The pH values of the sample were measured at 25°C ± 1°C using digital pH meter.[34]

Rheology

The rheological property of the emulsion was investigated using C75-1 spindle plate-plate type a Brookfield R/S-CPS+ Rheometer at 22°C ± 2°C. About 2 g of non-aqueous cream was placed at the center of lower and the upper plate. Flow properties were investigated using the dynamic viscosity (n, pa/s) as a function of time 150 s in addition to measurement of viscosity as a function of share rate (ranging from 1 to 100/s).[35,36]

Flow type

The flow type was determined using increased shear rate (1-100/s) linearly for 150 s. The measured viscosity versus shear rate curve indicates the flow type of anhydrous emulsion.[35,36]

Thixotropy

The dynamic viscosity of anhydrous emulsion was studied for resolute the thixotropic behavior of sample. The process parameters embrace the increased and decreased shear rate from 1-100/s and 100-1/s for 150 s.[35,36]

Viscosity

The viscosity of formulation was measured at changing shear rates from 1-100/s and 100/s with an equal stray.[35,36]

Drug content

The cholecalciferol anhydrous emulsion was evaluated for its drug content using methanol as blank (UV Visible Spectrophotometer, Shimadzu Corp., Japan) at 258 nm.[36]

Globule size analysis

Globule size analysis was carried out through Beckman coulter counter (Malvern Analyzer, Germany) based on the laser diffraction phenomenon. During a laser diffraction experiment, particles are illuminated in a collimated laser beam, causing the light to be scattered in a variety of directions. Larger globules brought a high intensity of scattering at low angles to the beam and smaller particles; create a low-intensity signal at far wider angles. This angular scattering was measured with specially-designed detectors and particle size distribution is resolute.[35,36] The polydispersity index (PI) of anhydrous emulsion gives an indication of the width of size distribution of particle population in the emulsion.

In vitro drug release

In vitro drug release was carried out using a vertically static type Franz diffusion cell (artificial membrane 0.1 µm).

Table 1: Formulation of SO/CO emulsion with cyclomethicone

Emulsion type	Surfactant conc. (%)	Phase volume ratio	Method of preparation and stability			
			Trituration (1 h)	Lab stirrer (2 h)	Bath sonication (6 h)	Probe sonication (50 min)
SO/CO	3	1:9	±	±	±	±
		2:8	±	±	±	±
		3:7	±	-	-	-
	7	1:9	±	±	±	+
		2:8	±	-	-	+
		3:7	-	-	+	++

SO: Silicone oil

Stability study

The chemical and physical stability of the anhydrous emulsion were subjected to stability study. The thermodynamic stability of anhydrous emulsion was resolute with centrifugation at 3500 rpm and 25°C ± 1°C for 30 min. Anhydrous emulsion was examine for changes in color, viscosity, and drug content for the period of 3-month.[37-40]

Agitation test

Accurately weigh 5 g of the anhydrous emulsion was carried over reciprocating shaker approximately at 60 cycles/min for 24 h. After stipulated period cream was observed for any signs of phase separation.

Centrifugation test

Accurately weigh 5 g of the anhydrous emulsion was carried over centrifugation approximately at 3500 rpm for 30 min. After stipulated period cream was observed for any signs of phase separation.

Freeze-thaw cycles

The anhydrous emulsion was kept at −10°C and 25°C for 48 h and observed for phase separation and viscosity after three freeze-thaw cycles.

Comparison with aqueous emulsion

The anhydrous emulsion was compared with conventional aqueous emulsion for verifying the stability of cholecalciferol in anhydrous emulsion system at 25°C. The both formulations were assessed for color, changes in physical appearance, drug content, viscosity, etc.

RESULT AND DISCUSSION

Formulation of SO/CO nanoemulsion with cyclomethicone

The components OOE system was selected as oils with more immiscibility with other oil. From immiscibility data, SO and CO had more immiscibility among other oils investigated. The surfactant selection was based on more miscibility in continuous phase than the dispersed phase. For screening of surfactant, individual non-ionic surfactants were screened out. PEG/PPG-18/18 Dimethicone cyclomethicone, Aqua gel 35 were selected for SO-CO emulsion based on miscibility in oil phases. In the formulation of preliminary batches with individual and combination of surfactant for phase volume ratio 3 and 7 (SO and CO) using probe sonication method, we had found that for surfactant combinations concentration (3-7%) Tween 80 and Span 60, Span 65 and Span 20, and Tween 20 and Span 80 phase separation occurred but for using cyclomethicone and PEG/PPG-18/18 Dimethicone for same concentration of surfactant phase separation occurs after 2 weeks and for PEG/PPG-18/18 Dimethicone after 2 months. Cyclomethicone at 3% concentration stable for 7 days and at 7% concentration stable up to 30 days and for PEG/PPG-18/18 Dimethicone at 3% concentration stable for 30 days and at 7% concentration stable for more than 60 days.

The SO and CO was prepared using different phase volume ratio, surfactant concentration and sonication time [Tables 1 and 2]. After the preliminary studies using different method of preparation, it is clear that 7% of the PEG/PPG-18/18 Dimethicone surfactant concentration at 1:9, 2:8, and 3:7 gives good stability against phase separation using probe sonication method as compare to other batches prepared with 3% of surfactant concentration. Based on the results obtained with preliminary formulations, the Box-Behnken experimental design was applied. A wide range of oils and surfactants were screened for the formulation of OOE.

Drug excipient compatibility

IR spectra were obtained by Agilent Fourier transform infrared (FTIR) spectrophotometer. IR spectra were obtained by Agilent FTIR spectrophotometer. Aliphatic C-H stretching, O-H stretching, CH_2 bending, CH_3 bending, and C-H out of plane bending of aromatic ring of pure cholecalciferol and the cholecalciferol formulation containing oils and surfactant were almost in the same region of wave number ranging from 4000 to 400/cm. The results proved that there were no

Table 2: Formulation of SO/CO emulsion with PEG/PPG-18/18 dimethicone

Emulsion type	Surfactant conc. (%)	Phase volume ratio	Method of preparation and stability			
			Trituration (1 h)	Lab stirrer (2 h)	Bath sonication (6 h)	Probe sonication (50 min)
SO/CO	3	1:9	±	±	-	-
		2:8	±	±	-	+
		3:7	±	-	+	++
	7	1:9	±	-	-	+++
		2:8	-	+	++	+++
		3:7	+	++	++	***

±: Unstable, –: 0-7 days, +: 8-15 days, ++: 15-30 days, +++: 30-45 days, ***More than 60 day, SO: Silicone oil, CO: Castor oil

significant interactions between the drug and all excipients [Figure 1].

Silicone emulsifier based anhydrous emulsion

Among several emulsion preparation methods, the high-pressure homogenization method was selected as that for the development of a new vehicle. In HPH method, OOE was prepared. Based on results obtained from preliminary evaluations, the Box-Behnken experimental design was applied for further investigation.

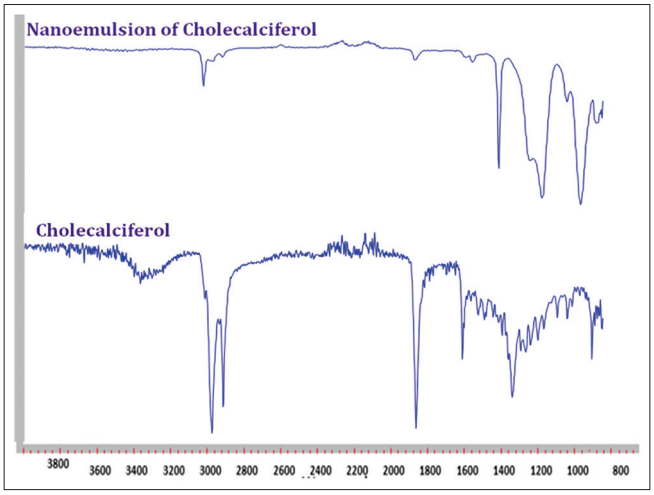

Figure 1: Drug – excipient compatibility by Fourier transform infrared study

Box-Behnken experimental design

Data analysis

The anhydrous OOE was developed and evaluated in the terms of viscosity and stability. Systematic optimization procedures are carried out by selecting an objective function, finding the most important or contributing factors and investigating the relationship between responses and factors. The Box-Behnken experimental design [Table 3] has the advantages of requiring fewer experiments (17 batches). The all selected dependent variables obtained at various levels of the 3 independent variables (X_1, X_2, and X_3) were subjected to multiple regression to yield a second order polynomial equation.

Effect of formulation variables

The results clearly indicate that viscosity and stability are strongly affected by the variables selected and a wide range of coefficients of the terms of the polynomial equation for Y_1. The main effects of X_1, X_2, and X_3 represent the average result of changing one variable from its low level to its high level.

Table 3: Box-Behnken experimental design

Run order	Independent variables		
	(X_1) Phase volume ratio (mL)	(X_2) Surfactant concentration (%)	(X_3) Sonication time
1	0	0	0
2	−1	−1	0
3	0	1	−1
4	1	0	−1
5	−1	0	1
6	1	1	0
7	0	1	1
8	−1	1	0
9	1	0	1
10	0	0	0
11	0	−1	1
12	0	0	0
13	−1	0	−1
14	1	−1	0
15	0	−1	−1
16	0	0	0
17	0	0	0

Independent variable	Low	Medium	High	Dependent variable
A: Phase volume ratio (mL)	1:9	2:8	3:7	Y_1=Viscosity (m.Pa.S)
B: Surfactant concentration %	6	8	10	Y_2=Stability (days)
C: Sonication time (min)	340	350	360	

The interaction terms (X_1X_2, X_1X_3, X_2X_3, X_{12}, X_{22}, and X_{32}) show how the viscosity changes when remained variables are simultaneously changed. The negative coefficients for all 3 independent variables indicate an unfavorable effect on the viscosity, while the positive coefficients for the interactions between 2 variables indicate a favorable effect on the viscosity. Among the three independent variables, the lowest coefficients value is for X_3, indicating that this variable is insignificant in the prediction of viscosity [Table 3].

Y_1 and Y_2 values measured for the different batches showed wide variation (values ranged from; 19 m.Pa.S to 54 for Y_1 and 19-76 days for Y_2) which clearly indicate that the Y_1 and Y_2 values is strongly affected by the variables selected and imitate by wide range of values for coefficients of the terms in equations. The main effects of X_1, X_2, and X_3 represent the average result of changing one variable at a time from its low level to its high level. The negative sign for the coefficients in the polynomial equation specify a negative effect on responses, while the positive sign specify a positive effect [Table 3].

$$Y_1=32-38X_1+14.38X_2-0.50X_3+0.25X_1X_2 -1.50X_1X_3-3.50X_2X_3+1.88X_{11}+1.38X_{22}+3.62X_{33}$$

$$Y_2=+35-0.62X_1+23.00X_2-1.62X_3-0.25X_1X_2-1.50X_1X_3 -5.25X_2X_3-3.552E-015X_{11}+8.25X_{22}+2.50X_{33}$$

Probability plots

Probability plots [Figure 2] explain, whether the residuals follow a normal distribution, in which case the points will follow a straight line. It was expected that some scatter even with normal data. Look only for definite patterns like an "S-shaped" curve, which indicates that a transformation of the response may provide a better analysis from this we say that plot shown by viscosity Figure 2a nearly follows straight line as compare to the effect shown by stability in Figure 2b. In the case of normal probability distribution, the blue spot indicates non-significant effect on variable while red dots indicates significant effect distributed around a straight line.

Plot of predicted versus actual values

The plot of predicted versus actual values [Figure 3] and it helps to detect a value, which is not easily predicted by the model. The plot is straight line if all the actual values are same that of predicted value in Figure 3a seen that all the data points almost goes through straight line indicating less is residual values; while in Figure 3b of stability was not seen this effect thus residual is more.

Interaction matrix

Interaction matrix shows effect of change in concentration of dependent variables on response it easy to interpret two factor interactions from this plot; If plot was appeared with two non-parallel lines indicating that the effect of one factor depends on the level of the other (Figure 4a-c for viscosity and Figure 4d-f for stability, respectively) indicating significance of effect on viscosity and stability.

ANOVA, pure error and lack of fit

Regression analysis was carried out to determine the regression coefficients. All the independent variables were found to be significant for all response variables. The linear model was found to be significant for Y_1 and Y_2. Hence, the result indicates that both the factors play an important role in the formulation of OOE.

The ANOVA reveals [Tables 4 and 5] that study was significant for all response variables and the effects like, the percentage of surfactant concentration and phase volume ratio

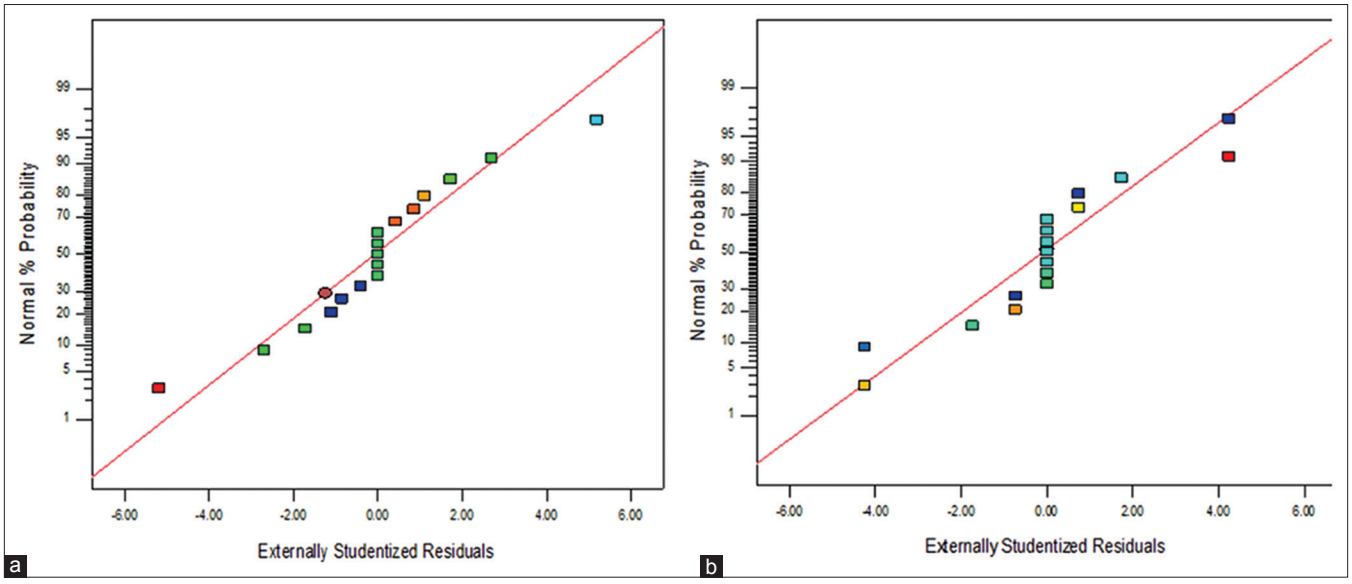

Figure 2: Normal probability plot for residual of (a) viscosity and (b) stability of oil-in-oil nonaqueous nanoemulsions respectively

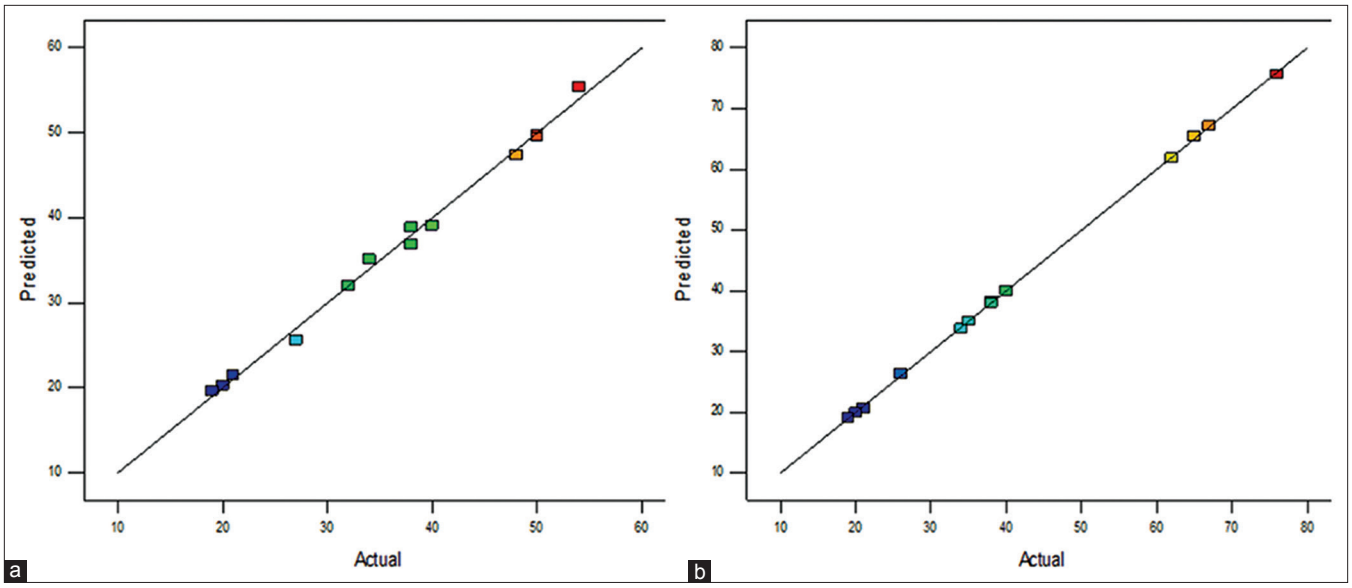

Figure 3: Plot of predicted versus actual for (a) viscosity and (b) stability of oil-in-oil nonaqueous nanoemulsions, respectively

Table 4: ANOVA for viscosity

Source of variation	*F* ratio	*P* value
Model	151.34	<0.0001
A: Phase volume ratio	0.85	
B: Surfactant conc.	1251.01	<0.0001
C: Sonication time	1.51	
AB	0.19	
AC	6.81	
BC	37.08	
A^2	11.20	
B^2	6.02	
C^2	41.87	
Residual	-	-
Lack of Fit	-	-
Pure Error	-	-

ANOVA: Analysis of variance

Table 5: ANOVA for stability

Source of variation	*F* ratio	*P* value
Model	4874.27	<0.0001
A: Phase volume ratio	29.17	
B: Surfactant concentration	39498.67	<0.0001
C: Sonication time	197.17	<0.0001
AB	2.33	
AC	84.00	<0.0001
BC	1029.00	<0.0001
A^2	0.000	
B^2	2674.74	<0.0001
C^2	245.61	<0.0001
Residual	-	-
Lack of fit	-	-
Pure error	-	-

ANOVA: Analysis of variance

were also found to be significant, along with its quadratic and interaction terms for all the dependent variables and have an important role for optimal concentration in OOE give rise to greater stability and optimum viscosity.

The pure error and lack of fit provide a mean response and an estimate of pure experimental uncertainty. The residuals values represent the differences between the observed and predicted values, given that computed *F* values were, respectively, lower than critical *F* values, which denotes non-significance with regard to the lack of fit. For lack of fit *P* values, we obtained 0.1904 for Y_1 and hence the current model provided a satisfactory fit to the data and had no lack of fit. The ANOVA studies [Tables 4 and 5] for Y_1 and Y_2. The statistical significance of each effect was tested by comparing the mean square against an estimate of the experimental error. It was noted that X_1, X_2, and X_3 with their interaction effect other than X_1X_2 and quadratic effect had $P < 0.05$, indicating significance effect of these variables in prediction of X, while linear effect X_1, interaction effect X_1X_3, and quadratic effect of X_{22}, X_{33} indicating non-significance effect of these variables in prediction of response Y_2 because of having $P > 0.05$, indicating significance of these variables in prediction of Y_1 and Y_2.

Furthermore, the difference between low critical and high critical level is one of evaluation parameters for the significant effect of independent variable on response, indicating that the difference between levels near to zero indicating non-significant effect on response, means interaction effect of X_1X_2 does not show significant effect on stability, more is the difference more is significant effect of that variable. The standard error indicates the standard deviation of coefficient.

The R^2 can be artificially inflated by simply continuing to add terms to the model, even if the terms are not statistically significant. The adjusted R^2 plateaus when insignificant terms

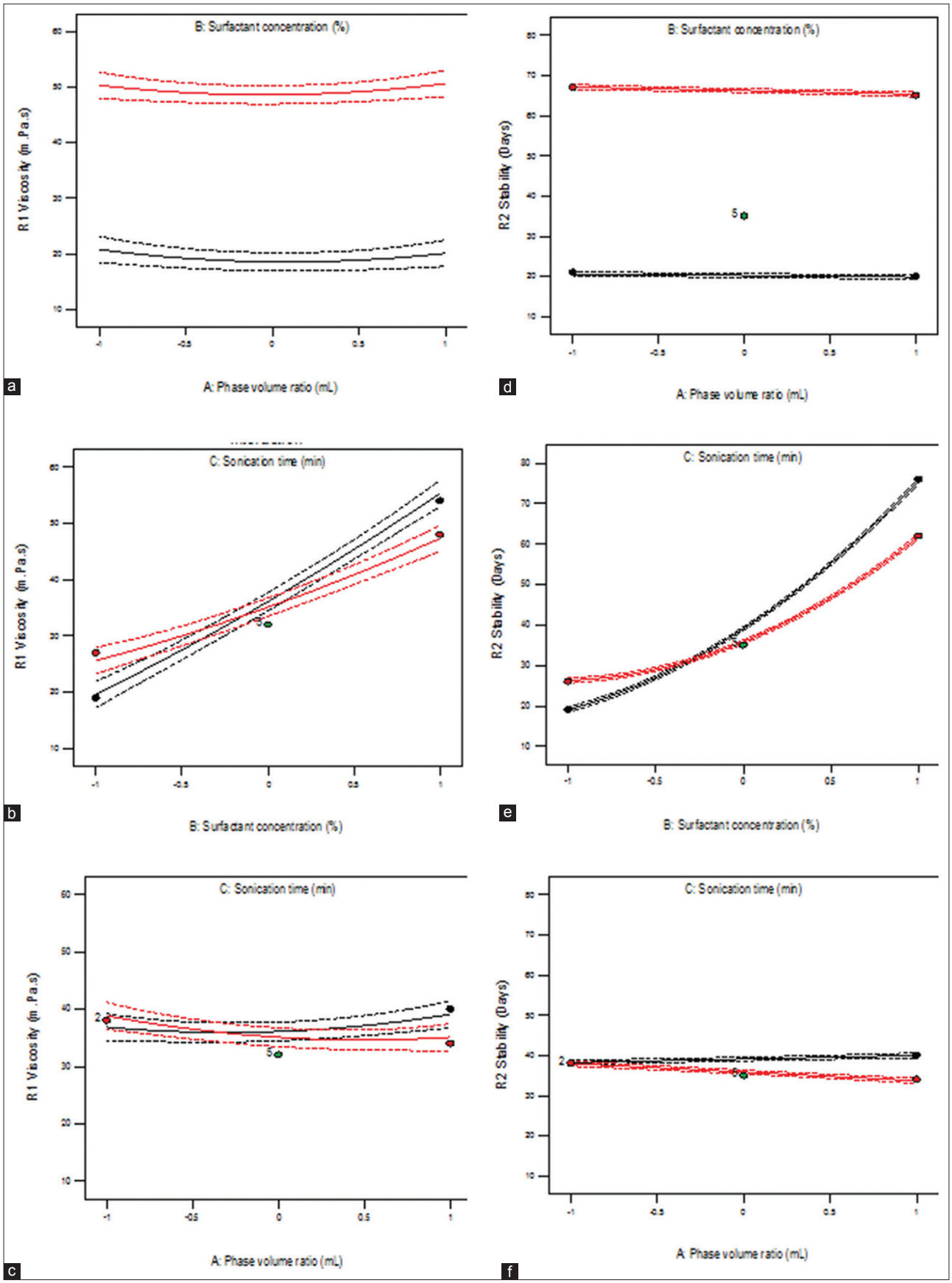

Figure 4: Effect of changes in (a and d) phase volume ratio and surfactant concentration (b and e) surfactant concentration and sonication time (c and f) phase volume ratio and sonication time on viscosity and stability of oil-in-oil nonaqueous nanoemulsions respectively

are added to the model, and the predicted R^2 will decrease when there are too many insignificant terms. If the model is significant, lack of fit insignificant, there is good agreement between adjusted and predicted R^2, adequate precision is over 4 and the residuals are well behaved; then the model provides good predictions for AVERAGE outcomes. A low R^2 indicates there is variation around the average predictions [Table 5]. From this information, the predicted R^2 decreased for response Y_2 denotes there is more insignificant effect of variable.

Contour plots and response surface analysis

Two-dimensional contour plots and three-dimensional (3D) response surface plots were plotted [Figures 5 and 6] for observing the interaction effects of factors on responses. All the relationships among the three variables are linear up to certain range; the effects of X_1 and X_2 with interaction effect on viscosity at a fixed level of X_3 [Figure 5] found linear up to 34 m.Pa.S, but below it, the plots were found to be non-linear indicating a non-linear relationship between X_1 and X_2.

In all the presented Figures 5 and 6, the third factor was kept at a constant level. All the relationships among the three variables are linear up to certain range of the effects of X_1 and X_2 with their interaction on stability at a fixed or zero level of X_3 [Figure 5a and b]. The plots were found to be linear up to 60 days, indicating a linear relationship between X_1 and X_2. Similarly, all values for remained dependent variables. It was determined from the contour plot that an optimum value of stability could be obtained with and X_1 level range from 50 to 60 and X_2 at 3:7 [Figures 5a and 6a]. It is evident from the contour plot that the higher level of the both X_1 and X_2 favors the stability of the formulation. When the coefficients values of two key variables, X_1 and X_2 were compared, the value for variable X_1 was found to be higher (Figure 6b and 6c), indicating that contributes the most to predicting the stability. Figure 5b and c shows the effect of X_1 and X_2 on the viscosity at a zero level of X_3. The plot shows a linear pattern which indicates X_2 and X_3 have a linear relationship.

From 3D response surface plot observed that the major effect on stability and viscosity was dependent on two factors as surfactant concentration and phase volume ratio [Figure 7]. As a phase volume ratio increases with decreasing concentration shown better stability also the viscosity of system was maximum. But as concentration of surfactant in emulsion increases stability decreases.

Checkpoint analysis

As a confirmation of this process, a new formulation was prepared at the optimum level of an independent variable and evaluated. The observed values of independent variables

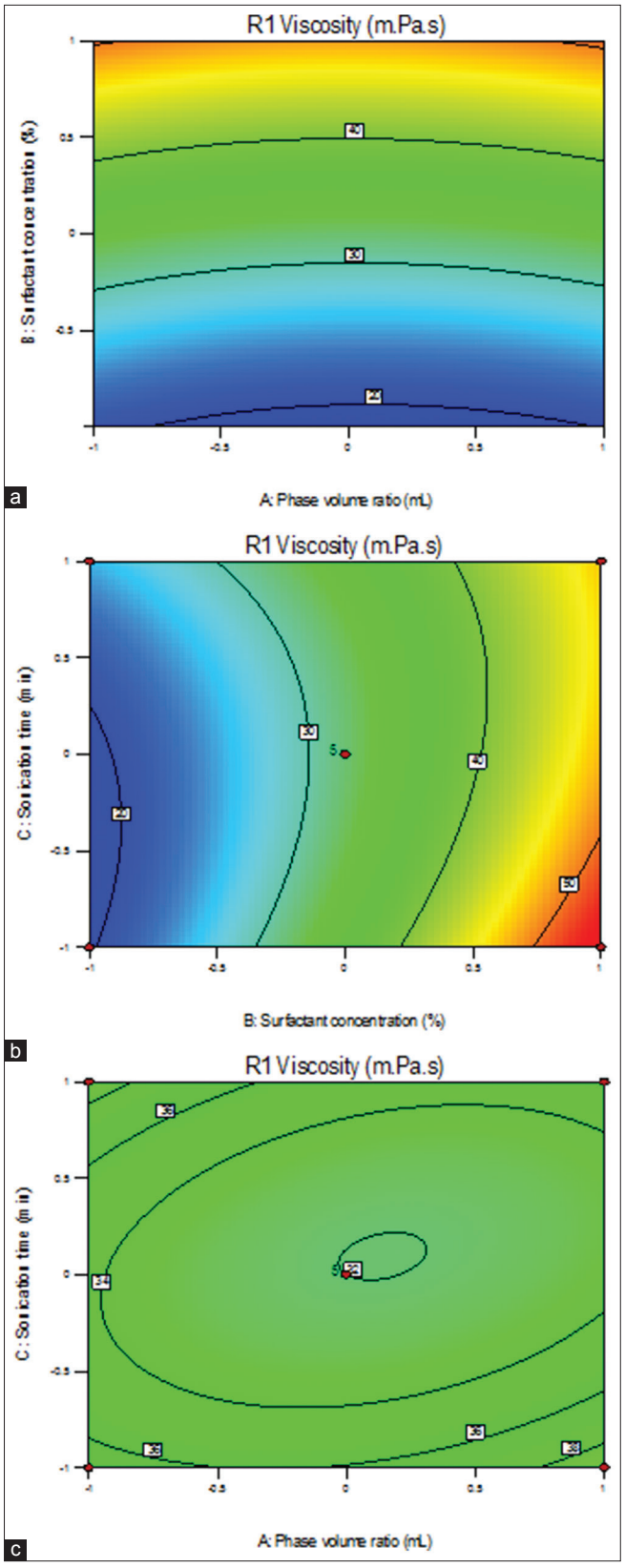

Figure 5: (a-c) Contour plot showing effect of (X1X2, X2X3, X1X3) phase volume ratio (X1) surfactant conc. (X2) and sonication time (X3) on viscosity of oil-in-oil nonaqueous nanoemulsions respectively

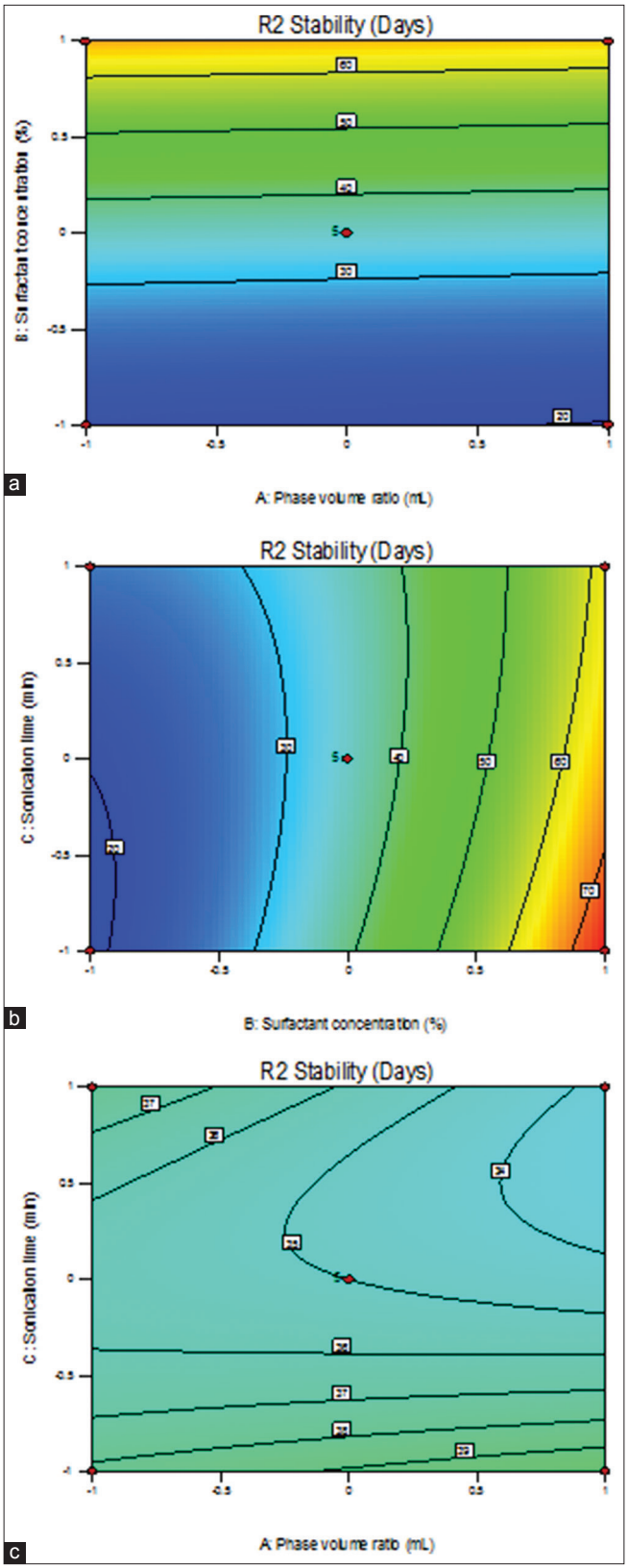

Figure 6: (a-c) Contour plot showing effect of (X1X2, X2X3, X1X3) phase volume ratio (X1) surfactant conc. (X2) and Sonication time (X3) on stability of oil-in-oil nonaqueous nanoemulsions respectively

shown in Table 6, which give a close agreement with predicted values. Three checkpoint batches were prepared and evaluated for viscosity [Table 6]. The result indicates that the measured values were as expected, when measured viscosity value was compared with predicted viscosity the difference were found to be insignificant. Thus, we can conclude that the obtained mathematical equation is valid for predicting viscosity.

Formulation of optimized OONE

The one optimal solution was suggested in terms of coded and actual values of independent variable for optimized formula, which clearly indicate that when sonication time increases viscosity of system first increases and then it get increased at 360 min. The same effect was showed on stability. In the case of phase volume ratio, it showed the linear effect on stability as well as on viscosity, but as surfactant concentration decreases shown maximum stability and viscosity.

As a rule of thumb, can assumed that fluid nanoemulsion result from low levels of the internal phase, whereas heavier nanoemulsion are the result of higher percentage of internal phase also a high internal phase ratio normally requires a high level of emulsifying agent, But in the system of nonaqueous emulsion this rule fail to explain that in the formulation of OONE, when there is increase in internal phase ratio from 1:9 up to 3:7 quantity of surfactant required is lower. For phase volume ratio 3:7 as per rule of thumb is more but it was found that when there is increase in the concentration of surfactant from 6% up to 10% system does not show stability, for 6% concentration and at 3:7 phase volume ratio system showed excellent stability. In preliminary study by taking a low level of internal phase volume ratio and lower surfactant concentration emulsion was prepared, then also system doesn't showed stability, this means rule of thumb is not applicable for such system. An optimized formula was obtained by State Ease DOE++ software.

Characterization of OONE

OOE was evaluated for its organoleptic properties appearing milky white, liquid, free from greasy less. The pH was found to be in the range 4.9-5.3.

Drug content

The cholecalciferol content of OONE was found to be 97.98 ±` 1.30 and 98.64 ± 0.3859 at 265 nm estimated using UV spectroscopy and high-performance liquid chromatography method.

Rheological characteristics

The optimized formulation of OONE was characterized for rheological parameters and found to be in the range [Table 7].

Flow type

The flow type of formulation determined by plotting a graph of viscosity versus shear rate from 1-100/s and 100-1/s

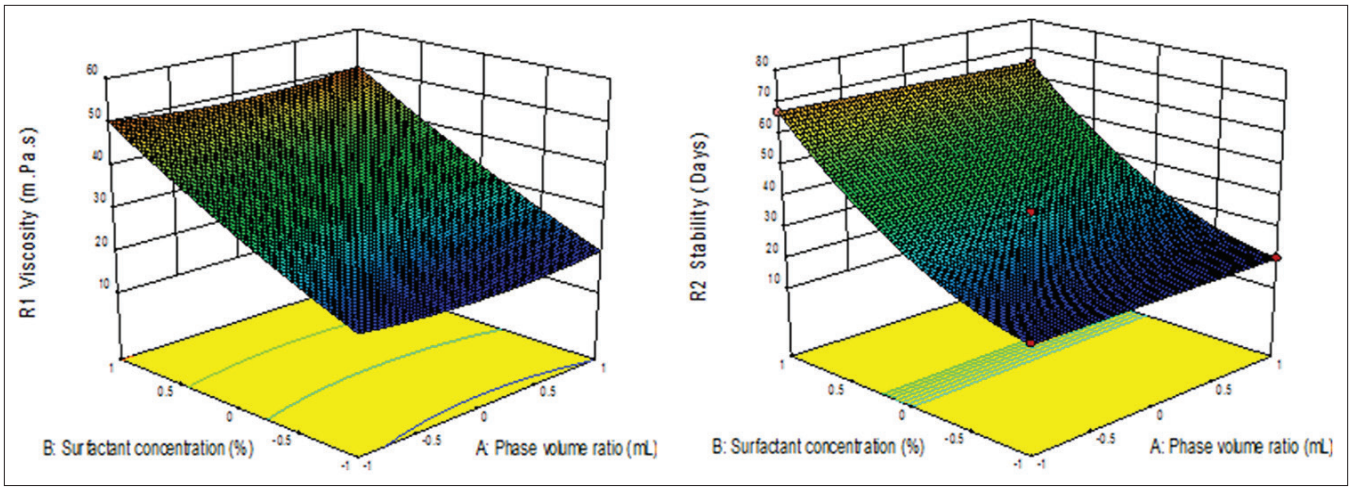

Figure 7: Response surface plot (three dimensional) showing the effect of phase volume ratio and surfactant concentration on viscosity and stability of oil-in-oil nonaqueous nanoemulsions respectively

Table 6: Checkpoint analysis

Response	Mean	Median	Observed	SD	n	SE prediction	PI low	Data mean	PI high
R1 viscosity	32	32	-	1.149	1	1.26	29.0	54.0	34.9
R2 stability	35	35	-	0.327	1	0.36	34.1	76.0	35.8

SD: Standard deviation, SE: Standard error, PI: Polydispersity index

Table 7: Parameters of optimized formulation

Parameter	Minimum	Maximum	Average
Viscosity (m.Pa.S)	0	69.8	54.9
Torque (mNm)	0	0.7530	0.3643
Shear stress (Pa)	0	6.8172	3.2986
Shear rate (1/S)	0.9900	99.9900	50.4899
Speed (1/min)	0.3300	33.3300	16.8300
Kinematic viscosity (m^2/S)	0.0000	0.0001	0.0001
Density g/cm^3	1.000	1.000	1.000
Angular velocity (1/S)	0.000	0.000	0.000

linearly for 150 s. It was observed that material becomes less viscous as the rate of shear is increased is referred to as pseudoplastic, a term more commonly employed. The term pseudoplastic is reserved for that type of flow which becomes less viscous upon an increase in shear rate. The relationship between shear stress and shear rate represented by graph called consistency curve or rheogram by plotting shear stress (F) on x-axis and shear rate (G) on y-axis, this curve provide information regarding flow property. In general, Newtonian flow shows linear relation in shear stress and shear rate, but in the case of non-Newtonian pseudoplastic material as shear stress increases progressively shear rate also increases but it is not in a linear manner. Hence, the viscosity of formulation was goes on decreases with increase in shear rate shown in Figure 8 and as shear stress increases there was an increase in shear rate, but it not observed in linear from the graph shown in Figure 9. Therefore, it was concluded that formulation exhibits (pseudo plastic) non-Newtonian flow property.

Viscosity

It is important parameter considered for evaluation of stability of system in relation to creaming, flocculation, and coalescence. The instability in the means of flocculation, globules comes to each other leads to form colonies in external phase this mainly depends on viscosity, globule size and surfaces charges, if the viscosity of system is more, causes immobilization the globule and thus flocculation prevented. If the viscosity of system is less them creaming observes, it may be downward or upward depending on density of internal phase. Hence, the viscosity of system is more which may lead to decrease in creaming. The viscosity of formulation was measured by increasing and decreasing shear rate from 100-1/s and 1-100/s linearly for 150 s, because it not possible to predict the viscosity at single value, so the average of the viscosity was taken by increasing (1-100/s) and subsequently decreasing (100-1/s) shear rate.

From preliminary study and experimental design, it was found that only cyclomethicone and with higher phase volume ratio gives stable emulsion with SO and CO while all other surfactants fail in formation of stable and viscous emulsions. It was observed that the surfactant concentration decrease and phase volume ratio increases viscosity of the emulsion increases. Depending on Surfactant concentration and phase volume ratio viscosity of OONE was varied from 0 to 70 m.Pa.S. The viscosity optimized of OONE formulation was found to be 54.9 m.Pa.S [Figure 10].

Thixotropy

Thixotropy is a property exhibited by non-Newtonian materials; they return to their original viscosity after lag time when applied shear stress is removed. This is the useful property for the topical formulations that ideally should have a high consistency in the container, yet pour or spread easily

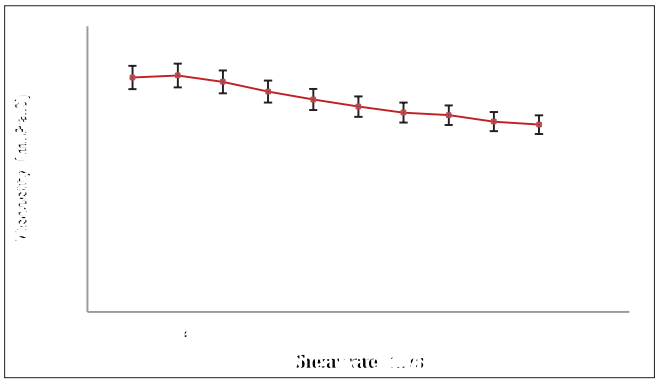

Figure 8: Schematic representation of structural change when shear applied

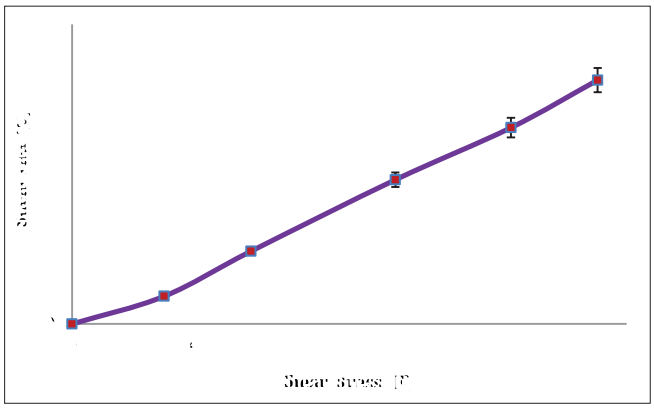

Figure 9: Consistency curve showing flow behavior of oil-in-oil nanoemulsion

Figure 11 shows that measured ascending and descending curves are combine showing that the formulation required less time for regaining its original viscosity. The NANE undergoes gel-to-sol transformation. The area between two curves (hysteresis area) defines the extent of the time dependent flow behavior. The smaller hysteresis area in Figure 11 shows less time is required for the regaining the original viscosity. After this lag time, droplets of the emulsion come into contact of each other by random Brownian movement and regain its original 3D network through numerous points of contact.

Globule size analysis

The size and size distribution analysis was performed on the selected formulation using Beckman Coulter nanosizer. A graphical representation of particle size distribution of freshly prepared oil-in-oil anhydrous emulsion [Figure 12] shows a broader globule size distribution and mean globule size intensity was 1155 nm. PI of system was found to be 0.675. Size is the small Brownian motion fast and scattering fluctuation is strong and size is large Brownian motion small and scattering fluctuation moderate. The PI 0.08-0.7 refers to a mid-range polydispersity, it is the range over which the distribution algorithm best operates over. Care should be taken in interpreting results as the sample may not be suitable for the technique, e.g., a sedimenting high size tail may be present.

Zeta potential

The significance of zeta potential is that its value can be related to the stability of colloidal dispersions. The zeta potential indicates the degree of repulsion between adjacent, similarly charged particles in the dispersion. For molecule and particles that are small enough, a high zeta potential will confer the stability, i.e. the attraction exceeds repulsion

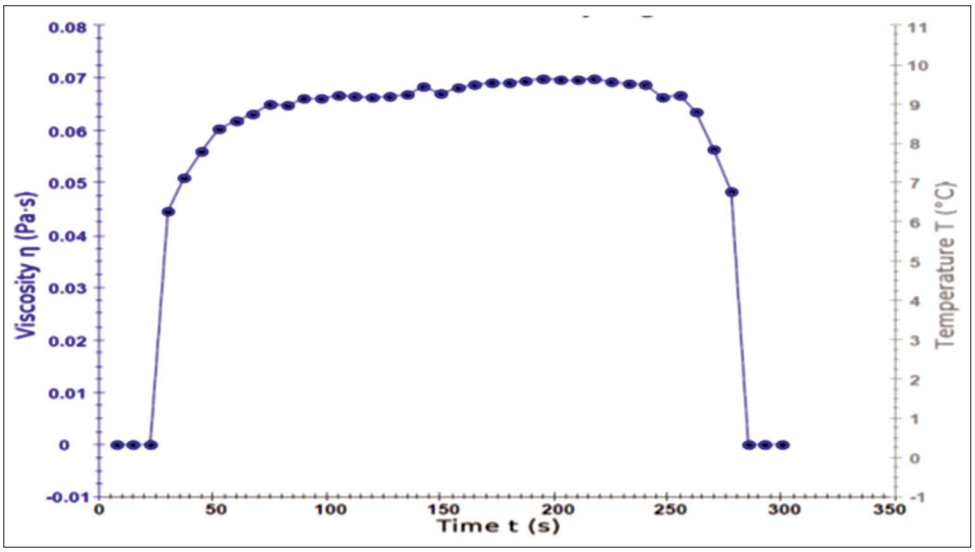

Figure 10: Viscosity diagram of optimized formulation

and the dispersion will break and flocculate. Hence, the NANE shows the zeta potential 0.164 having low potential but having better stability. The significance of zeta potential in NANE is that its value can be related to the stability of colloidal dispersion [Figure 13]. The zeta potential indicates the degree of repulsion between adjacent, similarly charged particles in the dispersion.

In vitro drug diffusion/permeation

The *in vitro* drug release was performed using vertical Franz diffusion cell. The drug release rate is very slow; only 6.69% of the cumulative drug is released within a period of 8 h in phosphate-buffered saline buffer pH 5.0 [Figure 14] while 9.44% cumulative drug diffused within a period of 4 h. The observed value of Permeability coefficient Kp (cm/h) through the membrane and Steady-state flux *Jss* (μg/cm²/h) was 3.073 ± 0.1596 and 0.1522 ± 0.022 within a period of 8 h and 4 h, respectively. Cholecalciferol having time-dependent solubility therefore release for 8 h in less as compare to 4 h which showed significant solubility and subsequently permeation. Formulation contains drug dissolved in oil phase/lipid also surfactant is present in it, if we consider *in vivo* condition presence of surfactant causes more solubility of the drug. The rate of drug release was depended on two factors; first, the rate of release from the SO internal phase to CO external phase, and second, the rate of release from CO to buffer system.

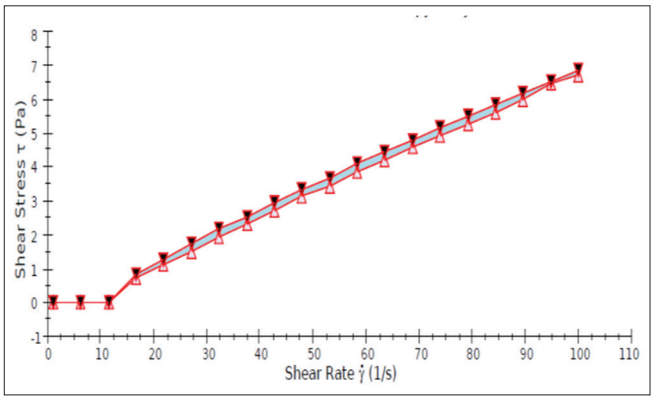

Figure 11: Flow curve of oil-in-oil nanoemulsion showing thixotropy behavior

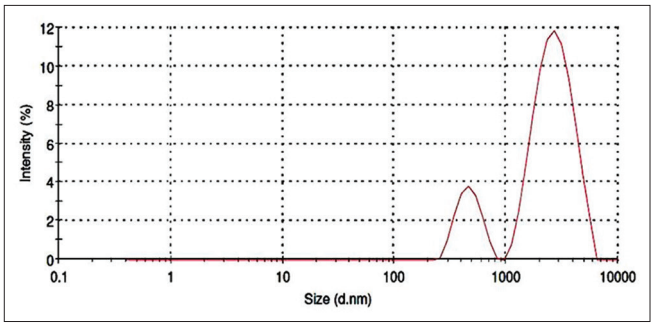

Figure 12: Globule size distribution of optimized anhydrous nanoemulsion

Drug retention study

To quantify the amount of cholecalciferol deposited in the dermis, drug retention study was carried out. Drug retention study shows that after 8 h 36% of drug was retained in the skin. This indicates that NANE decreases the permeation of cholecalciferol and enhances its retention in dermis which is useful for its topical effect on psoriasis. Retention of the cholecalciferol in skin may be due to the lipophilic nature of the NANE vehicle. CO in the external phase is present up to 50% of the total formulation. It has a greater affinity towards the dermis which is hydrophobic in nature. Hence, the vehicle is entrapped in the hydrophobic membrane which results in slow release of cholecalciferol and greater retention in skin.

Stability studies

Agitation test

Droplets of the emulsion exhibit Brownian movement and it is believed that no coalescence of droplets takes place unless droplets impinge upon each other owing to their Brownian movement. Agitation can contribute to the energy which impinges two droplets on each other. After agitation on a reciprocating shaker for 24 h, there was no phase separation hence were taken for centrifugation test

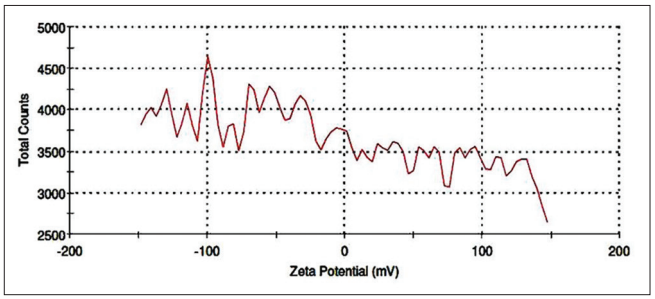

Figure 13: Zeta potential of anhydrous nanoemulsion

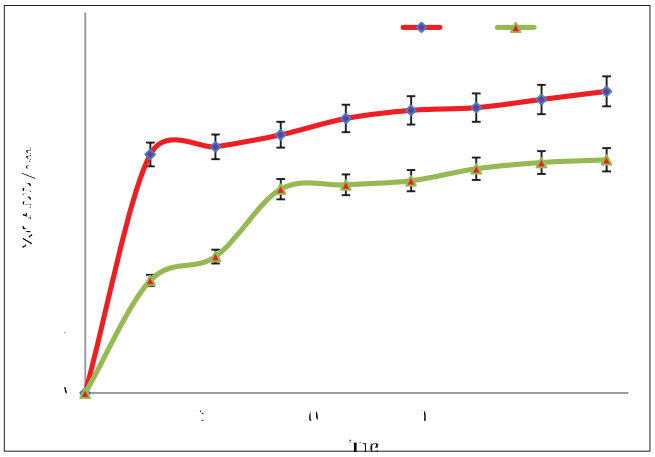

Figure 14: *In vitro* drug diffusion/permeation study of optimized formulation phosphate-buffered saline pH 5.0 for 8 h and 4 h

and concludes that it has good stability and can withstand the mechanical forces during the transportation and handling [Table 8].

Centrifugation test

Stokes' law shows that creaming is function of gravity, shows linear relation in gravity and rate of sedimentation, increase in gravity accelerates separation. Emulsions always contain materials of differing specific gravities. Creaming is one of the first signs of impending emulsion instability and should be taken quite seriously. A good test method to predict flocculation and downward creaming is centrifugation. Optimized formulations were centrifuged at 3500 rpm for 30 min and it was observed that the formulations that did not show any phase separation hence were taken for freeze-thaw stress test [Table 8].

Freeze thaw cycles

Freeze-thaw testing is conducted by exposing the product to freezing temperatures (approximately −10°C) for 24 h, then allowing to thaw at room temperature for 24 h. The sample is then placed in a higher temperature (approximately 45°C) for 24 h, and then placed at room temperature again for 24 h. The sample is analyzed for significant changes as the phase separation. This completes one cycle. The emulsion was repeated this test through 3 cycles [Table 8].

Accelerated stability study

The physical and chemical stability of oil-in-oil formulation was evaluated using accelerated stability studies. During studies, the formulation was kept at three temperature condition: Low temperature (5°C), moderate temperature (25°C/RT), and high temperature (45°C). The formulation was monitored for changes in color, viscosity and drug content for the period of zero/initial to three months.

Stability study at 5°C

It observed that formulation was quit sensitive to the low temperature. A large globule occurs before a period of 2-month. So stability study at low temperature was done up to 1 month after it was terminated [Table 9].

Stability study at 25°C

At low temperature, due to the property of CO becomes more viscous therefore emulsion becomes more viscous but particle size stay as it is or increases, if the time period goes

Table 8: Stability study of OONE under different stress condition

Test	Conditions	Duration	Observation				
			Phase separation	Creaming	Cracking	Phase inversion	Stability
Agitation	On rotary shaker 60 cycles/min	24 h	No	No	No	No	Yes
Centrifugation	2000 rpm	2 h	No	No	No	No	Yes
Freeze-thaw cycles	Three cycles between refrigerator temp.-RT–45°C	6 days	No	No	No	No	Yes

OONE: Oil-in-oil nanoemulsion

Table 9: Stability study of optimized formulation at different temperature condition

Temp condition	Duration (month)	Stability	Color	Viscosity (m.Pa.S)	Drug content %
5°C	Initial	Stable	Milky white	37.0	97.98±1.30
	1	Stable	Milky white	44.5	96.94±1.22
	2	Separation	Upper layer transparent	-	-
	3	-	-	-	-
RT	Initial	Stable	Milky white	54.9	97.98±1.30
	1	Stable	Milky white	44.5	96.98±1.25
	2	Stable	Milky white	42.7	96.81±1.20
	3	Stable	Milky white	37.0	96.75±1.17
45°C±2°C	Initial	Stable	Milky white	54.9	96.84±1.20
	1	Stable	Milky white	10.15	96.50±1.10
	2	Separation	Upper layer transparent	-	-
	3	-	-	-	-

Each value is average±SD (n=3). SD: Standard deviation

on increasing separation was observed means globule size increases.

Stability study at room temperature

At room temperature emulsion showed good stability over 3 months, the viscosity of emulation was slightly decreases and it indicates that there is slightly increase in the globule size. The drug content of formulation does not decrease significantly which indicates chemical stability of emulation does not changed [Table 9].

Stability study at 45°C

It observed that formulation was sensitive to the high temperature. A distinct phase separation occurs before a period of 2 month. So stability study at high temperature was done up to 1 month after it was terminated [Table 9]. As the temperature increase viscosity of emulsion decrease due to decrease in viscosity globules trying to come with each other's which may causes flocculation, also increase in temperature may destroy emulsifier film around the globule leads to increase globule size by decrease number of globules. As globule size increase distinct phase separation was observed after 1 month. From the stability studies, it was evident that OOE is stable at moderate temperature.

CONCLUSION

Pharmaceutically acceptable, non-irritating, and non-toxic excipients were selected based on potential screening and the result of screening of oils and surfactant, suggested SO as dispersed phase, CO as a continuous phase and cyclomethicone surfactant. The work depicts that it is possible to sustain effect of cholecalciferol by oil-in-oil anhydrous emulsion with castor and SO. Cholecalciferol loaded oil-in-oil anhydrous emulsion was successfully prepared by hand high-pressure homogenization method. Oil-in-oil anhydrous emulsion could be useful for where some drugs are either unstable in the presence of water or are insoluble in water and therefore cannot be incorporated into aqueous formulation. Based on stability and viscosity result oil-in-oil anhydrous emulsion was optimized and can be used at topical delivery with sustained effect. From the stability studies, it was evident that OONE is stable at room temperature and unstable at low and high temperature. The present investigation has shown that it is possible to topical effect of cholecalciferol by OONE with silicone and CO on psoriasis.

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