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Original Article

Self-Nanoemulsifying Isotretinoin Topical Formulation: Development, Optimization and Characterization, *in vitro* Permeation Study by Using Response Surface Methodology

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ABSTRACT

Aim/Background: The objective of this research was to develop a nano-emulsified formulation by using a central composite design for topical drug delivery. **Materials and Methods:** Isotretinoin-loaded nano-emulsified drug delivery system was prepared for topical application. Surface response methodology was used for the Design Of Experiment (DOE) using Central Composite Design (CCD). A combination of roseship and soyabean oil was used as the oil phase for the development of the formulation. Kolliphor® EL and Transcutol® P were used as surfactant and co-surfactant, respectively. The impact of independent parameters, percentage of oil phase (X1), and surfactant to co-surfactant ratio (X2) were checked at three levels of dependent variablesviz particle size and drug permeation. **Results:** *p*-value in ANOVA tables indicate a significant impact of both independent parameters on dependent parameters. R-sq and R-sq(adj) in both regressions is above 99%, confirming the model's suitability. The final formulation contains 22.07% of the oil phase. The ratio of surfactant and co-surfactant is 40:60. It helped to achieve a minimum particle size of ~280 nm and a maximum percentage permeation of ~ 70%. The optimized formulation was characterized for particle size, zeta potential, refractive index, polydispersity index, thermodynamic stability, and percentage drug permeation. **Conclusion:** The optimum percentage of the oil phase and surfactant to co-surfactant ratio improved the drug permeation when compared with the marketed formulation.

Keywords: Isotretinoin, Photostability, Solubility, *in vitro* drug Permeation.

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INTRODUCTION

Acne vulgaris is caused by the presence of comedones and is assumed to be a chronic disease. Comedones are formed due to the overproduction of sebum in the dermis layer of skin by sebaceous glands. Androgen, genes, bacteria, and other factors are responsible for the overproduction of sebum. Acne can be non-inflammatory or inflammatory lesions and be present in open or closed forms. Inflammatory lesions are referred to as papules, pustules, and nodules.[1-3]

Acne is most prevalent in adolescents and impacts nearly 85% of teenagers.[4,5] Adult or post-adolescent acne reduces to 12-14% and acne continues from adolescence to adulthood or sometime starts in the adult. Generally, the onset of acne is 11 years in girls and 12 years in boys.[6] Acne deteriorates the quality of life of the person. The negative impact on behavioural and social skills is more when compared with other medical conditions like asthma and epilepsy. The incidence of anxiety and depression is higher in individuals with acne as opposed to unemployed persons.[7] The common misbelief in the medical community and layman is that acne is only a teenage disease. Hence it is not considered as a chronic disease.[8]

A study has shown that 43% of the patients who consult any dermatologist have scars due to chronic acne. Retinoids are the basic treatment in acne therapy.[8] The target of acne treatment is minimizing scarring and reducing the duration of the disease. Acne treatment should also aim to reduce the psychological stress which is prevalent in 50% of acne patients.[9]

Retinoids' mechanism of action gained clarity post-discovery of nuclear receptors. Retinoids exhibit physiological effects on DNA transcription by binding with two specific receptors, namely Retinoic Acid Receptors (RAR} and Retinoid X Receptors (RXR}. For each of these receptors, there are three isotypes (α, β and γ). Different retinoids have different binding properties against these receptors. For example, tretinoin binds to RAR-y and RXR-a.

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recently, new-generation retinoids like adapalene and tazarotene have been found to be selective agonists of RAR~ and RARy. While trifarotene is selective for RARr agonist activity and has very less effect on RAR~ and RARa.[10,11]

Retinoid's efficacy in acne is due to their impact on intranuclear retinoid receptors. A retinoid is a molecule that binds to RAR receptorss and activates them. Activation of retinoic acid-responsive genes resulting in specific skin responses. Retinoids are known as the first-line agents to manage any abnormal follicular differentiation observed in acne. Abnormal follicular differentiation can cause the loosening of micro comedones. This results in sebum reaching the skin surface, eventually preventing pilosebaceous unit obstruction. Additionally, retinoids decrease the expression of Toll-Like Receptor (TLR}-2, which cause an increase in acne lesion. Retinoids can also lead to sebaceous gland atrophy, thus decreasing sebum production, thus stopping inflammation caused by sebum-dependent cutibacterium.[12,13]

Tretinoin is the first topical retinoid approved for clinical use. Some new retinoids are also used for acne, particularly adapalene and tazarotene. Adapalene can be sold as over the counter drug. Some fixed dose combinations are also available in the market like with clindamycin or benzoyl peroxide coupled with a retinoid have also been introduced. Due to the unique structure of adapalene's it is resistant to oxidation which eases its combination with benzoyl peroxide.[14]

Nanoemulsions are lipid-based and have the advantage of a colloidal drug delivery system which encloses, protects, and distributes lipophilic bioactive molecules.[15] Nano emulsion provide important advantages for the formulation of hydrophobic drug compound, by increasing the solubility and bioavailability.[16]

MATERIALS AND METHODS

Materials

Isotretinoin of analytical grade (CAS# 472-61-7) with a purity of 99.7%, was received as a gift sample from Nikhil chemicals. Kolliphor® EL (Macrogolglycerol Ricinoleate) and Transcutol® P (Ethoxydiglycol–CAS# 111-90- 0) were purchased. Roseship oil, soyabean oil, HPLC-grade methanol and ethanol were received from Thermo Fisher Scientific. Deionised water was produced using a Milli-Q RC apparatus (Millipore Corporation, Bedford, MA, USA).

Methods

Solubility Studies

Isotretinoin solubility was evaluated in different oil combinations and surfactant/co-surfactant mixtures. Oil phase and surfactant mixture which showed maximum solubility of Isotretinoin were used for further development of nanoemulsion.[17]

Experimental Design

Response surface methodology was used for formulation optimization. Experiments were designed by central composite design by using Minitab version 21 statistical software.[18,19] Impact of independent variable oil % w/w (X1) and surfactant to co-surfactant ratio (X2) on dependent variable particle size (Y1) and percentage drug permeation (Y2) was eavaluated. The experimental design summary and levels of independent variables and dependent variable with constraints are shown in Table 1.

Nanoemulsion Preparation

Isotretinoin was added to a combination of roseship oil, soyabean oil and ethanol in a homogenizer. Organic solvent ethanol was evaporated in roto evaporator at 75°C. Subsequently, the mixture of Kolliphor® EL and Transcutol® P was added to the previous blend. The Prepared mixture was sonicated for 30 min to enhance the solubility of isotretinoin. After the sonication prepared blend was mixed for 5 min. At last prepared dispersion was added dropwise to distilled water and nanoemulsion was formed at room temperature.[20,21]

Particle size measurement

Particle size was measured at room temperature by Dynamic Light Scattering (DLS) using Malvern zetasizer. The particle size was measured with a disposable capillary cuvette connected with electrode. Nanoemulsion was diluted to 100-fold with distilled water to circumvent multiple scattering. Dilution was done just before the measurement.[22]

In vitro Skin Permeation (IVPT) Study

In vitro permeation study was executed by using Strat-M® membranes. Strat-M® membranes are widely used as an alternative to human skin for penetration testing. A 25 mm Franz diffusion cell with a 20 mL receiver compartment was employed in this study. Strat-M was mounted on cells and sandwiched between the donor and receptor compartments with the help of locking nuts. About 2 mL of formulations were placed on a diffusion membrane. Receptor fluid was placed in the receptor chamber. Samples were collected at predefined time points (0.25, 0.5, 1.0, 2.0, 4.0, 8.0 and 12 hr) in scintillation vials to determine the amount of Isotretinoin permeated through Strat-M. Receptor media was replaced after each sample collection to maintain the sink condition. The temperature of the receptor chamber was maintained at 37°C.[23-25]

Optimization and evaluation of formulation

The degree of freedom, F-ratio, and *p*-value for all factors and their interactions were determined by using Minitab software to analyse the variance of models computed for the measured responses. The most appropriate model was chosen according to

the data obtained. A *p*-value, below 0.05 indicates the significant impact of independent variables on responses. The determination coefficient, predicted (R^2), adjusted (R^2) and %CV (Coefficient of variance) values have been used to determine model suitability. In this research, the criteria used to identify the optimized formulation were minimal particle size and maximum drug permeation.[26,27]

Characterization of optimized formulation

Particle Size and Polydispersity Index

Nanocarriers must be characterized for particle size distribution or "Polydispersity Index" (PDI). It is also known as the heterogeneity index. PDI is a number calculated using a two-parameter fit in the correlation data. An index value smaller than 0.05 is mainly seen with highly monodispersed standards. Particle size was measured at room temperature by Dynamic Light Scattering (DLS) using Malvern zetasizer.[28]

Zeta potential measurement

An electrophoretic mobility study was conducted to determine the superficial charge of the optimal formulation. This parameter predicts the phase separation potential of a spontaneously emulsified system. Zeta potential was measured with Malvern Zetasizer Nano ZS which can measure the potential between -120 to 120 mV. The zeta potential was measured at a temperature of 25°C and a steady angle of 90°. Samples were diluted about 100 times before measurement.[29]

Refractive Index

The refractive index of nanoemulsion is determined by using an abbes type refractometer. The refractive index of each sample was measured three times and the mean value and standard deviation were calculated.[30]

Thermodynamic Stability Studies

Thermodynamic stability test was conducted to assess the physical stability of the emulsion. Freeze thaw cycles were carried out three times. Samples were heated at a temperature of 40°C and cooled at 4°C. This was followed by centrifugation at 3500 rpm for 30 min. The formulation was observed for phase separation after each freeze-thaw cycle.[30]

RESULTS

The nano-sized transdermal drug delivery system enhances drug penetration in the skin via intracellular pathways and depots the drugs in the epidermis and stratum corneum.Solubility studies

Isotretinoin solubility was evaluated in various oils, oils combinations, surfactants and co-surfactants. The results of solubility studies are depicted in Figure 1. Oil phase, surfactants and co-surfactant were selected based on isotretinoin solubility. Maximum isotretinoin solubility (24.62 mcg/mL) was observed in a combination of roseship oil and soyabean oil among all oils tested for stability studies. If comparing the solubility of Isotretinoin in surfactants, solubility in Kolliphor® EL (42.21 mcg/mL) was found maximum and followed by transcutol P (32.87 mcg/mL).

Response surface analysis

Response surface methodology has shown good results in the statistical optimization of various parameters to obtain the desired response. Two parameters i.e., oil phase percentage (X1) and the ratio of surfactant and co-surfactant (X2) were selected as independent variables and investigated at three different levels. The observed responses of dependent variables (Y1 and Y2) against different levels of independent variables (X1 and X2) are given in Table 2. Table 2 depicts a complete 2^2 factorial design with four axial points and one centre point in the axial. The number of experiments required (N) were given by the expression 2^k (2^2 = 4; star points) + 2 k (2 x 2 = 4; axial points) + 1 (centre point). For the response surface method involving CCD, a total of 9 experiments were conducted for the two factors at three levels with one centre point. Polynomial regression modelling was performed on the responses of the corresponding coded values of the two different process variables and the results were evaluated.

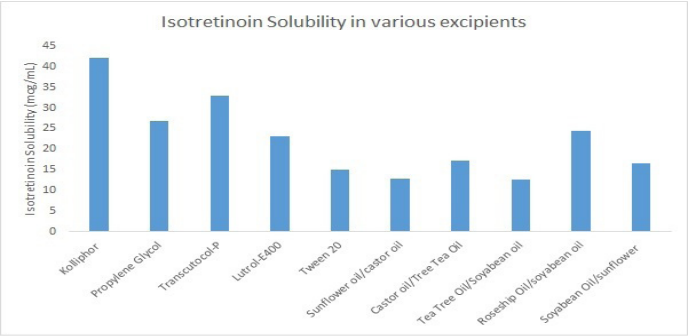

Figure 1: Isotretinoin Solubility in various excipients.

Table 1: Different levels of independent parameters.

Independent Variable	Levels			Dependent Variables	Constraints
	-1	0	1		
Oil phase %w/w (X1)	10	15	20	Particle Size (Y1)	Minimize
Surfactant and co-surfactant mixture (X2)	30:70	40:60	50:50	Percentage drug permeation (Y2)	Maximize

Table 2: The observed responses of dependent variable (Y1 and Y2) against different level of independent variable (X1 and X2).

Pattern	X1	X2	Y1	Y2
+−	1	-1	380.73	57.46
−−	-1	-1	621.38	31.11
0a	0	-1.414213562	543.43	41.21
0	0	0	354.91	63.35
A0	1.414213562	0	284.44	74.30
0A	0	1.414213562	308.59	52.36
a0	-1.414213562	0	535.47	38.72
−+	-1	1	365.23	45.65
++	1	1	269.41	64.31

Table 3: Relative Error for Design Precision.

Experiment No.	Percentage drug permeation (Y2)			
	Experimental Value	Experimental Value	Predicted value	Relative Error (%)
1	380.73	57.46	59.83	3.96
2	621.38	31.11	32.16	3.26
3	543.43	41.21	39.92	-3.23
4	354.91	63.35	63.53	0.28
5	284.44	74.30	72.35	-2.70
6	308.59	52.36	52.35	-0.02
7	535.47	38.72	38.65	-0.18
8	365.23	45.65	44.29	-3.07
9	269.41	64.31	65.28	1.49

$$\text{Relative Error (\%)} = \frac{\text{Predicted Value} - \text{Experimental Value}}{\text{Predicted value}} \times 100$$

Table 4: ANOVA regression model for Particle Size (PS) and percentage Drug Permeation (%DP).

Source	Degree of Freedom		Sum of squares		Mean square		*F* value		*p*-value	
	PS	% DP	PS	% DP	PS	% DP	PS	% DP	PS	% DP
Model	5		129507	1561.47	25901.4	312.29	219.55	60.09	0.000	0.003
X1	1		59768	1135.92	59768.1	1135.92	506.61	218.56	0.000	0.001
X2	1		61177	172.59	61177.2	172.59	518.56	33.21	0.000	0.010
X1*X2	1		5244	14.78	5243.9	14.78	44.45	2.84	0.007	0.190
X1*X1	1		1866	44.79	1865.6	44.79	15.81	8.62	0.028	0.061
X2*X2	1		3236	224.58	3235.8	224.58	27.43	43.21	0.014	0.007

The regression equation characterizing the influence of the two variables on particle size and percentage drug permeation was obtained using equations 1 and 2, respectively. A positive sign indicates a synergistic effect whereas a negative sign indicates an antagonistic effect.

The selected experimental design was validated by comparing the experimental values with predicted values and calculating relative error (%). The following equation was used to calculate the relative error. The results of relative errors are shown in Table 3. The relative error was in the acceptable range (±5%), confirming the design delivered precise results,

$$\text{Relative Error (\%)} = \frac{\text{Predicted Value} - \text{Experimental Value}}{\text{Predicted value}} \times 100$$

Particle size

The Analysis of Variance (ANOVA) values for the quadratic regression model obtained from CCD for particle size is presented in Table 4. The model was found to be statistically significant at 95% confidence level, with *F*-value of 512.59 and a very low *p*-value of < 0.001 for particle size. The interactions between X1 and X2 were significant at *p*=0.007 for particle size.

The obtained determination coefficients, R^2 and R^2 (Adj) for particle size were 99.73% and 99.27% respectively. It represents the model fitting reliability for the models. This showed about 99.99% and 99.75% of the respective variance is accredited to the variables and indicates a good level of significance for the models.

Equation 1: Particle size (Y1)= 354.9.223 + (-86.44)X1 + (-87.45)X2 + 36.21X1*X2 + 25.32 X1*X1 + 33.35 X2*X2

Response (Y1) was significantly affected antagonistically from both tested parameters. The above polynomial equation may be used to estimate the response from any certain value of independent variables. Both parameters had almost the same impact on particle size as their coefficients were almost the same (~ 86). The increase in particle size is equivalent to less co-surfactant, which means that increasing the co-surfactant proportion would result in decreased particle size.

Contour, 3D-surface, and cube plots are depicted in Figure 2 to demonstrate the impact of independent variables on particle size.

The optimization process was done to get the optimum level of X1 and X2 for particle size by using Minitab software. The anticipated goal for each parameter (percent content of oil phase (% w/w) and ratio of surfactant/co-surfactant) was selected "within" the studied range. The ideal solutions for the input variables, along with optimization plots are shown in Figure 2. Using the Response surface optimizer, the maximum response at 1.414 level of X1 was 284.44 nm for particle size and 74.30% drug

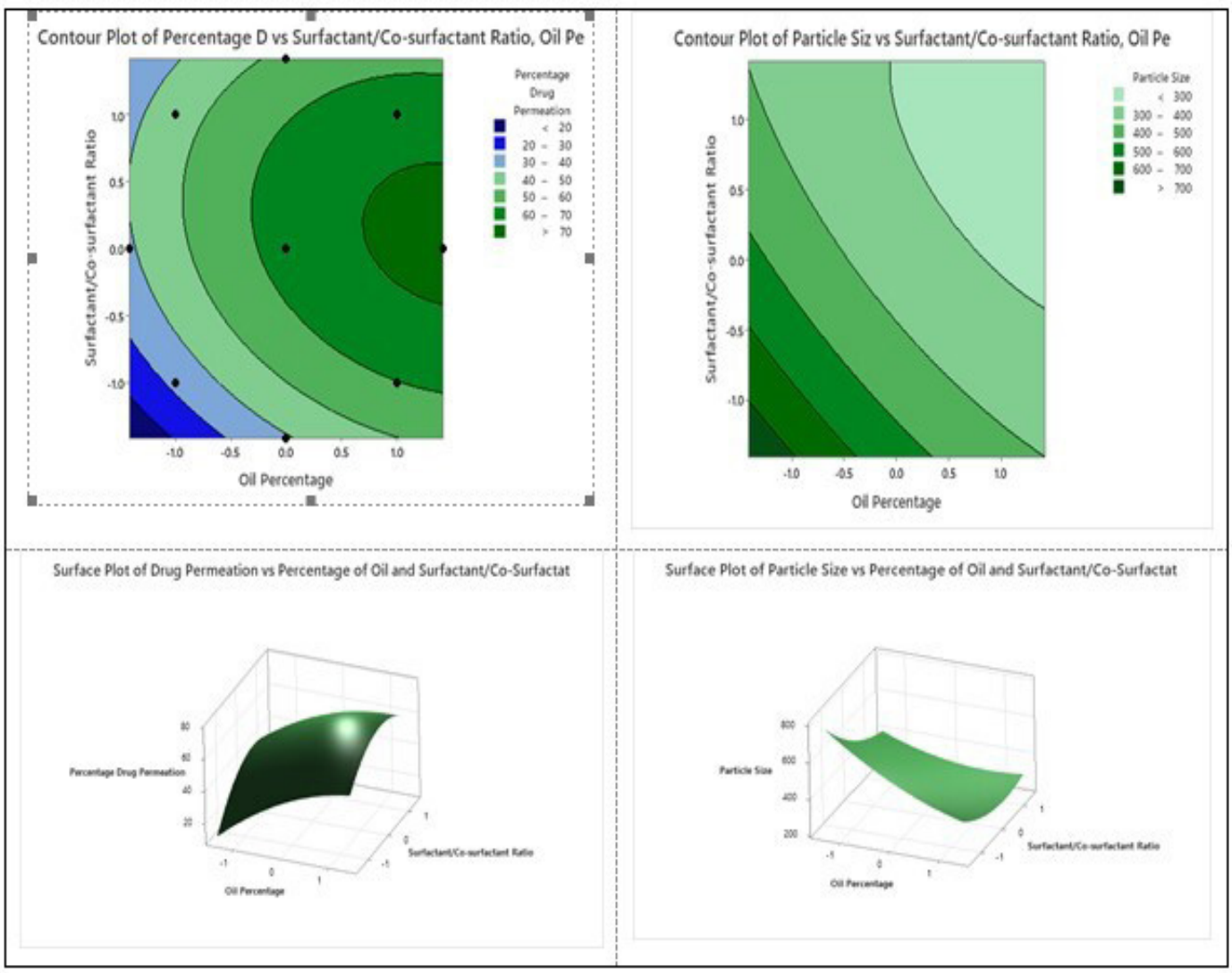

Figure 2: Contours and surface plots.

permeation. To confirm the suggested values, the formulation was prepared with recommended levels of independent variables. The values of the X1 and X2 from the response optimizer suggestion were calculated and optimized formulation was prepared by using them. The observed value of particle size was 279.27 nm and the percentage drug permeation was 71.19 in the developed formulation.

Percentage drug permeation

The Analysis of Variance (ANOVA) values for the quadratic regression model obtained from CCD for particle size is presented in Table 4. The model was found to be statistically significant at a 95% confidence level, with F-value of 60.09 and a very low *p*-value of < 0.001 for percentage drug permeation. The interactions between X1 and X2 were not significant at *p*-value 0.190 for percentage drug permeation.

The obtained determination coefficients, R^2 and R^2 (Adj) for particle size were 99.01% and 97.36% respectively. It represents the model fitting reliability for the models.

Equation 2: Percentage drug permeation (Y2)= 63.35 + 11.92 X1 + 4.65 X2 + (-1.92) X1*X2 + (-3.92) X1*X1 + (-8.79) X2*X2

Response (Y2) was significantly affected synergistically by both independent parameters. The above polynomial equation may be used to estimate the response from any certain value of independent variables. Oil percentage was the most relevant parameter as its coefficient (11.92) is bigger than the surfactant and co-surfactant ratio (4.65). The drug permeation is directly proportional to the oil percentage in the optimized formulation.

Contour, 3D-surface, and cube plots are depicted in Figure 2 to demonstrate the impact of independent variables on particle size.

The optimization process was done to get the optimum level of X1 and X2 for drug permeation by using Minitab software. Using the Response surface optimizer, the maximum response at 1.414 levels of X1 and at 0 levels of X2 was 284.44 nm for particle size and 74.30% drug permeation. To confirm the suggested values, the formulation was prepared with recommended levels of independent variables. The values of the X1 and X2 from the response optimizer suggestion were calculated and formulation was prepared by using them. The experimental value of percentage drug permeation was obtained as 71.19% which is like the predicted value. Hence the predicted and experimental values under optimized conditions are in close agreement

Table 5: Characterisation of optimized formulation.

Parameters	Particle Size (nm)	PDI	Zeta Potential	Refractive Index	Thermodynamically Stability	% Drug Release
1	279.47	0.0031	24.63	1.52	No phase separation	74.48
2	281.94	0.0022	23.04	1.55		67.31
3	282.32	0.0008	24.19	1.54		69.44
4	289.41	0.0009	25.57	1.52		71.51
5	274.63	0.0018	24.38	1.51		72.08
Mean	281.55	0.00176	24.36	1.53	NA	70.96
SD	5.35	0.00096	0.91	0.02		2.71
Min.	281.94	0.0008	23.04	1.51		67.31
Max.	289.41	0.0031	25.57	1.55		74.48

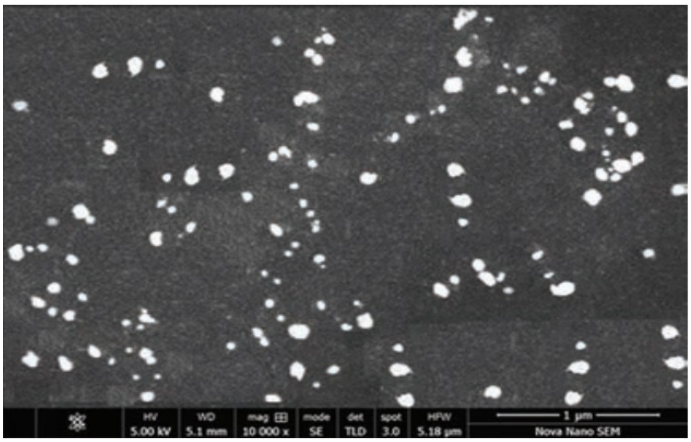

Figure 3: SEM micrograph for optimized nano formulation.

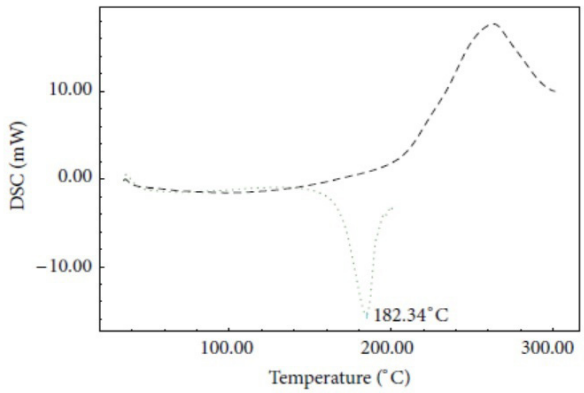

Figure 4: DSC thermograms of pure isotretinoin and Nanoemulsion.

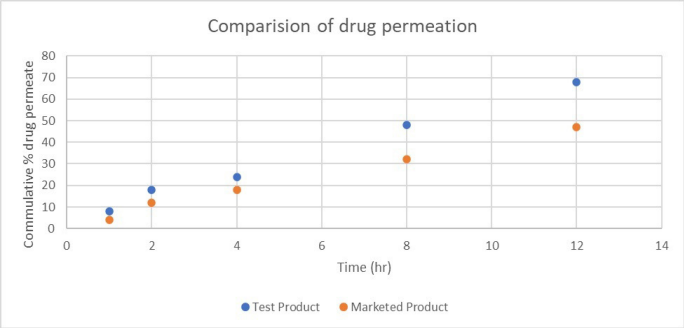

Figure 5: Percentage drug permeation comparison of optimized formulation and marketed formulation.

Characterisation optimized formulation

Particle Size and Polydispersity Index

The particle size and PDI were measured by 5 replicates after the preparation of the optimized formulation. The prepared formulation exhibited uniform size distribution. Developed formulations had a particle size below 285 nm and with a polydispersity index of less than 0.005, indicating a narrow distribution pattern and homogeneity. Results are shown in Table 5.

Zeta Potential

The zeta potential of formulation was determined after dilution with distilled water and results are summarised in Table 5. The relatively low value of zeta potential in the prepared nano emulsifying formulations may be due to the presence of a relatively higher amount of transcutol-p used in formulations so that the surface charge was contributed only by the co-surfactant.

Scanning Electron Microscopy (SEM)

Morphological analysis of prepared nano emulsion confirmed that particles were in nano metric range, authenticating the results of zeta sizer analysis. SEM micrograph exposed perfectly spherical shaped, smooth surfaced particles confirming the conversion of particles to less crystalline form (Figure 3).

DSC

Isotretinoin showed an endothermic peak at about 182.34°C, near to its melting point 173-178°C (Figure 4). However, peak of the drug was not observed in the optimized formulation, which means the drug was present in molecularly dissolved form.

Refractive Index

The Refractive index of the optimized formulation is shown in Table 5. The refractive index of each sample was measured three times and the mean value was 1.53 with a standard deviation of 0.02 indicating the fewer denser and clear formulation.

Thermodynamic Stability Studies

After the completion of three freeze-thaw cycles, samples were centrifuged. Samples were checked for their physical appearance. Formulations maintained their homogeneity and no phase separation was observed. Results are shown in Table 5.

In vitro permeation comparison with marketed formulation

In vitro, permeation comparisons between marketed and developed formulations were repeated five times. A comparison of mean drug permeation is shown in Figure 5. The results indicated that the permeability of the optimized formulation is significantly higher ($p<0.05$) in comparison to the marketed formulation.

DISCUSSION

The therapeutic potential of ISN has been investigated for a wide range of conditions. Administration routes investigated include oral and topical delivery platforms. Topical administration to the skin has been primarily for managing acne. Isotretinoin has shown great efficacy in the reduction of sebum from the sebaceous gland. In the current research, a central composite design from response surface methodology was adopted in developing and optimizing for topical treatment of acne. As independent variables, two parameters the percentage of the oil phase (X1) and the ratio of the surfactant to the co-surfactant (X2) were chosen and looked at three distinct levels. Both parameters showed an antagonist effect on the particle size at the same magnitude. The percentage of drug release was affected synergistically by both independent parameters which are oil phase percentage in formulation and surfactant to co-surfactant ratio. The surface plot indicates the curvature for both variables. The *p*-value for the statistical model applied was found to be < 0.001 which signifies the impact of independent variables on the observed responses. The obtained determination coefficients, R^2 and R^2 (Adj) for particle size and percentage drug release was more than 99% for both the responses. The optimum oil percentage of 22% w/w) with a surfactant-co-surfactant mixer ratio of 40:60 has shown the best results. The Optimised Obtained particle size was approx. 285 nm with approximately 70% drug permeation at the above-said level of the independent variable. The model was also validated by calculating the relative error of experimental and predicted values which is within the acceptable range (±5%). The developed formulation was also checked for thermodynamic stability of nano-emulsion after multiple freeze-thaw cycles and found positive results. The optimized formulation has a particle size of approximately 280-290 nm. The prepared formulation exhibited uniform size distribution with a polydispersity index of less than 0.005, indicating a narrow distribution pattern and homogeneity. The relatively low value of zeta potential in the prepared nano emulsifying formulations was due to the presence of a relatively higher amount of transcutol-p used in formulations

so the surface charge was contributed only by co-surfactant. Morphological analysis of prepared nanoemulsion confirmed that particles were in nanometric range, authenticating the results of zeta sizer analysis. *In vitro* permeation study was executed by using Strat-M® membranes. The percentage drug permeation was almost 70-75% in the finalised formulation. The formulation developed in the current research was compared with the marketed formulation. The graph was plotted for the comparison which shows test formulation has better skin permeability when compared with the marketed formulation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

ABBREVIATIONS

ANOVA: Analysis of variance; **CCD:** Central composite design; **CV:** Coefficient of variation; **DLS:** Dynamic light scattering; **DNA:** Deoxyribo nucleic acid; **DOE:** Design of experiment; **DSC:** Differential scanning calorimeter; **ISN:** Isotretinoin; **IVPT:** *In vitro* Skin Permeation Study; **PDI:** Polydispersity Index; **RAR:** Retinoic acid receptors; **RXR:** Retinoid X receptors; **SEM:** Scanning electron microscope.

SUMMARY

Nanoemulsion formulation was developed to increase the permeability of Isotretinoin. Independent variables were identified and experiments were designed by using central composite. A combination of roseship and soyabean oil were used as oil phase for development of the formulation. Kolliphor® EL and Transcutol® P were used as surfactant and co-surfactant, respectively. Impact of independent parameters, percentage of oil phase (X1) and surfactant to co-surfactant ratio (X2) were checked at three levels on dependent variables, like particle size and drug permeation. *p*-value in ANOVA tables indicate significant impact of both independent parameters on dependent parameters. R-sq and R-sq(adj) in both regression is above 99% which confirms the suitability of the model. Final formulation contains 22.07% of oil phase. Ratio of surfactant and co-surfactant is 60:40. It helped to achieve a minimum particle size of ~280 nm and a maximum percentage permeation of ~ 70% (Table 5). The optimised formulation was characterised for particle size, zeta potential, refractive index, polydispersity index, thermodynamic stability, and percentage drug permeation. Optimum percentage of oil phase and surfactant to co-surfactant ratio improved the drug permeation when compared with marketed formulation.

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