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STATISTICAL OPTIMIZATION OF TOPICAL CREAM CONTAINING VITAMIN D3 NANOPARTICLES FOR ANTI-AGING EFFECT: BOX-BEHNKEN DESIGN

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ABSTRACT: Aging is defined as decreased maximal function and reserve capacity in all body, organs, resulting in an increased likelihood of disease and death. One of the reasons for Premature Aging is deficiency of Vitamin D_3. The main aim of the research work is to develop and evaluate the Nanoparticle based Anti-aging cream of Vitamin D_3 for Premature aging. Six formulations were prepared by Nano precipitation method using Poloxamer 407 as polymeric stabilizer. It was then incorporated into cream base. FT-IR studies were carried out to determine the drug-excipient compatibility. The prepared Nano-suspension was subjected to evaluations including particle size, zeta potential, drug entrapment efficiency and drug content. The formulated cream was tested for pH, Spreadability, Viscosity and *in-vitro* drug release. From the preliminary trial, F_4 was found to be the best formulation. It was then fitted to Box-Behnken design with three independent variables including concentration of Stearic acid (X_1), KOH (X_2), Glycerine (X_3) and two dependent variables – Spreadability (Y_1) and *In-vitro* drug release (Y_2). Among the 15 runs, R_2 was found to be the optimized formulation. Stability studies revealed there is no change in the spreadability and *ex-vivo* permeation from the cream base. The optimized Vitamin D_3 loaded nanoparticle anti-aging cream provides satisfactory release with improved therapeutic effect.

INTRODUCTION: Skin, like any other organs, usually undergoes progressive decline in its physiological, morphological, and functional features during aging. The phenomenon of aging is common, natural and genetically predisposed [1]. Aging process is classified into two distinct types, i.e. "sequential skin aging" process and "photo-aging" process. Sequential skin aging is universal and predictable process which is often characterized by physiological alteration in the skin functions. On the other hand, photo aging is caused by over exposure to UV rays emitted from sunlight. It is characterized by the appearance of dry, pale and shallow skin, displaying fine wrinkles as well as deep furrows caused by the disorganization of epidermal and dermal components associated with elastosis and helio dermatitis [2]. Vitamin D is the sunshine vitamin which has been produced on this earth for more than 500 million years. During exposure to sunlight 7-dehydrocholesterol in the skin absorbs UV B radiation and is getting converted to pre vitamin D_3 which in turn isomerizes into Vitamin D_3.

Pre vitamin D_3 and Vitamin D_3 also absorbs UV B radiation and are converted into a wide variety of photoproducts some of which have unique biologic properties [3]. Telomeres are the tail ends of linear chromosomes, of several thousand bases in length that comprises of tandem repetitive DNA sequences. The presence of telomeric repeats protects the chromosome from photo degradation

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or fusion. It has also been determined that the length of telomeres reduces by up to 150 base pairs during every cell division. When telomeres become 'critically shorter, cells enter proliferative senescence and thus telomeres appear to serve as the biologic clock of skin aging process, informing cells that they are young or old [3, 4]. Although VD_3 effectively prevents skin photo aging process, it is sensitive to air, light and high temperature, which is not conducive to storage, transportation and use. VD_3 also rapidly degrades in the traditional carrier such as water/ethanol[5]. Nanotechnology may cause a significant impact on drug delivery systems. The drug loading allows the optimization parameters in controlled drug delivery. By encapsulation of potential drugs inside Nano carriers it is possible to intensify their therapeutic index by enhancing their drug release time and also their pharmacokinetic profile. Besides, the solubility and the stability of the drug is improved. Vitamin D is lipo-soluble; thus, it is essential to enhance its solubility in water [6, 7]. In this research, we optimized the nanoparticle formulation of VD_3 cream using Box-Behnken design to improve encapsulation efficiency (EE %) and to enhance transdermal absorption and stability of VD_3.

MATERIALS AND METHOD:

Materials: Vitamin D_3 pure was purchased from Supreem Pharmaceuticals Mysore Pvt Ltd. Ethyl cellulose, Poloxamer 407, Acetone, Stearic acid, Lanolin, Glycerine, Triethanolamine, Methyl Paraben, Propyl Paraben and Rose oil were purchased from Yarrow Chem Products, Mumbai. All other chemicals and reagents were of analytical grade.

Drug-excipient Compatibility Studies: The compatibility of Vitamin D_3 with Ethyl cellulose and Poloxamer-407 were analysed with Fourier Transform Infrared Spectroscopy (FT-IR) at transmittance mode over wave number range of 4000 to 400cm^{-1}[8].

Method of Preparation:

Procedure for Preparation of Vitamin D_3 Loaded Nano-suspension: The Nano suspension loaded with Vitamin D_3 was prepared by Nano precipitation technique using Poloxamer 407 as polymeric stabilizer [9]. The previously formulated organic phase was injected to the magnetically stirred aqueous stabilizer solution, and subjected to probe sonication. The first three formulations were prepared with drug: polymer ratio as 1:1, 1:5, 1:10 keeping the polymeric stabilizer 0.25g. The next three formulations were prepared with drug: polymer ratio as 1:1, 1:5, 1:10 keeping the polymeric stabilizer 0.50g. The prepared Nano suspension were subjected to evaluation parameters like entrapment efficiency, drug content, particle size determination, and zeta potential.

TABLE 1: COMPOSITION OF VITAMIN D_3 LOADED NANO-SUSPENSION

Sl. no.	Ingredients	Formula (g)					
		F1	F2	F3	F4	F5	F6
1.	Vitamin D_3	0.025	0.025	0.025	0.025	0.025	0.025
2.	Ethyl Cellulose	0.025	0.125	0.25	0.025	0.125	0.025
3.	Poloxamer 407	0.025	0.025	0.025	0.050	0.050	0.050
4.	Acetone	2mL	2mL	2mL	2mL	2mL	2mL

Formulation of Vitamin D_3 loaded Nanoparticle Cream: The formulated Vitamin D_3 loaded nanoparticles were then uniformly dispersed in the cream base by mechanical stirring for 30 min to get nanoparticle loaded cream. Evaluations including pH, Viscosity, Spreadability, *In-vitro* drug release and *Ex-vivo* permeation studies were performed.

TABLE 2: COMPOSITION OF VITAMIN D_3 NANOPARTICLE CREAM

Sl. no.	Ingredients	Quantity(g)	Category
1.	Stearic Acid	18g	Emulsifying agent
2.	Lanolin	2g	Emollient
3.	Glycerin	3ml	Humectant
4.	Triethanolamine	1ml	Emulsifying agent
5.	Methyl Paraben	0.18g	Preservative
6.	Propyl Paraben	0.02g	Preservative
7.	Rose Oil	q.s	Perfume
8.	Distilled water	75.8ml	Solvent

Optimization of Vitamin D_3 Loaded Nanoparticle Cream: Response surface methodology is employed to develop and statistically optimize the drug delivery systems. Box-Behnken statistical design is an independent, rotatable or nearly rotatable, quadratic design [10]. The treatment combinations are placed at the midpoints of the edges and at the centre of the process space. This design requires less experimental runs and time. Therefore, it is considered as a cost-effective technique than other usual processes of formulation and optimization of dosage forms. Vitamin D_3 Nanoparticle cream was statistically optimized using Box Behnken experimental design using Minitab of 3- factor 3 – level wherein 15 formulations were developed.

Characterization of Vitamin D_3 Loaded Nano suspension

Entrapment Efficiency: For determination of drug entrapment, the amount of drug present in the clear supernatant after centrifugation was determined (w) by UV spectrophotometer at 265 nm.

Percentage entrapment efficiency = Total drug (W) - Drug in supernatant liquid (w) / Total drug (W) ×100

Drug Content: After the centrifugation of Nano suspension, 5 mL of supernatant was diluted with 100 mL methanol, and the amount of unbound drug was measured by taking the absorbance of the diluted supernatant solution at λmax=265 nm.

Drug content = Total amount of drug- amount of unbound drug / Total amount of drug initially taken × 100

Determination of Particle size, Zeta Potential and Poly Dispersity Index: Particle size was calculated by using Malvern size analyser. For a stable Nano suspension, it should have low particle size and higher zeta potential.

Optimization of Nanoparticle Cream:

Primary Evaluation of Polymeric Nanoparticle Cream:

Appearance and Homogeneity: Visually, cream was inspected in terms of colour, texture, phase separation as well as grittiness.

Determination of pH: The Digital pH meter was calibrated using standard buffer solution. About 0.5 g of cream was weighed and dissolved in 50.0 ml of distilled water and its pH was measured.

Determination of Viscosity: The viscosity of Nano cream was determined using Brookfield viscometer using spindle 4. Spindle was allowed to move freely in the cream and reading was recorded. All measurements were made in triplicate using fresh sample each time.

Determination of Spreadability: An excess amount of cream was placed between the glass slide and a 100g weight was placed on to the glass slide so as to compress the sample to a uniform thickness. The weight was then removed and weight was placed into the upper pan and time taken to completely separate the two slides was recorded. Spreadability was then calculated by following formula [11].

Spreadability = M.L / T

Where, S = Spreadability in g.cm/s, M = Weight tied to upper slide, L = Length of glass slide, T = Time in seconds

In-vitro **Drug Release:** The Vitamin D_3 loaded topical cream was permeated through an artificial cellophane membrane. 3g of topical cream was placed in the donor compartment. The receptor medium was filled with pH 6.8 Phosphate buffer. Samples were analysed spectrophotometrically at 265nm.

TABLE 3: LAYOUT OF INDEPENDENT VARIABLES FOR OPTIMIZATION

Factors (Independent Variables	Levels		
	+1	0	-1
X1 (Concentration. of Stearic acid)	20	18	16
X2 (Concentration. of Glycerin)	5	3	2
X3 (Concentration of KOH)	0.15	0.10	0.05

TABLE 4: LAYOUT AND RESULTS OF VITAMIN D_3 LOADED NANOPARTICLE CREAM-DEPENDENT VARIABLES

Runs	X1	X2	X3	Y1	Y2
R1	-1	-1	0	5.00±0.10	59.21
R2	1	-1	0	5.96±0.32	62.50

R3	-1	1	0	4.72±0.13	52.82
R4	1	1	0	6.15±0.21	61.66
R5	-1	0	-1	6.95±0.16	65.23
R6	1	0	-1	6.00±0.43	58.02
R7	-1	0	1	5.80±0.76	59.73
R8	1	0	1	4.91±0.42	62.66
R9	0	-1	-1	6.20±0.33	58.35
R10	0	1	-1	6.61±0.42	60.82
R11	0	-1	1	5.35±0.71	64.43
R12	0	1	1	6.92±0.16	62.53
R13	0	0	0	6.31±0.07	57.95
R14	0	0	0	5.50±0.11	60.32
R15	0	0	0	4.95±0.31	63.52

Note: Y_1=Spreadability (gm.cm/sec), Y_2= In-vitro drug release (%).

Characterisation of Optimised Formulation:

Drug Release Kinetics: *In-vitro* release data of optimized formulation (R_5) was analysed by various kinetic models like Zero order ($C=k_0t$), first order (Log C= Log C_0 – $k_ft/2.303$), Higuchi's model ($Q=Kt^{1/2}$) and Korsemeyer Peppas model ($M_tM_N= Kt^n$) to check the release pattern and mechanism of drug release of the prepared formulation.

***Ex-vivo* Absorption Study:** The goat skin was cut into small circular pieces and allowed to hydrate. It was then mounted carefully onto the diffusion cell facing the donor compartment. After clamping the donor and receiver compartments together, the receiver compartment was filled with pH 6.8 Phosphate buffer and magnetically stirred. The samples were withdrawn at various time intervals was analysed by UV spectrophotometer at 265nm.

Scanning Electron Microscopy: The morphology of the optimized nanoparticles (R_2) was analysed using SEM (S-4800, Hitachi technologies corporation, Japan). The scanning electron microscope is operated at an acceleration voltage of 1.5kv [12].

Stability Study: A short term (1month) stability studies were performed for the final optimized best formulated topical cream loaded with Vitamin D_3 Nanoparticles. The temperature was maintained at 40°C/75% RH. Entrapment efficiency and *in-vitro* drug release study was performed.

RESULT AND DISCUSSION: The results of the study indicate FT-IR spectrum of drug and excipients did not differed with major peaks of Vitamin D_3, i.e; all the major peaks of the drug appeared on the blend indicate that there is no possible interaction between drug and excipients. The Nanoparticles were prepared by Nano precipitation technique and then incorporated into cream. The λ max of the formulation was found to be 265 nm using pH 6.8 Phosphate buffer. The melting point of the pure drug was found to be 85°C. The prepared Nanoparticle suspension was subjected to primary evaluation studies. The particle size was in the range of 150-780nm. The least size was in the F_4 formulation due to the less amount of (0.025g Ethyl cellulose), because the polymers tends to swell when comes to contact with the aqueous phase. The zeta potential values were in the range of -26 mV to -14.28 mV. The entrapment efficiency of the formulations were in the range of 68.17–85.60 %. When concentration of polymer is increased, the platform for binding the drug to the core is increasing. The F_4 formulation was found to have the best drug content of 86.4%.

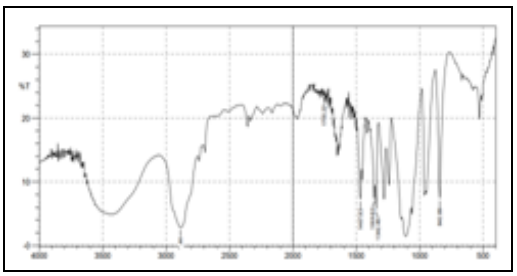

FIG. 1: FT-IR SPECTRUM OF VITAMIN D_3 PURE

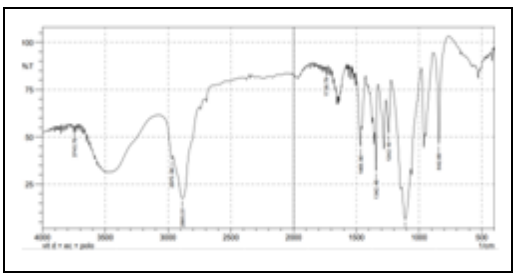

FIG. 2: FT-IR SPECTRUM OF VITAMIN D_3 + ALL EXCIPIENTS (ETHYL CELLULOSE+ STEARIC ACID)

TABLE 5: RESULTS OF EVALUATION PARAMETERS

S. no.	Parameters	Formulation Code					
		F1	F2	F3	F4	F5	F6
1.	Appearance and homogeneity	White, smooth and homogenous	White, smooth and homogenous	White, smooth and homogenous	White, smooth and homogenous	White, smooth and homogenous	White, smooth and homogenous
1.	pH	5.6±0.8	6.1±0.1	6.4±0.6	6.6±0.4	5.8±0.2	5.9±0.3
2.	Viscosity (Cp)	1256 ± 6	1459±3	2481±2	1459±7	2830±4	3026±2
3.	Entrapment efficiency (%)	64.17±0.5	71.65±0.4	73.17±0.1	86.02±0.6	78.21±0.2	84.42±0.4
4.	Particle size (nm)	642 ± 3	521.4 ± 2	480.7 ± 5	266.9 ± 4	715.5 ± 6	589.2 ± 7
5.	Polydispersibility Index	0.698±0.21	0.731±0.43	0.819±0.11	0.606±0.80	0.883±0.60	0.757±0.82
6.	Zeta Potential (mV)	-19.8±0.41	-16.5±0.22	-18.11±0.57	-26.6±0.68	-14.28±0.96	-20.78±0.43
7.	Drug content (%)	74.3±0.34	76.2±0.51	77.9±0.29	86.4±0.72	84.2±0.11	84.9±0.50
8.	Spreadability (g.cm/sec)	5.0±0.61	5.96±0.25	4.72±0.87	6.95±0.44	6.0±0.36	6.15±0.82
9.	*In-vitro* drug release (%)	47.91	48.99	50.21	58.95	56.99	57.09

The formulated cream was smooth in texture with no grittiness. pH of all the formulations were in the range of 5.6-6.6, thus non-irritating to the skin surface. F_4 exhibited maximum spreadability of 6.95g.cm/sec. The *in-vitro* drug release of the F_4 formulation was found to be 58.95% at the end of 8hrs. Rheological studies were performed through Brookfield Viscometer and an inverse relation was observed between shear and viscosity. This type of behaviour is termed as shear thinning behaviour.

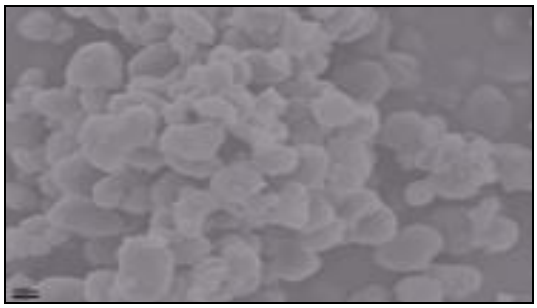

FIG. 3: SCANNING ELECTRON MICROSCOPIC IMAGE OF OPTIMIZED FORMULATION R_2

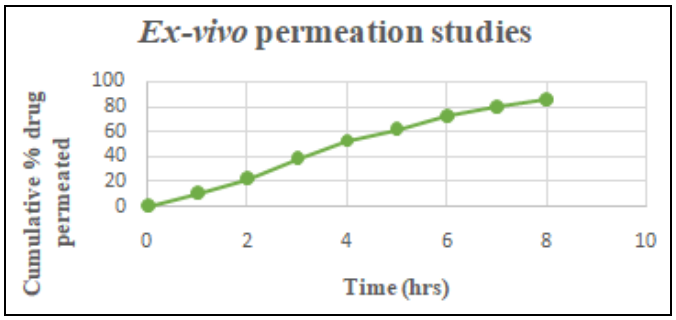

FIG. 4: *EX-VIVO* PERMEATION STUDIES OF OPTIMIZED FORMULATION (R_2)

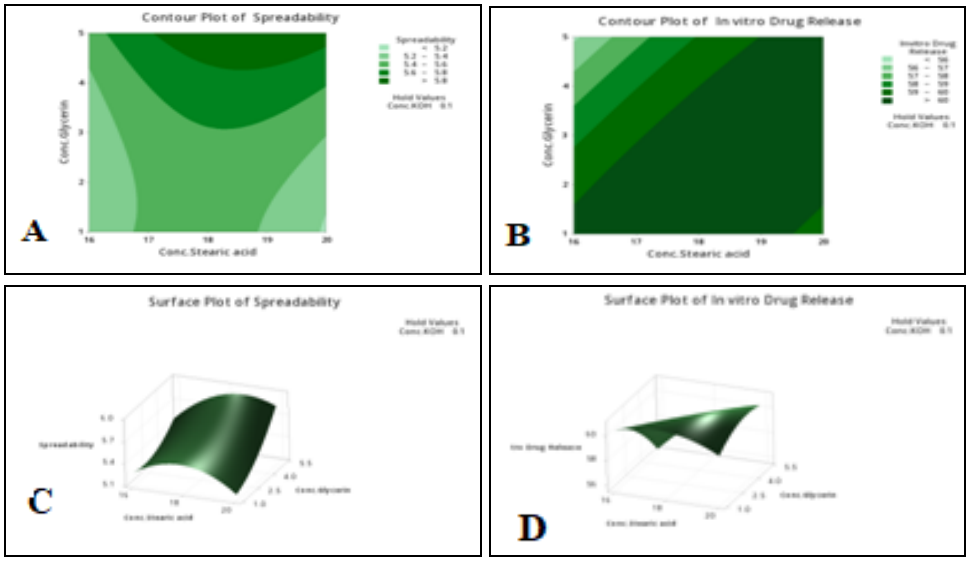

FIG. 5: CONTOUR PLOT OF (A) SPREADABILITY (B) *IN-VITRO* DRUG RELEASE SURFACE PLOT OF (C) SPREADABILITY (D) *IN-VITRO* DRUG RELEASE

From the preliminary evaluation studies, F_4 was found to be the best formulation. F_4 was then fitted into Box-Behnken design [15]. Formulations were prepared to determine the effect of independent variables of Concentration of Stearic acid (X_1) and Concentration of Glycerine (X_2) and Concentration of KOH (X_3) on the Spreadability (Y_1) and *in-vitro* drug release (Y_2) profile.

Effect on Spreadability (Y_1):

Spreadability = $-9.2+ 2.12\ X_1 -0.87\ X_2-63.5\ X_3-0.061\ X_1*X_2 + 0.029\ X_2 * X_2 +228\ X_3^* X_3 + 0.029\ X_1^* X_2 + 0.12\ X_1^* X_3 + 2.90\ X_2^* X_3$.

The equation reveals that there is a significant effect (p = 0.001) of the Concentration of stearic acid, KOH, Glycerine on the Spreadability. The thermo physical behaviour of the cream was found to be dependent on the proportion of independent variables. Greater the concentration of Stearic acid and KOH, smaller the Spreadability. Increased concentration of glycerine resulted in increased Spreadability.

Effect on *In-vitro* Drug Release:

In-vitro drug release = $46+ 4.9\ X_1 -4.50\ X_2-538\ X_3-0.221\ X_1^* X_1-0.166\ X_2^* X_2 + 679\ X_3^* X_3 + 0.347\ X_1^* X_2 + 25.3\ X_1^* X_3 - 11.9\ X_2^* X_3$

The equation reveals that there is significant effect (p = 0.02) on the Concentration of Stearic acid and KOH while the Glycerine concentration does not influence the drug release pattern from cream. The optimized formulation R_2 was subjected to kinetic study, *Ex-vivo* study, SEM and stability study. The kinetic study indicates that the formulation is controlled release with Case II transport as the n value was found to be 0.650. Scanning Electron Microscopic image indicated that particles are spherical with smooth surface. Stability study showed that there are no significant changes in the dissolution pattern. The results of the Box Behnken design revealed that R_2 formulation exhibited better therapeutic effect against premature aging.

CONCLUSION: Vitamin D_3 effectively prevents photo aging but needs to be additionally supplied for skin protection. Among the six formulations prepared, was found to be the best with a particle size of 200nm, Zeta Potential of -27.35mV, *Ex-vivo* permeation of 85.41% at the end of 8 hrs.

The formulated cream was found to be non-irritant, homogenous with improved therapeutic activity.

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