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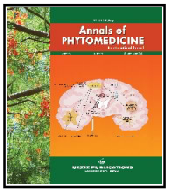

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Preformulation and synthesis of retinol acetate nanoemulsion for skin disorder

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Abstract

The objective of this study is to perform preformulation and synthesis of nanoformulation of drug for skin disorder. Preformulation study helped in qualitative estimation of drug and excipients used. The formula optimized by Box Behnken design. The formula matched with predicted and observed values obtained with $p<0.001$. About 19 formulations of nanoemulsion made and characterized by various parameter like zeta potential, particle size distribution and FESEM analysis gave information about globule size and shape for nanoemulsion delivery. Optimized formula undergone biological evaluation including skin irritation test and concluded that nanoemulsion had nonirritating, non-itchy, compatible, smooth and potential to deliver drug at targeted side, with minimum side effects.

1. Introduction

The life span of normal cells are about 44 days but in disease condition like skin disorders and psoriasis, it shade off in 1-2 days due to autoimmune reaction. Skin disorders affect not only physical but also mental and psychological health. The annual economic cost of moderate-to-severe plaque psoriasis is $1.7 billion all over the world (Subramonian and Walter, 2021). The drug retinol acetate had low molecular weight, 328.49 dal, low melting point, 57-58 degree celsius, bioavailability was 70-90%. These properties made suitable candidate for nanodrug delivery for skin disorders. Retinol acetate is first line of drug and had potential to treat skin disorder. Systemic administration of vitamin A derivates has moderate effects, but its topical form acts synergistically when combined with phototherapy (Orfanos *et al.*, 1979). Many retinoids and its derivatives like retinol, retinaldehyde, and retinyl palmitate used not only on skin disorder but also antiaging effects. They found in various research studies that the tazarotene and adapalene showed antiageing effects (Milosheska and Roskar, 2022), vitamin A bound and activated retinoid receptors (RARs) and induced cell differentiation and apoptosis. It plays an essential role including proper functioning of the retina, growth and differentiation of target tissues (Hughes *et al.*, 1997). Retinoids regulate the cellular mechanism against degradation and inhibiting metalloproteinases activity (Zasada and Budzisz, 2019). It binds and activates retinoid receptors, thereby inducing cell differentiation and apoptosis (Nolan *et al.*, 2010). Retinoic acid (RA), a retinol (vitamin A) metabolite, binds to nuclear RA receptors (RARs), which regulate chordate animal development (Ghyselinck and Duester, 2019). Use of newer drug therapies like liposomes, solid lipid nanoparticles (SLNs), microemulsions, and nanoemulsions (Katare *et al.,* 2010). The diverse systemic medications like retinoids, cyclosporine and methotrexate were the choice of drugs for topical and systemic therapy (Lebwohl and Ali, 2001). Calcipotriol was more effective than calcitriol in psoriasis (Ashcroft *et al.*, 2000). Monotherapy using UV or systemic medications for the treatment of skin disorders including psoriasis, (Torsekar and Gautam, 2017; Nakamura *et al.*, 2016) focused on recent advancements in targeted phototherapy methods for scalp psoriasis. Nanotechnology is a new dimension and has great potential to treat disease condition at targeted and specific site. The types like nanoparticles, nanocrystals, nanorods, nanocubes, quantum dots, *etc.,* delivered various drugs. Nanoemulsions and their synthetic processes evaluated in this review (Elzayat *et al.,* 2021). Ashaolu (2021) explored nanotechnology with all safety measures. Souto *et al.* (2022) reported that the drugs can be delivered over lipophilic barriers using nanoemulsion and microemulsions. Advances in material design and engineering on nanomaterials well explained by Fu and Kao (2010). Dash *et al.* (2010) provides an overview of the mathematical models and the kinetics of drug release from drug delivery systems. Mukherjee (2013) described the targeted and pharmacokinetics of drug delivery system. Srinivasan and Murali (2023) researched on creating a new route at nanoscale for effective drug delivery.

2. Materials and Methods

2.1 Drugs

The retinol acetate was pale yellow to orange crystals, empirical formula was $C_{22}H_{32}O_2$ with 99% purity with HPLC grade and purchased from sigma aldrich, Mumbai, India for nanoemulsion preparation. The nanoemulsion of retinol acetate was pre-optimized by Box Benkhen design prepared by emulsification method using probe sonication method. Water used in experiments was double filtered deionized.

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2.2 Excipients

Excipients like formaldehyde, span 20, span 80, tween 20, tween 80, hydrochloric acid, sodium hydroxide, sodium chloride, methanol (HPLC grade), octanol, soybean oil, methyl paraben, sodium dihydrogen phosphate, potassium hydrogen phosphate, formalin standard irritant, moisturizing cream and many more purchased from Loba Chemie Private Limited, Mumbai, Maharashtra, India.

2.3 Preformulation study

Retinol acetate was selected as drug, soybean oil for oil phase, span 20 and span 80 in the ratio of 8:2 and methyl paraben as preservative was selected for the formulation. The formula was optimized by response surface methodology using Box Behnken design with design expert software version 12.

2.3.1 Organoleptic properties of drug

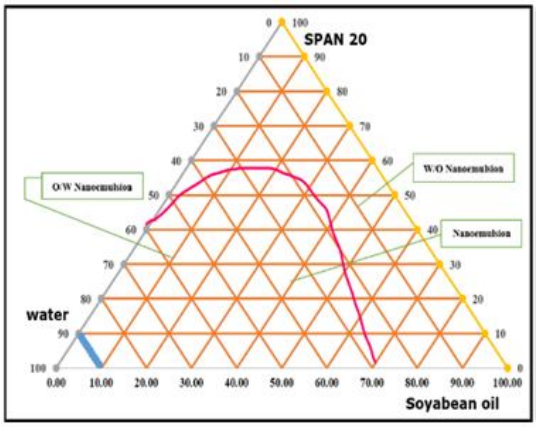
Organoleptic properties like color, odor, taste, and nature were observed physically. Retinol acetate was light yellow in colour, odourless, bitter in taste and rod like granules was given in Table 1.

Table 1: Organoleptic properties of retinol acetate

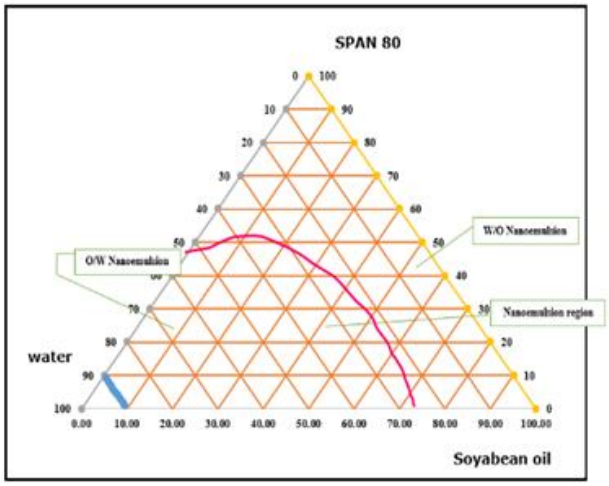
S. No.	Properties	Organoleptic characters
1	Colour	Light yellow to orange colour
2	Odour	Odourless
3	Taste	Bitter
4	Nature	Rod like granules

2.3.2 Ternary phase diagram

Further, the effectiveness of surfactants overserved in making ternary phase diagram. The concentration of surfactants and oil determine for globule size and self-emulsification time. For the selection of surfactants, pseudoternary phase diagram mends the plot between 3 phases span 20, water and soybean oil. The ternary diagram showed nanoemulsion regions where right side of ternary showed w/o nanoemulsion region while left side of Figure 1 (A) showed o/w emulsion in which the blue dots represented the region of Figure 1, o/w nanoemulsion (B) (Gurpreet and Singh, 2018). In the pseudoternary phase diagram given in Figure 1, there are 3 variables, one was SPAN 20 depicted in (A) while span 80 in (B), second was soybean oil and third was water.



(A)



(B)

Figure 1: Pseudoternary diagram of SPAN 20 A; SPAN 80 B with soybean oil and water.

The variables must be either 1% or 100%. Two variables can be plotted in a two-dimensional plot. With the help of this plot, we can easily find where the nanoemulsion formed. In the diagram, the pink link indicates the region of nanoemulsion. In the right side of the plot o/w nanoemulsion formed while in opposite side w/o nanoemulsion formed. The blue dotted lines showed the formation of o/w nanoemulsion towards the right side of the plot as shown in Figure 1.

2.3.3 Partition coefficient

In this the lipophilic and hydrophilic characteristics of drug were determined. The partition coefficient of drug retinol acetate determined using shake flask method. Equal quantity of octanol and water taken in flask added 100 mg of drug sample separately in flask rapid shaking for 1 h and stay for 24 h. After that aliquot of oil and water samples were analyzed separately spectrometrically compared with standard curve. Log Po/w value of retinol acetate was found to be 6.081. The value showed that the drug was lipophilic in nature, have stratum corneum permeation ability and best candidate for skin disorder study referred to in Table 2.

2.3.4 Melting point

It was determined by capillary method in which the melting point of retinol acetate was found to be 58.3 ± 0.30°C, matched with standard drug 58°C. The calculated values of SD ± 5 given in Table 2.

2.3.5 High performance liquid chromatography (HPLC)

HPLC spectrum of retinol acetate using methanol and water (95:5) v/v as a mobile phase and the retention time of retinol acetate was found to be 8.41 min compared with standard drug shown in Table 2 and Figure 2 at λ_{max} 325 nm. The potency of retinol acetate was 500000 IU. The spectrum run on HPLC against methanol and water as a blank using C18 column. The flow rate was 1 ml/min.

Table 2: Quality control testing of retinol acetate

S. No.	Drug sample	Melting point		Partition coefficient		Inference
1	Retinol Acetate	Observed values °C	Standard values °C	(Log P o/w) observed	Standard value	Hydrophobic in nature
		58.3 ± 0.30	57.3 - 58.4	6.08 ± 0.03	6.10	
				HPLC study		
		Mobile phase	Column size	Pore size	Flow rate (ml/min)	Retention time (min) at 325 nm
		Methanol: Water (95:5) v/v	C18 column	5 µ, 4.6 x 250 µ	1 ml/min	8.41 min

± SD values (n=5)

2.3.6 Box Benkhen design

Behnken design is used for optimization for pharmaceutical formulations like nanoemulsion. There are 3 independent variables like quantity of oil A, quantity of surfactant and water B and quantity of drug C and 2 levels high and low. In response surface methodology, 7 responses; namely, percentage drug content, *in vitro* drug release at 10,30,60,90,120,150 min time interval were studied given in Table 3.

Table 3: Polynomial analysis of independent variables and responses

Study type	Response surface methodology	Runs		19
Design type	Box Behnken	Design model		Quadratic
Factors	Independent variables	Unit	Low	High
A = X1	*Quantity of oil	g	1	9
B = X2	**Quantity of surfactant A and B	Ratio	1	8
C = X3	Quantity of drug (retinol acetate)	mg	100	200
Responses				
R1	Percentage drug content	%		
R2	Percentage *invitro* drug release at 10 min	%		
R3	Percentage *invitro* drug release at 30 min	%		
R4	Percentage *invitro* drug release at 60 min	%		
R5	Percentage *invitro* drug release at 90 min	%		
R6	Percentage *invitro* drug release at 120 min	%		
R7	Percentage *invitro* drug release at 150 min	%		

*Oil = Soybean oil, **Surfactant A = SPAN 20, Surfactant B = SPAN 80

For optimization independent variables quantity of oil, A quantity of surfactant A (SPAN 20) and B SPAN 80), quantity of drug (retinol acetate) C with 7 responses % drug content and % *in vitro* drug concentration at different time interval.

Table 4: Optimization of combination of soybean oil, surfactant A and B and retinol acetate

Number of batches	A	B	C	R 1	R2	R3	R 4	R 5	R 6	R 7
F1	1	1	150	80	5.4	10.5	25.2	45	66	79
F2	9	4.5	100	75	4.2	9.5	25.5	43	76	80
F3	5	4.5	150	94	3.6	9.2	26	42	65	78
F4	5	1	100	90	5.2	9.1	21	54	66	80.1
F5	9	4.5	200	87	4.8	9.4	20.2	48	70	80.2
F6	5	4.5	150	78	6.7	8.88	19.5	49.6	61.3	64.3
F7	5	4.5	150	67	7.3	12.3	22.5	48.6	64	79.5
F8	5	4.5	150	89	4.2	10.4	23	51	80	85

F9	5	4.5	150	85	3.6	12.5	24	51.3	76	79
F10	9	1	150	87	6	16.5	25.3	51.4	75	76.6
F11	1	4.5	100	86	5.3	14.3	25.4	52.5	82	83.5
F12	1	8	150	83	3.2	12.1	25.6	52.3	65	69.4
F13	9	8	150	82	5.4	13.2	26.3	44.6	74	79.3
F14	5	4.5	150	95	5.8	12.1	25.4	44.7	65	69.5
F15	5	8	200	97	6.2	15.3	31.4	45.3	67	70.32
F16	5	8	100	80	6.3	16.5	32.5	44.3	73	79.5
F17	5	1	200	76	6.4	16.5	38	42	74	79.6
F18	1	4.5	200	87	6.3	13.5	21.3	43	70	80.12
F19	5	4.5	150	75	6.2	15.6	30.2	44.5	55.4	75.7

Their values keep in Box Behnken model, total 19 combinations were made and showed in Table 4 and for ANOVA values referred to in Table 5.

Table 5: ANOVA for drug content

Source	Sum of squares	df	Mean square	F-value	*p*-value	
Model	351.50	6	58.58	4.02	0.001	Significant
A-amount of oil	78.13	1	78.13	5.37	0.043	
B-surfactant	98.00	1	98.00	6.73	0.026	
C-Drug	1.12	1	1.12	0.077	0.786	
AB	81.00	1	81.00	5.56	0.040	
AC	12.25	1	12.25	0.841	0.380	
BC	81.00	1	81.00	5.56	0.040	
Residual	145.56	10	14.56			
Lack of fit	102.76	6	17.13	1.60	0.337	Not significant

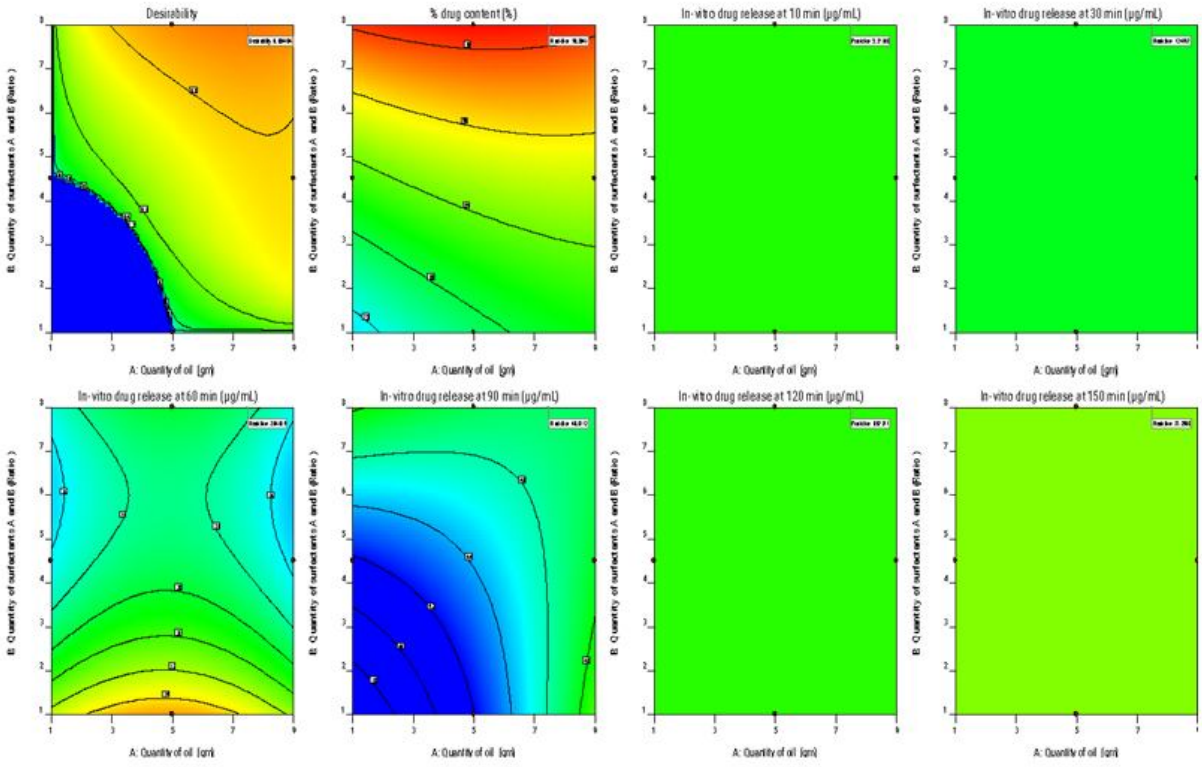
$R^2 = 0.707$, Adjusted $R^2 = 0.531$, Predicted $R^2 = 0.079$.

There is a 33.78% chance that a Lack of fit F-value, this large could occur due to noise. Non-significant Lack of fit is good, we want the model to fit. The F-value was found to be in the range of 0.3 to 2.45, the highest seen in % *in vitro* drug release at 60 min, the *p*-values were found to be <0.001 the values were significantly shown in Table 6.

Table 6: Comparison of *p*-values and F-values with various responses

Responses	Sum of square	df	Mean^2	F-value	*p*-value	Inference
Response 1: % drug content						
Lack of fit	102.76	6	58.58	4.02	<0.001	Significant
Pure error	649.43	6	17.13			
Cor total	1070.53	18				
Response 2: % *in vitro* drug release at 10 min						
Lack of fit	10.32	12	0.8600	0.3686	<0.001	Significant
Pure error	14.00	6	2.33			
Cor total	24.32	18				
Response 3: % *in vitro* drug release at 30 min						
Residual	133.11	18	7.40			
Lack of fit	100.97	12	8.41	1.57	<0.001	Significant
Pure error	32.14	6	5.36			
Cor total	133.11	18				

Response 4: % *in vitro* drug release at 60 min						
Residual	148.88	9	16.54			
Lack of fit	81.95	3	27.32	2.45	<0.001	Significant
Pure error	66.93	6	11.16			
Cor total	381.92	18				
Response 5: % *in vitro* drug release at 90 min						
Residual	101.00	12	8.42			
Lack of fit	21.69	6	3.62	0.2735	<0.001	Significant
Pure error	79.31	6	13.22			
Cor total	284.11	18				
Response 6: % *in vitro* drug release at 120 min						
Residual	821.37	18	45.63			
Lack of fit	388.08	12	32.34	0.4478	<0.001	Significant
Pure error	433.29	6	72.22			
Cor total	821.37	18				
Response 7: *in vitro* drug release at 150 min						
Residual	498.48	18	27.69			
Lack of fit	213.15	12	17.76	0.3735	<0.001	Significant
Pure error	285.15	6	47.56			



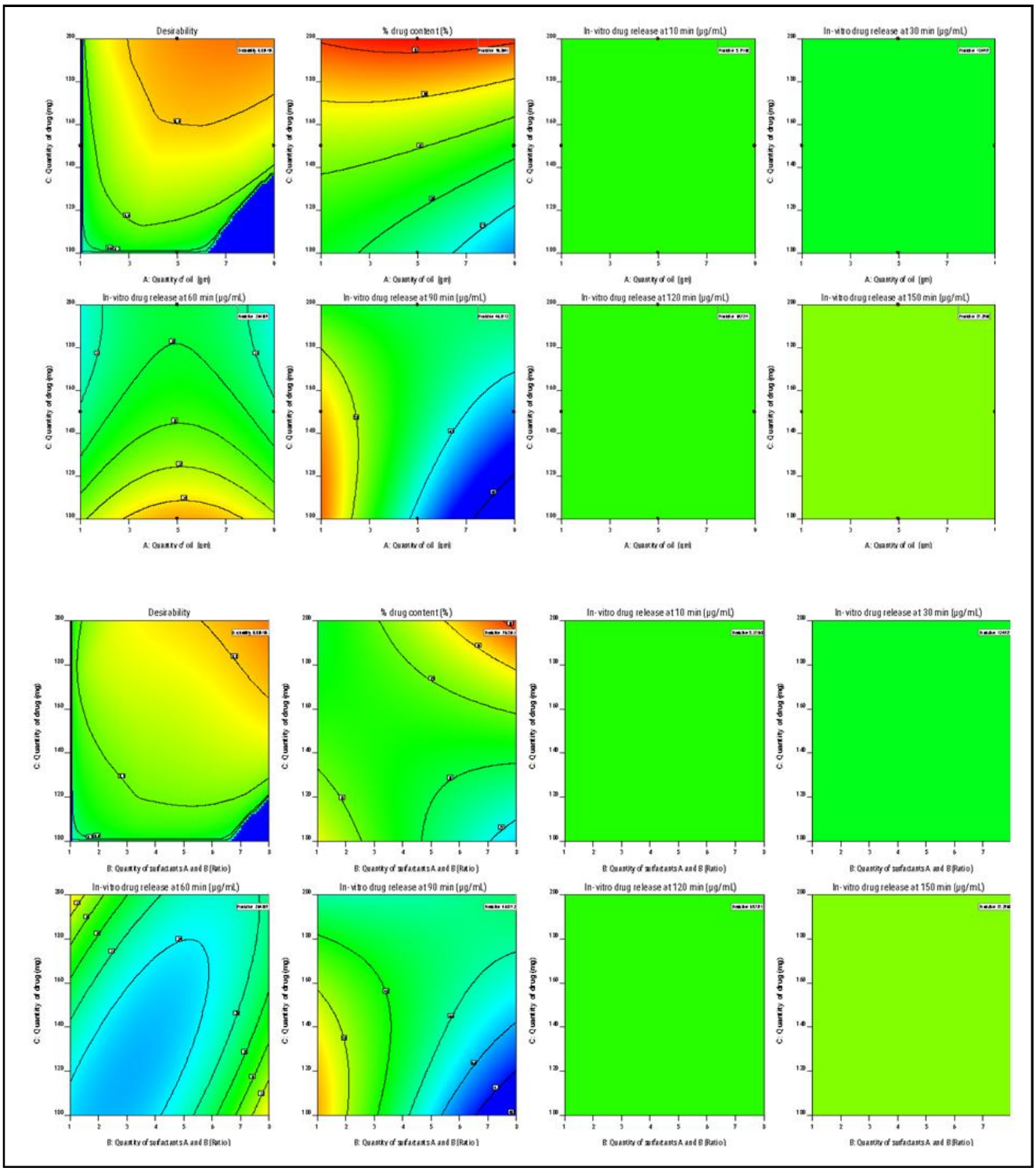


Figure 2: Contour images showed effects of variables AB, AC, BC against responses.

In Figure 2, contour images showed the effects of independent variables like quantity of drug, quantity of surfactants A, B and quantity of oil against each response. Bright yellow and green colours image showed the acceptance region while red region shows some limits were higher. Green color picture indicates the optimized parameters are in limit. The flag values indicated the predictive value which was compared with experimental values. In RANE nanoemulsion, all predicted values compared with experimental values shown in Table 7. The highest values in drug contents were 80.24 ± 1.14, *in vitro* drug release obtained at 150 min 75.32 ± 5.26.

Table 7: Predicted and observed experimental values of RANE

Responses	Predicted mean	Observed value	% Predicted error
Percentage drug content	83.3 ± 3.37	80.24 ± 1.14	3.81
In vitro drug release at 10 min	05.37 ± 0.27	05.20 ± 1.16	3.27
In vitro drug release at 30 min	12.49 ± 0.62	13.46 ± 2.71	7.21
In vitro drug release at 60 min	24.37 ± 1.54	23.90 ± 4.06	1.96
In vitro drug release at 90 min	47.38 ± 1.21	48.23 ± 2.90	1.76
In vitro drug release at 120 min	69.72 ± 1.55	67.88 ± 6.75	2.71
In vitro drug release at 150 min	77.29 ± 1.21	75.32 ± 5.26	2.61

± SD (n=3)

2.4 Synthesis of nanoparticles

Nanoparticles prepared by emulsification method using probe sonication method shown in Figure 3 (Song and Lin, 2021).

2.4.1 Retinol acetate nanoemulsion by probe sonication method

Formulations were prepared by mixing of defined quantity of soybean oil, surfactants like span 20 and span 80 (8:2) with retinol acetate prepared both oil phase A and water phase B as per Figure 4. In oil phase A, oil, drug, and appropriate concentration of surfactants dissolved and in phase B, water and preservative dissolved. Both phases maintained at 75°C, then B was poured into A dropwise with high stirring 4000 rpm for 20 min to prepare microemulsion. Then, this microemulsion was processed with nanoemulsion using sonics probe sonicator with threaded probe having 13 mm, 630-0220 probe number, amplitude 30%, pulse 3 at the temperature 30°C, elapsed time was 3 min and made oil globules in the range of 200-800 nm as shown in Figure 3. Prepared nanoemulsion stored at 15-25°C in suitable container for further evaluation.

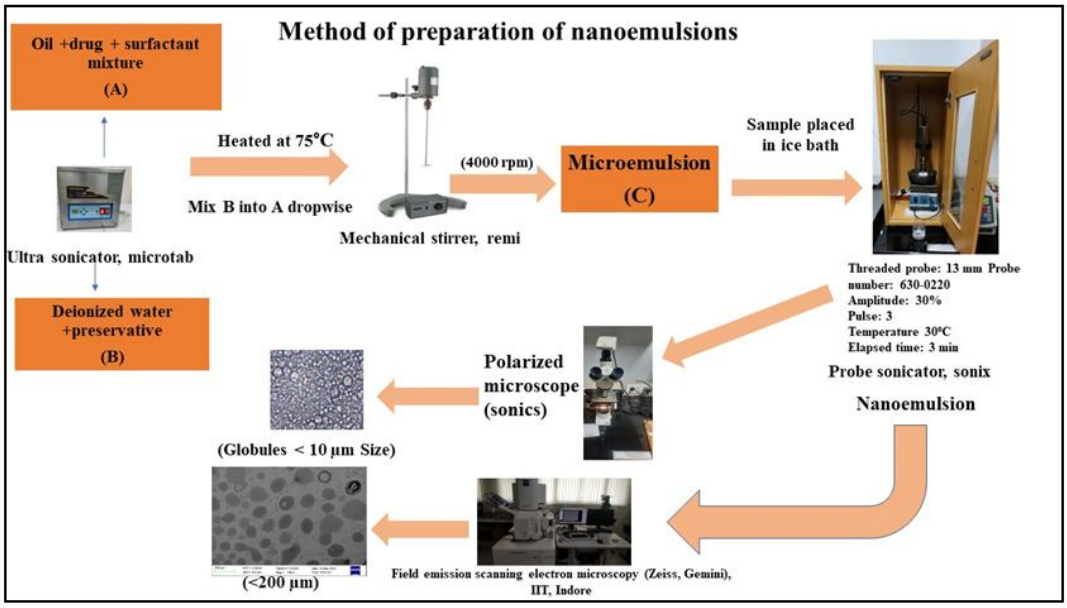


Figure 3: Method of preparations of RANE nanoemulsions.

2.5 Statistical analysis

All data analysed statistically, using prism graphpad software, 2021 and design expert software version 12. The mean ± standard deviation of n = 5 measurements used to conduct data analysis and the significant difference was set at $p \geq 0.05$.

2.6 Characterization

Nanoformulations of retinol acetate were characterized by field emission scanning electron microscopy (FESEM), zeta potential and size distribution analysis.

2.6.1 Field emission scanning electron microscopy

FESEM of optimized formulations F7 was compared with blank formulation F15.The particle size was observed in ZEISS FESEM in range of 200 µm to 200 nm. The magnification of F7 was 49.41 KX and width was 8 mm. FESEM provided topographical and elemental information at magnifications of 10X to 300,000X, clearly indicated that particle size of retinol acetate was at nanometric range 100-1000 nm particles used for medical therapeutic purposes referred to in Figure 4.

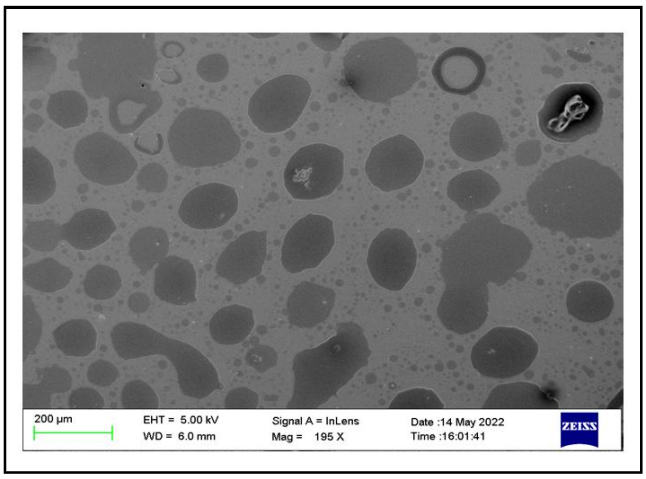


Figure 4: FESEM image of retinol acetate nanoemulsion F7.

2.6.2 Zeta potential and size distribution

This study included zeta potential and particle size distribution from malvern instrument limited, zeta sizer ver. 8.01.4906 serial no: mal 1023461 used for analysis at temperature 25°C in clear disposable zeta cell with 2 mm, dispersant was water with refractive index 1.330. The dielectric constant was 78.5. The graph plot between % intensity vs size (d, nm). The below diagram of zeta potential of optimized formulations F7 lied in -19 value means the nanoemulsion showed moderate stability. This confirmed that nanoemulsion was stable. For particle size analysis with dispersant water with refractive index 1.330, the average viscosity of formulations was lied in range of 0.8872 cP. and average globule size found to be in the range of 200 nm to 800 nm, shown in Figure 5.

2.7 Skin irritation test

As per the guidelines of the committee for the purpose of control and supervision of experimental animals (CPCSEA), this experiment was carried out with reference number 779/CPCSEA/IAEC/2022/001 approved by IAEC of DAVV. The protocol for *in vivo* experiments of skin irritation was performed by a modified method of Draize *et al.* (1944). The animal (albino mice) was kept under observation for 7 days prior to the experiments. The temperature of the room was maintained at 22 ± 1°C and the humidity was maintained at 36-46 per cent relative humidity. Each rat was individually weighed and their hair were removed on the previous day, marked as a circular area (~3 cm^2) with a felt tip marker on the dorsal surface. The animals were divided into 4 groups, each group consists of 5 animals (n=5); namely, Group I control, Group II test I, Group III normal diet and Group IV standard were observed daily for general health. Body weights were recorded for each animal at pre-treatment (100-200 g). The animals were treated with an optimized retinol acetate nanoemulsion formulation (F7) and formalin solution as standard irritant. Dermal observations for erythema and edema were recorded at 1, 24, 48, and 72 h. Finally, the treated skin was examined visually for erythema and edema. The skin irritation (erythema and edema) was evaluated by visual observations using PASI scoring given in Table 8 and Figure 6.

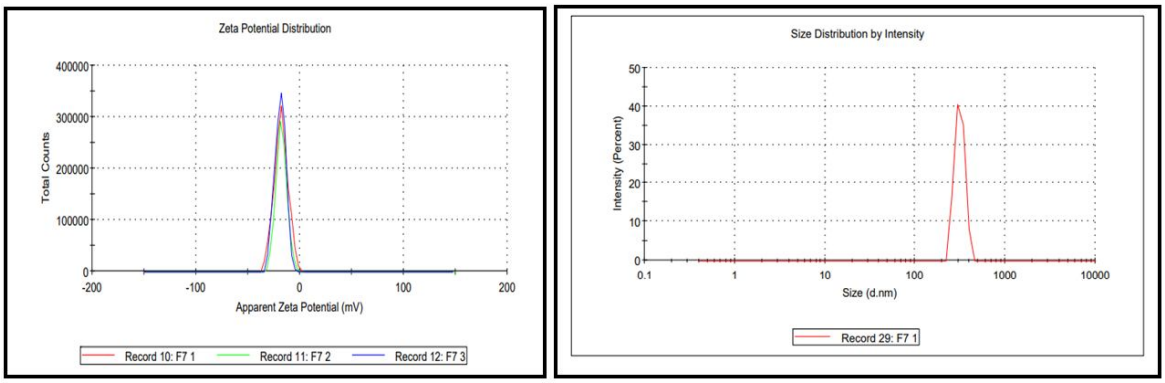


Figure 5: Zeta potential and size distribution of F7 retinol acetate nanoemulsion.

Table 8: Skin irritation study for RANE using PASI score

S. No.	Groups	General information	Erythema (redness) and edema				Induration (thickness)	Desquamation (scaling)
			1 h	24 h	48 h	72 h		
1	I	Control group + cream	4 ± 0.01	4 ± 0.5	3 ± 0.01	3 ± 0.7	0	0
2	II	Test I RANE + treated with standard irritant	4 ± 0.03	4 ± 0.6	4 ± 0.5	4 ± 0.05	3 ± 0.1	2 ± 0.12
3	III	Normal diet	0	0	0	0	0	0
4	IV	Standard	4 ± 0.5	4 ± 0.45	4 ± 0.50	4 ± 0.34	4 ± 0.65	4 ± 0.43

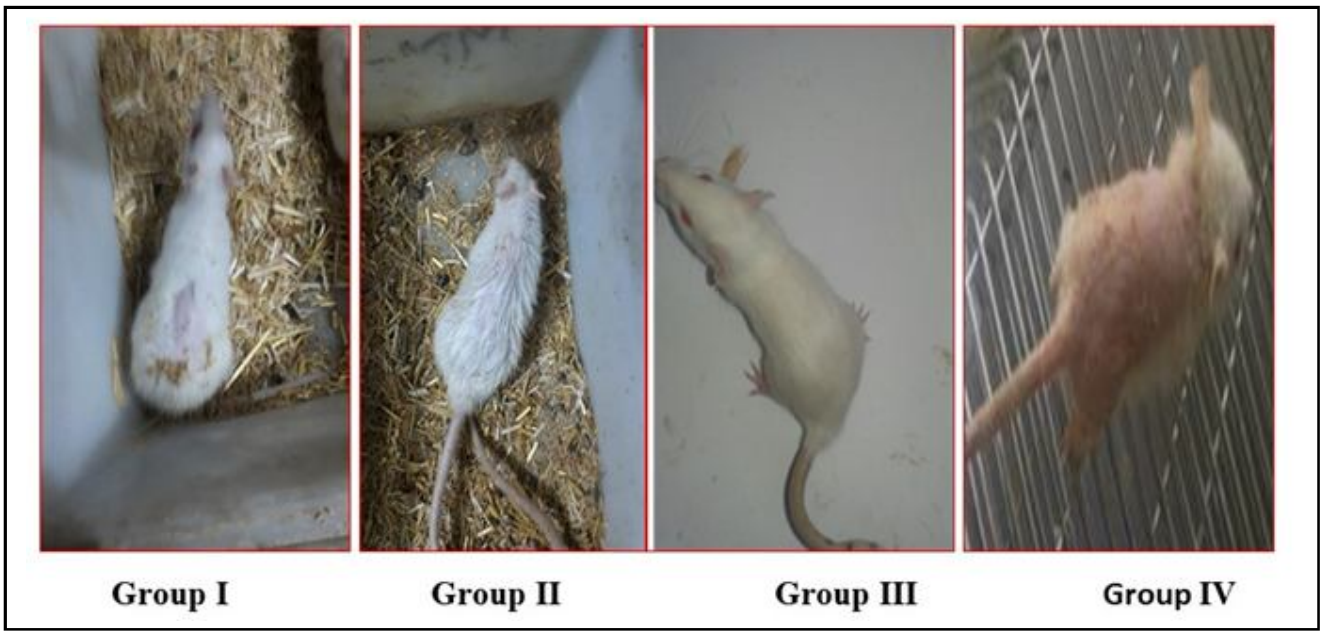


Figure 6: Skin irritation study of retinol acetate nanoemulsion F7.

3. Results

The drug retinol acetate used in nanoemulsion had orange to yellow, bitter, odorless and rod like granules. The partition coefficient, log Po/w value of retinol acetate was found to be 6.081. The soybean oil was selected as oil phase while span 20 and span 80 in ratio of 8:2 selected as per ternary phase diagram. The melting point of retinol acetate was found to be 58.3 ± 0.30°C. The retention time of drug was found to be 8.41 min. The box benkhen design used to optimized nanoformulations using design expert software version 12. For compatibility of retinol acetate nanoformulation undergone skin irritation test using albino wistar rats both sexes. It was found that the prepared optimized nanoemulsion F7, Group II was effective and had no irritation and no redness, erythema, edema, Induration desquamation seen after 72 h study as compared to standard irritant formalin in Group IV.

4. Discussion

Organoleptic properties evaluated for the given drug sample retinol acetate was found to be orange to yellow, odorless, bitter and rod like granules. The partition coefficient, Log Po/w value of retinol acetate was found to be 6.081. This value showed that the drug was lipophilic in nature, have stratum corneum permeation ability and best candidate for dermal study. It helped in drug distribution in oil and water phases and also helped in finding the nature of drug, *i.e.*, lipophilic character. The melting point of retinol acetate was found to be 58.3 ± 0.30°C matched with standard value 58°C of retinol acetate standard drug determined by capillary method. In HPLC study, the retention time of drug was found to be 8.41 min using methanol: water (95:5 v/v) as a mobile phase at a rate 1 ml/min, using C18 column with pore size 5 µ, 4.6 x 250 µ matched with standard value of retention time of pure drug shown in Table 2 at λ_{max} 325 nm. The potency was found to be 500000 IU. It proved that drug was retinol acetate and it was in a pure form, with the help of ternary phase diagrams emulsifier selected. For the optimization of surfactant mixtures different ratios of surfactant, oil and water mixture mixed and check the stability of emulsion. The span 20 was best suited for emulsification process. The o/w type nanoemulsion and w/o type nanoemulsion formulation area was segregated by line and well defined in ternary phase diagram. For stability of nanoemulsion for a long time the ratio of emulsifier selected. The ratio of span 20 and span 80, 8:2 was best among all. Probe sonication method is adopted and prepare 19 different formulations using response surface methodology, Box Behnken design with contour plots for optimization of nanoformulations. Out of which, only one F7 showed optimized results compared predicted and observed values. The retinol acetate nanoemulsion was characterized by FESEM, the size of many oil globules <200 µm while zeta potential -19 mV showed that moderate stability of F7 formulation. The size distribution was found to be 200-800 nm and ideal for therapeutic use. There was no skin irritation observed in Group II. It was found that the prepared optimized nanoemulsion Group II, had no irritation and no redness, erythema, edema, induration desquamation seen after 72 h study as compared to standard irritant formalin in Group IV.

5. Conclusion

Preformulation study concluded that the drug retinol acetate data matched with standard drug. This value showed that the drug was lipophilic in nature, had stratum corneum permeation ability and best candidate for skin disorder. Due to low melting point easily disperse in oil phase and helped in formulation. HPLC study showed that the retention time 8.41 min using mobile phase methanol: water (95:5) v/v at λ_{max} 325 nm with potency 500000 IU, International unit. It proved that drug was retinol acetate and it is in pure form. Ternary phase diagram helped in selecting of soybean oil and surfactant. For enhancing the stability of nanoemulsion combination of emulsifier like span 20 and span 80 in ratio of 8:2 showed prolonged stability and prepared stable o/w nanoemulsion with probe sonication method using response surface methodology. For compatibility of retinol acetate nano formulation over skin, skin irritation test was performed in albino wistar rats. It was

found that the prepared optimized nanoemulsion was effective and had no irritation. Optimized retinol acetate nanoemulsion F7 has strong potential to combat skin disorders due to exhibit physiochemical properties. In future, use of retinol acetate nanoemulsion in permeation study of psoriatic cell lining can helpful in treatment and cure.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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