This article can be cited before page numbers have been issued, to do this please use: A. Shah, S. Boldhane, A. P. Pawar and C. Bothiraja, Mater. Adv., 2020, DOI: 10.1039/D0MA00298D.

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Advanced development of non-ionic surfactant and cholesterol materials based niosomal gel formulation for the topical delivery of anti-acne drug

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Abstract

The hypothesis of the present investigation was to develop adapalene (ADP), a high lipophilic, and low solubility anti-acne drug-loaded niosomal topical gel formulation in order to improve its therapeutic efficacy. ADP niosomes were prepared using Span 60 and cholesterol by the modified ethanol injection method. A design of experiment (DOE) study was used to optimize ADP loaded niosomes. The potential of ADP niosomes were investigated for in-vitro release and ex-vivo skin permeation studies. Additionally, ADP niosomes were loaded into Carbopol 934 gel and studied for its skin irritation, skin deposition and ex-vivo skin permeation potential. Developed ADP niosomes showed mean particle size, zeta potential, and entrapment efficiency of 278 nm, -17.99 mV, and 86 %, respectively. Optimized ADP niosomes showed controlled drug release up to 12 h. While, niosomal gel displayed controlled drug release up to 24 h with a reduction in skin irritation in the Wistar rats. An in-vivo skin deposition study showed 2.5-fold higher ADP retention in the stratum corneum layer as compared to commercial ADP formulation. ADP niosomal gel would be a safe and valuable option to the conventional delivery systems for the treatment of acne.

Keywords- Adapalene, niosomes, carbopol gel, skin permeation, skin irritation, acne vulgaris
1. Introduction

Acne vulgaris is the most common and chronic skin disease in teenagers and adults. It is associated with an elevated rate of sebum secretion which shows as mild-severe inflammatory lesions on areas of the face, back and chest enclosing large numbers of sebaceous follicles. It is categorized as mild, moderate or severe based on the number and type of lesions. Treatment choices differ with the phase and intensity of the disease. Usually, topical treatment is favored for mild and moderate acne while systemic therapy is utilized to treat severe cases. Topical therapy mainly contains retinoids (vitamin A), antibiotics and combinations thereof [1,2].

Adapalene (ADP) is chemically 6-[3-(1-adamantyl)-4-methoxyphenyl]-2-naphthoic acid. ADP is a synthetic third generation retinoid compound and a broad-spectrum anti-acne agent approved by the USFDA in 1996. Though, the precise mechanism of action is unknown, it binds to certain retinoic acid receptors in the nucleus leading to specific gene expression. It accelerate skin growth through modulation of cellular differentiation, keratinization of follicular epithelial cells and inflammatory processes [3,4]. ADP is a BCS class II drug with log P and pKa of 8.6 and 4.2, respectively. ADP showed poor therapeutic applications clinically because it causes irritation of skin, dryness, burning or stinging sensation, peeling/exfoliation and photosensitivity with pigmentation. To overcome these limitations, various approaches have been studied such as liposomes [5], TyroSpheres [6], polymeric micelle [7] and hydrogel matrix [8] to control and improve the ADP permeation. Nano-vesicles have showed huge potential in the topical delivery due to several advantages such as prolong therapeutic effect, avoid entry of drug into the systemic circulation and therefore controlling side effects. Furthermore, these nano-vesicles have been shown to provide efficacy, tolerability, compliance and cosmetic acceptability [1,2].
Lipid vesicles based delivery systems have established notable attention by scientific community due to various delivery advantages. Niosomes are non-ionic surfactant-based vesicles with a similar structure to that of phospholipid vesicles liposomes [9-13]. The unique arrangement of niosomes makes it capable of encapsulating both lipophilic and hydrophilic drug molecules. In recent years, the capability of niosomes as a drug carrier has been extensively investigated. They are enabling to deliver the drug at target site in a controlled and/or sustained manner and have long shelf life [14]. According to their size and structure, niosomes can be administered by various routes such as intravenous, intramuscular, intraperitoneal, subcutaneous, pulmonary, ocular, oral and transdermal also. They are also biocompatible, biodegradable and non-immunogenic in nature with low toxicity [15]. Moreover, inclusion of these vesicles in gel matrices enhances drug uptake and decreases skin irritation because of their particle size and surface composition [16].

The aim of the study was to design, optimize and characterizes ADP loaded niosomes. A $3^2$ factorial design was used to evaluate the effect of the variables such as non-ionic surfactant and lipid concentration on mean particle size and entrapment efficiency. The drug-loaded niosomes were characterized for entrapment efficacy, particle size, zeta potential, fourier transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), transmission electron microscopy (TEM), in-vitro drug release study, ex-vivo permeation study and one month stability. Additionally, optimized niosomes were incorporated in to Carbopol gel for suitable topical application. Niosomal gel was investigated for in-vitro drug release study, ex-vivo permeation study. Additionally, niosomal gel was evaluated for in-vivo skin penetration and skin irritation effects in Wistar rats and compared with commercial formulation.
2. Materials and methods

2.1 Materials

Adapalene was gifted by Abbott Healthcare Pvt. Ltd., India. Span 60 and cholesterol was purchased from Loba Chemie Pvt. Ltd., India. Carbopol 934 NF was obtained from S.D. Fine Lab, Mumbai, India. Adaferin® gel (0.1 %, Galderma India Pvt. Ltd.) was purchased from local market. All other chemicals and reagent were of analytical grade.

2.2 Animals

Wistar male rats (National Toxicology Centre, Pune, India) weighing between 180-200 g were utilized for skin permeation, skin irritation and skin penetration studies. All animals were kept in specific plastic cages under standard conditions of temperature (24 ± 1 °C) and relative humidity (45-55 %) and 12 h duration light/dark cycles maintained throughout the study. Rats had free access to commercially available normal pellet diet (Pranav Agro 7 Industries, Maharashtra, India) and clean water ad libitum unless or else declared. The complete experiment was implemented in harmony with the guidelines of the Committee for the Purpose of Control and Supervision on Experimental Animals (CPCSEA). An experimental procedure was agreed by the Institutional Animal Ethics Committee (IAEC) of Poona College of Pharmacy, Pune (CPCSEA/26/2014).

2.3 Preparation of adapalene loaded niosomes

Adapalene loaded niosomes (ADP-NM) was prepared by modified ethanol injection method [17]. Briefly, different ratios of non-ionic surfactant (Span 60), cholesterol and adapalene (10
mg) were accurately weighed, dissolved in 10 mL of acetone and ethanol (1:1) using bath
sonication at 60 °C. The clear organic phase was quickly injected into the aqueous phase at 60
°C under continuous stirring of 500 rpm (Remi magnetic stirrer) utilizing a Teflon-coated bead.
The aqueous phase instantly transformed into milky phase indicated niosomes formation. The
whole system was subjected to evaporation at 60 °C under vacuum for 15 min to eliminate
ethanol and stirring was continue up to 12 h. Water was added to maintain the volume of
niosomal suspension up to 10 mL. The prepared niosomal dispersion was filtered through 2-20
μm filter (Ultipor GF Plus®, Pall Life Sciences, CA, USA) to obtain a uniform size distribution
and refrigerated for 2 h for efficient vesicle sealing. Besides, aqueous dispersion of ADP (ADP-
AD) was formulated by dispersing accurately weighed 10 mg of ADP in 3 mL of Millipore water
containing 0.5 % of carboxyl methyl cellulose using magnetic stirrer (Remi Ltd., Mumbai, India)
at 500 rpm under ambient environmental conditions [18].

2.4 Effect of variables

Based on previous understanding and preliminary data, the $3^2$ factorial design was utilized to
optimize the amount of Span 60 ($X_1$) and cholesterol ($X_2$), recognized as the independent
variables affecting the mean particle size ($Y_1$) and drug entrapment efficacy ($Y_2$). The coded and
actual values of the experimental design are given in Table 1. The data analysis of values
achieved from various batches for mean particle size and drug entrapment efficacy were
subjected to multiple regression analysis using statistical software Unistat® (statistic Version 3,
US).

The equation fitted was,

$$Y = \beta_0 + \beta_1 X_1 - \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_{12} - \beta_{22} X_{22} \ldots \ldots \ldots \ldots \ldots \ldots (1)$$
Where, Y is the dependent variable, $\beta_0$ is the arithmetic mean response of the nine runs, $\beta_1$ and $\beta_2$ are the estimated coefficient for the independent factors $X_1$ and $X_2$ respectively. The main effects terms ($X_1$ and $X_2$) symbolize the average outcome of varying one factor at a time from its low to high value. The interaction terms ($X_1X_2$) exhibit how the response alter when two factors are concurrently altered. The consequence of the variables are clarified considering the degree of coefficient and the numerical indication it carries.

3. Characterization of adapalene loaded niosomes

3.1 Determination of encapsulation efficiency

Encapsulation efficiency was estimated by isolating non-encapsulated ADP from niosomal dispersion by centrifugation at 25,000 rpm for 20 min at 25°C. The supernatant was suitably diluted with dimethyl sulfoxide (DMSO) and free ADP content was calculated using a UV spectrophotometer (Jasco, V-530, Japan) at 270 nm. The entrapment efficiency (EE) was determined using the following equation [19];

$$EE = \frac{The\ total\ amount\ of\ drug - The\ total\ amount\ of\ drug\ in\ supernatant}{The\ total\ amount\ of\ drug} \times 100$$

3.2 Determination of particle size and zeta potential

The ADP-NM size was estimated using a dynamic laser diffraction system (Hydro 2000 SM, Malvern Instruments, UK) at a 90° scattering angle in normal room temperature with samples aptly diluted with dispersant water. Per sample, the mean diameter ± S.D. of three determinations
was estimated using multimodal analysis [20]. The surface charge was measured in triplicate with the laser doppler electrophoretic mobility measurements via Zetasizer (NanoBrook ZetaPALS) using 90 plus particle sizing software (ver. 394) at a temperature of 25°C [18].

3.3 Fourier transform infrared spectroscopy

To study the possible chemical interactions between ADP, excipients and formulation, fourier transform infrared (FTIR) spectra were run on the Jasco V5300 FTIR (Tokyo, Japan). Samples were mixed with KBr to construct pellets by employing a pressure of 150 kg/cm². FTIR spectra were scanned in the range of 4000-400 cm⁻¹ at a resolution of 2 cm⁻¹ [21].

3.4 Differential scanning calorimetry

Constant heating rate of 10 °C/min was utilized over a temperature range of 20-340°C for the lyophilized ADP-NM and their individual excipients components with nitrogen purging (50 mL/min). Indium standards were utilized to standardize the enthalpy scale and temperature. Approximately 3-4 mg of samples were used for the differential scanning calorimetry (DSC) study [22].

3.5 Transmission electron microscopy

Surface morphology of the ADP-NM was determined by utilizing transmission electron microscopy (TEM; Philips CM-200, Eindhoven, Netherlands). The ADP-NM (6-10 µL) was placed over Formvar® coated copper grids Philips CM-200 (Ted Pella, Inc., Redding, CA) for 5 min, adsorbed using filter paper then a dried at room temperature for 1 h. The dried grid
containing the ADP-NM was visualized using TEM coupled with digital micrograph and soft imaging viewer software (Olympus, Singapore).

3.6 In-vitro drug release study

The in-vitro release of ADP-NM was investigated at physiological temperature with ethanol: water (80:20) as a dissolution medium using dialysis bag diffusion method [23]. Briefly, ADP-NM equivalent to 4 mg of ADP was placed into a dialysis bag (cellulose membrane, mol. wt. cut off 12-14 0000 Da) hermetically closed and dipped into 200 mL of ethanol: water (80:20) media. The whole system was kept at 37 ± 2 °C with constant magnetic stirring at 100 rpm. At predetermined time slots, sample was collected and subsequently filtered through 0.45-mm membrane filter, if required suitably diluted and the concentration of ADP was estimated spectrophotometrically at 270 nm.

3.7 Ex-vivo skin permeation study

The ex-vivo skin permeation of ADP from ADP-NM was studied by using the Franz diffusion cell method [24]. The abdominal skin of the rat was removed, carefully excised and defatted so as to remove the subcutaneous fat. The defatted skin was cleaned several time with purified water. Skin permeation experiment were performed with vertical Franz diffusion cells with surface area of 2.54 cm² and reservoir capacity of 19 mL. The defatted skin was mounted on the Franz diffusion cell with the dermal side facing the receptor solution containing mixture of ethanol and water (80:20) and the epidermal side in contact with donor compartment and allowed to equilibrate for 30 min at 37 °C. The receptor solution was magnetically stirred at 100 rpm. Skin was treated with ADP alone and optimized ADP-NM (Batch 5) equivalent to 4 mg ADP.
The donor compartments were sealed carefully with parafilms to avoid evaporation of the solution. At predetermined time points aliquots of the receptor medium (1 mL) were withdrawn, subsequently filtered through 0.45-mm membrane filter and replaced with fresh acceptor medium. The collected aliquots was diluted and quantitatively analyzed by UV spectrophotometer at 270 nm. All measurements were performed in triplicate and their means were reported.

3.8 Stability study

ADP-NM were stored at room temperature (25 ± 5 °C and 60 ± 5 % RH) and at 4 °C for 1 month. Particle size and encapsulation efficiency were investigated. Additionally, ADP-NM were visually evaluated for presence of any precipitations. The samples were stored in USP type-1 flint vials and hermetically sealed with bromo butyl rubber plugs with aluminum caps [25].

4. Preparation of gel

Niosomal gel was formulated according to previously published method [26]. Gel of optimized ADP-NM (Batch 5), was formulated by incorporating the niosomal dispersion in 1 % (w/w) Carbopol® 934 NF gel base. Briefly, sufficient amount of Carbopol® 934 NF was gradually added to water and kept for 24 h for complete hydration of polymer chains. Triethanolamine (0.1 mL) was utilized to cause gelling as well as to neutralize the pH of the gel to 6-7. Optimized ADP-NM (Batch 5) and ADP alone was added slowly to the hydrated carbopol solution to obtained ADP-NM loaded gel (ADP-NM-G) and ADP gel (ADP-G) with a final concentration of 0.1 % (w/w) of ADP, respectively. Besides, in similar manner placebo carbopol gel was also formulated without addition of ADP-NM or ADP.
5. Characterization of gel

5.1 pH, drug content and appearance

The pH of the prepared ADP-NM-G was determined by using digital pH meter. Drug content was determined by dissolving ADP-NM-G in DMSO followed by dilution with ethanol: water (80:20). The ADP concentration was estimated spectrophotometrically at 270 nm [27]. The prepared ADP-NM-G inspected visually for their appearance, color and homogeneity.

5.2 Viscosity

Viscosity of the ADP-NM-G was measured using a stress control rheometer (Viscotech Rheometer, Rheologica Instrument AB, Sweden) [27]. Accurately weighted 1 g of ADP-NM-G was placed on the viscometer plate with a diameter of 2.9 cm and roto cone with 2.8 cm in diameter. The viscosity were calculated through a rheologic basic software (Version 5; Rheologica Instrument AB, Sweden) and the average of three readings was utilized to determined the viscosity at normal room temperature.

5.3 Spreadability

Spreadability study of ADP-NM-G was analyzed by wooden block and glass slide apparatus. Accurately weighted 5 gm of ADP-NM-G placed on the lower side of block and movable upper slide was placed on the top of the gel and time was noted for the upper slide to separate completely from the assembly [28]. The spreadability was determined by the formula;

$$S = \frac{m \times l}{t}$$
Where, $S$ is spreadability, $m$ is weight tied to upper slide, $l$ is length travel by upper slide and $t$ is time taken to separate slide.

5.4 In-vitro release studies

In-vitro release were carried out utilizing an artificial cellophane membrane (Membra-Cel® MD 34-14; cut-off: 12 kDa; Viskase Co, MS, USA). For this study, a vertical Franz diffusion cell with a reservoir volume of 32 mL and a surface area of 2.54 cm$^2$ was utilized. The artificial cellophane membrane was firmly mounted between the two halves of the diffusion chamber. The receptor chamber contained ethanol: water (80:20). The whole system was kept at $37 \pm 2$ °C with constant magnetic stirring at 100 rpm. Accurately weighted 1 gm of the ADP-NM-G and gel commercial gel was placed on the donor chamber, respectively. The donor compartments were sealed carefully with parafilms to avoid evaporation of the solution. At predetermined time points aliquots of the acceptor medium (1 mL) were withdrawn, subsequently filtered through 0.45-mm membrane filter and replaced with fresh acceptor medium. The collected aliquots was then properly diluted and quantitatively analyzed by UV spectrophotometer at 270 nm. All measurements were performed in triplicate and their means were reported. The release kinetics of ADP from ADP-NM-G and commercial gel was compared. Analysis of the data was carried out using PCP Disso software, version 3 (Poona College of Pharmacy, India).

5.5 Ex-vivo permeation study

The ex-vivo permeation study was carried out on excised Wistar rat skin according to the study protocol approved by the IAEC constituted under the CPCSEA. The abdominal skin of the rat was removed, carefully excised and defatted so as to remove the subcutaneous fat. The defatted
skin was cleaned several times with purified water. Further experimentation was similar to method described in the ‘Ex-vivo skin permeation study’ section. Measurements were performed in triplicate and their means were reported.

5.6 Skin irritation study

The skin irritation potential of ADP-NM-G was performed using Draize patch test on rats [29]. Skin irritation test of developed ADP-NM-G was compared with the commercial gel and ADP-G. The present study was employed in the three groups of rats (n = 6) to evaluate skin irritation. The area on the back of each rat was shaved prior to the experiment. The first, second and third group was applied with ADP-G, optimized ADP-NM-G and commercial gel, respectively. An accurately weighed 1 g of each formulation was applied to the hair-free skin of rats by equally distributing within an area of 4 cm². The skin was observed for any visible change such as erythema (redness) at 24, 48 and 72 h after the application of formulations. The sensitivity was scored as 0, 1, 2 and 3, for no reaction, slight patchy erythema, patchy erythema and severe erythema with or without edema, correspondingly [30]. Additionally, the primary irritation index (PPI) was calculated method described by Jain et al (2011) [31].

5.7 In-vivo skin permeation study

The in-vivo skin permeation of ADP was studied by using the tape-stripping method [32]. The animals were divided into three groups (n=6) to evaluate skin penetration potential. In brief, the dorsal skin of rats was shaved prior to the experiment and first, second and third group was applied with ADP-G, optimized ADP-NM-G and commercial gel, respectively. A formulation equivalent to 1 mg was applied to the hair-free skin of rats by equally distributing within an area
of 2 cm². The in-vivo trials were performed in an examination room under controlled environmental conditions of temperature 25 ± 1 °C and 40 ± 5 % RH. At the end of the 12 h treatment, excess formulation was removed, and the stratum corneum (SC) surface was blotted totally dry with absorbent pad. The site was then left untouched for 1 h before stripping. Then the SC was serially stripped from the same area, 15 times with adhesive tapes (Scotch Book Tape; 3M Tegaderm) in accordance with the already published data. ADP in the tape strips was consequently extracted with 5 mL of DMSO at controlled environmental conditions. Samples were filtered through 0.45 μm membrane filter prior to UV analysis. The sample was then appropriately diluted and quantitatively analyzed by UV spectrophotometer at 270 nm.

5.8 Stability Study

Developed ADP-NM-G was packed in USP type-1 flint vials and hermetically sealed with bromo butyl rubber plugs with aluminum caps and subjected to stability for 6 months at room temperature (25 ± 5 °C and 60 ± 5 % RH). The samples were observed periodically for any change in the physicochemical parameters including color, appearance and drug content.

5.9 Statistical analysis

All the experiments were performed in triplicates and displayed as the mean ± standard deviation (SD). Results obtained was subjected to Student’s t-test and statistical analysis was carried out using online QuickCalcs GraphPad software. Data analyzed in following manner; *P > 0.05 (no statistical significant), **P ≤ 0.05 (statistical significant) and # p ≤ 0.001 (very statistical significant).
6. Results and discussion

High surface area of skin (1.5-2.0 sq. m) and advantages such as noninvasive nature, bypassing first-pass metabolism, controlled delivery of medicament, reduced dosing frequency and improved patient compliance have recognized the topical route as a better option to drugs with limited oral bioavailability. Several studies showing the potential of various novel nanocarriers in combating the barrier nature of stratum corneum further increased the interest of formulation scientists in investigating the newer ways to maximize the drug delivery through topical route.

Among these various novel nanocarriers, niosomes were selected in the present investigation for their better stability, less toxicity and cost-effectiveness with an aim to enhance the therapeutic efficacy and reduced systemic toxicity of ADP and the results were compared with commercial ADP topical formulation [1,19, 33].

6.1 Factorial design

In present study, niosomes have been formulated and studied as topical delivery carriers for ADP. The $3^2$ factorial design was performed using Design-Expert® (Ver. 9.0, Stat-Ease, Inc., USA) program. Literature review and preliminary trials were utilized to choose the initial variables with the suitable levels [34]. The factorial design were used to determine the variables which have significant effect on mean particle size and entrapment efficiency EE) using two levels with center point in numeric variables. A classical niosomes vesicle would consist of non-ionic surfactants with different HLB values that affects the EE which is also stabilized by the addition of the optimum amount of cholesterol to develop stable vesicles. Further increase of cholesterol content beyond certain limit decrease the EE because of the disruption of the normal
bilayer arrangement; so factorial design were use to study the most suitable ratio of surfactant and cholesterol.

As per the $3^2$ factorial design, nine different batches were formulated using modified ethanol injection method. Response surface plot was construct using Design-Eexpert® program demonstrating the effect of the two significant variables as a surface in three-dimensional space are shown in Figure 1. Whereas the coded levels and actual values of the variables along with the calculated responses are revealed in Table 1. The data obtained were subjected to multiple regression analysis using PCP Disso software and fitted with the following Equation

$$Y = \beta_0 + \beta_1 X_1 - \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 - \beta_{22} X_2^2 \ldots . . . . . (1)$$

where, $X$ is the level of the factors, $Y$ is the measured response and $\beta$ is the coefficient computed from the responses of the formulations. The outcomes of the multiple regression analysis for particle size and EE are as follows;

$$Y_{ps} = 299.11 + 72.00 X_1 - 32.50 X_2 \ldots . . . . . . (2)$$

$$Y_{EE} = +84.96 + 7.94 X_1 - 2.73 X_2 \ldots . . . . . . (3)$$

Particle size is the key variable that determines the rate and extent of drug release. Moreover, it also has significant impact on permeation and biodistribution of drug. The particle size, uniformity and zeta potential (Fig. 2) of optimized ADP-NM batch was 278 nm, 0.727 and -17.99 mV, respectively.
Figure 1 - Response surface plots of (a) particle size and (b) entrapment efficiency.
Figure 2 - Zeta potential of ADP-NM.
The EE of the optimized ADP-NM batch was 86.07 % (Table 1). It was observed that increase in the surfactant concentration increases the EE. It may be due to high surfactant fraction controlled the diffusion rate of drug from concentrated exterior surfactant phase. Eventually, it offers more time for the droplet formation and thus, enhance the EE [23]. The mean particle size ($Y_{ps}$) and entrapment efficiency ($Y_{EE}$) were in the ranges of 218-479 nm and 72.44-94.23 %, respectively. The multiple regression analysis of the mean particle size and EE of the factorial batches revealed a good fit ($R^2 = 0.9796$ for particle size and $R^2 = 0.9727$ for EE), suggesting strong influences of the selected variables. After interpretation of the developed surface plots, it was observed that Span 60 and cholesterol at the middle level (0) is better respect to mean particle size and EE. The optimized ADP-NM (batch 5) contains Span 60 (60 mg) and cholesterol (20 mg). The results displayed that the observed values of the optimized formulation were well similar to the predicted values. It has been well reported that the electrostatic attraction between the carboxylic -OH and positively charged particles affect the charge distribution and therefore causing a reduction in zeta potential of whole system. This nature clearly suggest the existence of strong electric charges on the particles surfaces to obstruct the agglomeration [35]. Value of zeta potential displayed that formulated ADP-NM have an adequate charge and mobility to hinder aggregation of particles. This optimized ADP-NM was preferred for further investigation.
Table 1- Optimization of non-ionic surfactant and cholesterol concentration by $3^2$ factorial design for the preparation of ADP-NM.

| Batches | Coded levels (X1, X2) | Non-ionic surfactant (mg) (X1) | Cholesterol (mg) (X2) | Particle size (nm) (Y1) | Entrapment efficiency (%) (Y2) |
|---------|-----------------------|-------------------------------|----------------------|------------------------|---------------------------------|
| B1      | -1,-1                 | 40                            | 10                   | 200 ± 11.45            | 80.54 ± 10.99                   |
| B2      | 0,-1                  | 60                            | 10                   | 349 ± 12.01            | 88.34 ± 11.67                   |
| B3      | +1,-1                 | 80                            | 10                   | 479 ± 11.87            | 94.23 ± 12.01                   |
| B4      | -1,0                  | 40                            | 20                   | 259 ± 12.54            | 76.26 ± 13.39                   |
| B5      | 0,0                   | 60                            | 20                   | 278 ± 13.09            | 86.07 ± 11.19                   |
| B6      | +1,0                  | 80                            | 20                   | 274 ± 12.01            | 92.43 ± 12.09                   |
| B7      | -1,+1                 | 40                            | 30                   | 218 ± 12.23            | 72.44 ± 12.01                   |
| B8      | 0,+1                  | 60                            | 30                   | 279 ± 12.34            | 84.08 ± 13.76                   |
| B9      | +1,+1                 | 80                            | 30                   | 336 ± 13.55            | 90.23 ± 12.55                   |

Date are mean ± SD, n = 3.

6.2 Fourier transform infrared spectroscopy

The FT-IR spectra of ADP, cholesterol, Span 60 and lyophilized ADP-NM are shown in Figure 3. The ADP showed characteristic hydroxyl group peak at 2903 cm$^{-1}$ while ADP-NM showed a absorption peak at 2918 cm$^{-1}$, corresponding to the hydroxyl group peak of ADP. Furthermore, ADP also showed characteristic stretching bands within the carbonyl region (1689 cm$^{-1}$) which was slightly shifted to the higher side (1737 cm$^{-1}$) in the ADP-NM. This could be accredited to interaction of carbonyl group with the hydroxyl groups of cholesterol or Span 60.
Figure 3 - FTIR of Span 60, cholesterol, ADP and ADP-NM.

6.3 Differential scanning calorimetry

Differential scanning calorimetry (DSC) thermograms of ADP, cholesterol, Span 60 and lyophilized ADP-NM are revealed in Figure 4. The DSC thermogram showed that ADP
represents a single endothermic peak at 329 °C representing its crystalline nature, while cholesterol and Span 60 was melted at 150.45 and 59.20 °C, respectively. Melting endotherm for ADP was not observed in thermogram of lyophilized ADP-NM demonstrating the ADP was completely encapsulated within the cholesterol matrix of niosomes in amorphous form.

**Figure 4-** DSC thermograms of ADP and ADP-NM.
6.4 Transmission electron microscopy

The TEM image of ADP-NM was shown in Figure 5. TEM analysis represents that ADP-NM are spherical in shape. The surface of niosomes is smooth and size was found to be < 200 nm which was in equivalence with the measured particle size.

![Figure 5 - Representative TEM micrographs of optimized ADP-NM (batch F5).](image)

6.5 In-vitro drug release study

The release pattern of ADP from ADP-NM depends on various experimental parameters such as particle size, dissolution media and of the drug and polymer in media. The in-vitro release pattern of ADP from ADP-NM was shown in Figure 6. The release of ADP from ADP-AD was found to be more rapid and reached 99 % in 12 h. However, ADP release from ADP-NM exhibited a biphasic release pattern with an initial burst release (26 %) within 1 h followed by a sustained release at up to 12 h with 73 % release of ADP. This considerable difference of ADP release performance in both the cases might be described by the fact that release of ADP from the niosomal membrane like more controlled which used to improve the penetration when applied topically.
Figure 6 - Comparative in-vitro release profile for free ADP and ADP-NM in ethanol: water (80:20) as a dissolution media using dialysis bag diffusion method. All data are means ± SD (n = 3).

6.6 Ex-vivo skin permeation study

The ex-vivo permeation study was carried out for ADP-AD and optimized ADP-NM (Batch 5) in order to provide better permeation profiles through rat skin. Amount of drug permeated at the end of 24 h was 36.08 % for ADP-AD while it was 64.31 % for the ADP-NM (Fig. 7). The drug permeation through the skin was slight higher in case of niosomes compared to ADP-AD indicating the availability satisfactory drug concentration at target site which is advantageous for effective therapy of acne.
Figure 7 - Comparative ex-vivo permeation study for free ADP and ADP-NM in ethanol: water (80:20) as a dissolution media using Franz diffusion method. All data are means ± SD (n = 3).

6.7 Stability study

The stability study of optimized ADP-NM was studied by subjecting the formulations two different environmental conditions and observing the changes in particle size and encapsulation efficacy as key markers for stability. The results of stability study was showed in Table 2. The prepared ADP-NM was found stable during storage over the period of 1 month. No major change in the physicochemical parameters such as appearance, color and no precipitation were seen during storage. There was no statistical significant (P > 0.05) difference were observed in the particle size and encapsulation efficacy.
Table 2- Stability study of optimized ADP-NM (n=3)

| Condition                  | Particle size (nm) | Entrapment efficiency (%) |
|----------------------------|--------------------|---------------------------|
| Initial                    | 278 ± 13.09        | 86.07 ± 11.19             |
| 25 ± 5 ºC (1 month)        | 302 ± 11.97*       | 76.26 ± 11.08*            |
| 4 ± 1 ºC (1 month)         | 294 ± 12.73*       | 79.23 ± 10.12*            |

* P > 0.05 (no statistical significant) when compared to initial analysis.

7. Evaluation of gel

7.1 pH, drug content and appearance

ADP-NM-G was successfully prepared using film hydration technique. Developed ADP-NM-G remained in the pH range of skin to evade any risk of irritation upon the application to the skin. Drug content was found to be 72 ± 2.9 %. Furthermore, developed ADP-NM-G was white in color, uniform and had translucent matrix structure (Table 3).

7.2 Viscosity and spreadability

The ADP-G and ADP-NM-G showed viscosity of 22.01 and 23.97 Pa.s, respectively. Inclusion of ADP-NM in to Carbopol® 934 NF gel base showed satisfactory improvement in viscosity profile as compared to ADP-G. Cholesterol particles of niosomes leads to formation of colloidal network that aligns itself in the direction of applied shear, which cause in an enhance in viscosity compared to ADP-G. Moreover, viscosity of topical gels is one of the essential physical constraint which is inversely proportional to the degree of permeation if diffusion through vehicle is the rate-limiting step. Generally, an enhance in viscosity of system would basis a more
rigid structure and lessen the drug release rate and permeation. Spreadability of ADP-G and ADP-NM-G was found to be 11.37 and 18.75 g.cm/s. The ADP-NM-G showed satisfactory improvement in spreadability as compared to ADP-G. Result show that ADP-NM-G is easily spreadable by applying a small amount of shear. This might be due to the unique gel matrix structure of ADP-NM-G because of presence of cholesterol vesicles.

7.3 In-vitro release studies

ADP release pattern from ADP-NM-G and commercial (Adaferin®) gel was represented in Figure 8. ADP-NM-G showed higher release rate (48 %) of ADP compared to Adaferin® gel formulation (43 %). While ADP-G exhibited higher release rate of ADP compared to ADP-NM-G (data not shown). The delayed release rate from ADP-NM-G was attributed to encapsulation of the ADP into niosomes vesicles. The solid matrix of the ADP-NM-G was responsible for drug immobilization and subsequent lower drug release than ADP-G. With the fact that niosomes encapsulation controls ADP release from ADP-NM-G means that niosomes integrity is not influenced by the gel structure in which niosomes are dispersed [16]. Similar release kinetics were seen for etodolac containing topical niosomal gel system [28]. This controlled release profile of ADP into the skin can modify its percutaneous absorption. Simply, they would stay on the skin surface and slowly releasing their content over time, resulting in better safety of the applied drugs [36].
Figure 8 - Comparative in-vitro release profile for ADP-NM-G and ADP commercial gel in ethanol: water (80:20) as a dissolution media using a vertical Franz diffusion cell method. All data are means ± SD (n = 3).

7.4 Ex-vivo permeation studies

The ex-vivo permeation study was carried out for ADP-NM-G and commercial (Adaferin®) gel to present better comparisons between permeation behavior through rat skin. The amount of drug permeated per unit skin surface area was plotted against time (Fig. 9). Amount of ADP permeated through the skin at the end of 24 h was 46.49% from ADP-NM-G whereas it was 43% from the Adaferin® gel. The drug diffusion through the skin was slight higher in case of ADP-NM-G as compared to Adaferin® gel indicating the availability satisfactory drug concentration at target site which is advantageous for effective therapy of acne.
Figure 9 - Comparative ex-vivo permeation study for ADP-NM-G and ADP commercial gel in ethanol: water (80:20) as a dissolution media using Franz diffusion method. All data are means ± SD (n = 3).

7.5 Skin irritation study

The outcomes of skin irritation studies were based on visual observation of erythema (redness). Repetitive uses of ADP-NM-G exhibited negligible skin irritancy on rat skin (Fig. 10). It is recommended that the niosomal gel formulation shows no irritation therefore, enhancing suitability for safe topical application. The earlier study showed controlled release of ADP for therapeutic action, whereas the present study showed the controlled release with nonirritant nature of the niosomal gel formulation [37]. Additionally, PPI score was showed in Table 4. ADP-G and commercial formulation showed PPI of around 1 while ADP-NM-G showed PPI of less than 1. Briefly, all three groups showed PPI value less than 2 indicating that there are no signs of skin irritation [38].
Figure 10 - Comparative skin irritation study for ADP-G, ADP-NM-G and ADP commercial gel using Draize patch test. Where, after 24 h, A: control, B: ADP commercial gel, C: ADP-NM-G, D: ADP-G and after 7 day, A': control, B': ADP commercial gel, C': ADP-NM-G, D': ADP-G.

7.6 In-vivo skin penetration study

In-vivo skin penetration study results are showed in Table 5. After 12 h the amount of ADP recovered per area in the strips was 112, 84.4 and 46.10 μg/cm² in SC of rats treated with ADP-NM-G, ADP-G and Adaferin® gel, respectively. The ADP recovered from strips were considerably different at 1, 6 and 12 h (data not shown). During in-vivo skin penetration study, ADP-NM-G showed significant difference of 1.32 and 2.10-fold in ADP SC residence as compared to ADP-G (P ≤ 0.05) and Adaferin® gel (P ≤ 0.01), respectively. This indicated that the niosomal gel improved the drug residence of the skin. This outcome is in agreement with earlier investigations verifying that the use of vesicular delivery enhanced the drug residence in
the SC without modifying transdermal drug transport [36]. This enhanced dermal retention of ADP was also credited to improved contact with corneocytes and sustained release properties of niosomes. Because of their small particle size, niosomes create closer contact with an apparent junctions of corneocyte groups and channels nearby between corneocyte surfaces and leads accumulation for several hours [29]. Moreover, the high concentration of the ADP in SC after application of ADP-NM-G might be described by the occlusive effect, since ADP-NM-G construct a film on the skin surface that controls transepidermal water loss and favors drug penetration into the SC [39]. Therefore, we can conclude that the superior ADP retention in skin is essentially credited to the niosomal carriers, particle size and bioadhesive characteristics of ADP-NM-G [29].

7.7 Stability Study

Similar to ADP-NM, ADP-NM-G was also subjected to stability study. The prepared ADP-NM was found stable during storage over the period of 3 month. No major change in the physicochemical parameters such as appearance, color and no precipitation were seen during storage. There was no notable difference was observed in drug content (72.07 %) of ADP-NM-G after during three months storage as compared to initial drug content (76.43 %).

8. Conclusion

ADP-NM with a high EE and low particle size were prepared effectively using the modified ethanol injection method. It was found that the concentration of surfactant and cholesterol plays a important role in particle size and drug entrapment. Microscopy studies confirmed spherical and smooth surface niosomes in the nanometer size range. There was sharp decreased
crystallinity of the ADP when loaded in niosomes, which was verified by DSC studies representing the complete incorporation of ADP into the niosomal vesicles. ADP-NM-G demonstrated better rheological properties and control drug release up to 24 h as compared to commercial gel. The higher amount of ADP deposited in the stratum corneum from the ADP-NM-G shows that niosomes improved ADP residence in skin. Additionally, ADP-NM-G showed no irritation to the skin as compared to ADP-G and commercial formulation. Our study shows the potential of niosomal gel for treatment of acne. Therefore, design and development of topical ADP-NM-G can be a novel and effective option to the commercial formulations.

Acknowledgements

The authors would like to thanks Abbott Healthcare Pvt. Ltd., India for Adapalene gift sample. The authors would like to acknowledge the support from Bharati Vidyapeeth Deemed to be University, Poona College of Pharmacy, Pune, Maharashtra, India.

Research involving human participants and/or animals

The present work involves animal study. The whole animal study was performed according to the guidelines of the CPCSEA (CPCSEA/26/2014). Besides, all animal study protocols were agreed by the IAEC of Poona College of Pharmacy, Pune.

Disclosure of potential conflicts of interest

Authors do not have any conflict of interest regarding present work.
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