RESEARCH ARTICLE

Formulation Development and Characterization of Acitretin Proniosomal gel: *In-vivo* Exploration against Imiquimod-induced Psoriasis in Experimental Animals

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Received: 15th September, 2023; Revised: 18th October, 2023; Accepted: 15th November, 2023; Available Online: 25th December, 2023

ABSTRACT

The main purpose of this study was to prepare and evaluate acitretin proniosomal gel (APG) and find out if the antipsoriatic formulation could prevent Imiquimod (IMQ) induced psoriasis in experimental animals. APG was developed and by coacervation phase separation method. Cholesterol and soya lecithin was used as membrane modifiers and tween 80 and span 60 as non-ionic surfactants. Characterization of the prepared proniosomal gel revealed that the optimized batch APG3 presented excellent formulation characteristics like the viscosity of 9710 ± 1.24 cps, rate of spontaneity as 13 ± 0.86, %entrapment efficiency of 94.91 ± 0.51, average hydrodynamic diameter of niosomes as 345.7 nm, polydispersibility index as 0.159 and ZP of -2.3 mV. *In-vivo* animal study of the optimized batch APG3 was performed along with biochemical estimations of oxidative parameters as glutathione (GSH), superoxide dismutase (SOD), malondialdehyde (MDA) and catalase; also, TNF-α, IL-6 were estimated using ELISA sandwiched. Histopathological estimation along with psoriasis area severity index (PASI) scores in the IMQ-induced psoriatic model presented a significant fall in the scoring after treatment with APG3 compared with a psoriatic group. Primary dermal irritation scoring was also evaluated on Sprague Dawley rats. The results presented a considerable decline in skin irritation score compared with a psoriatic group. Also, the levels of GSH, SOD and catalase were increased in APG3 treated group as compared with a psoriatic group. While MDA level is decreased after topical treatment with APG3 compared with a psoriatic group. In conclusion, the optimized APG3 showed promising pharmacological effects in the treatment of psoriasis.

Keywords: Acitretin proniosomal gel, Psoriasis, *In-vivo* exploration, Superoxide dismutase, Glutathione, Malondialdehyde.

International Journal of Pharmaceutical Quality Assurance (2023); DOI: 10.25258/ijpqa.14.4.29

How to cite this article: Gudalwar BR, Shukla TP. Formulation Development and Characterization of Acitretin Proniosomal gel: *In-vivo* Exploration against Imiquimod-induced Psoriasis in Experimental Animals. International Journal of Pharmaceutical Quality Assurance. 2023;14(4):1011-1016.

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

Worldwide the era of psoriasis has been too long. It is a type of chronic inflammatory dermatological disease. Globally approximately 1 to 3% of the population is suffers from psoriasis.[1] Skin lesions, like erythematous plaque and lamellar scales are the major characteristics of psoriasis.[2] Several factors like metabolic disturbances, obesity, immunological parameters, genetic modifications and stress all have an influence and can contribute in the progression of psoriasis.[3] The most affected areas in psoriasis are elbows, knees as well the scalp. Common symptoms for psoriasis are scaling, itching, erythema, burning and bleeding.[4]

Initially, for the treatment of psoriasis some systemic medicines like methotrexate, and cyclosporine are the first choice of drugs.[5] Those agents control psoriasis but they have some serious side effects.[6] That's why there is a need of alternative topical medicines as well as the less toxic compounds for overcome of psoriasis. There is no proper treatment for the psoriasis. It is a lifelong disorder that particularly affects the mental and social lifestyle of the patient. The given treatment only manages and controls the psoriatic symptoms.[7]

A second-generation monoaromatic retinoid named acitretin is an active metabolite of precursor etretinate. It shows the antipsoriatic effect by controlling keratinocyte growth in the epidermis.[8,9] It inhibits cell division in psoriasis plaques and stimulates cell division in healthy normal skin. It decreases the desquamation, erythema, and overall lesion thickness of psoriatic skin.[8] It may also activate polymorphonuclear leukocytes and suppress chemotaxis along with modification of T-cell response. Acitretin is a drug of choice for the treatment of dermal psoriasis and is primarily administered via oral route. However, its severe systemic side effects limit its use. It

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is BCS class II drug that is practically insoluble in water.[10-12] Its topical formulation can be developed which can help reduce its systemic side effects and its application at psoriatic patches can also reduce the drug dose.[13-15] But its photosensitivity and skin irritant property limits the preparation of its topical formulation.[15] Proniosomal gel-mediated topical drug delivery is one of the best options to prepare topical formulation of acitretin.[16] Better stability and higher entrapment efficiency make proniosomal gel unique in formulation compared to other vesicular drug delivery systems.[17] Entrapment of acitretin in vesicular form may avoid direct skin contact and reduce skin irritant effect.

MATERIALS AND METHOD

Antipsoriatic acitretin was received from Kivi Labs, Baroda. Cholesterol and soya lecithin were purchased from Sigma Aldrich, India. Surfactants tween 80 and span 60 were purchased from BASF, Mumbai and Oxford Laboratory, Mumbai respectively. Other excipients required for the formulation of proniosomal gel were of analytical grade.

Preparation of APG

First of all, acitretin was dissolved in dimethyl sulphoxide (DMSO) and then added to methanol to get a concentration of 1-mg/5mL and take the same in a glass vial. Then add surfactants tween 80: span 60 (1:1), cholesterol and soya lecithin in the vial. The vial was heated on magnetic stirrer with hot plate at (55–60ºC) at 40 rpm for 5 to 10 minutes to completely solubilize all ingredients. Phosphate buffer of pH 7.4 was heated to (55–60ºC) and added to the previously formed solution until a yellowish translucent proniosomal gel is formed.[18,19] After formulation of proniosomal gel it was incorporated in 1.5% carbopol 934 base. The prepared batches from APG1 to APG8 were kept in dark room for further use and characterization with the reference to Table 1.

Characterization of APG

Viscosity

In this test, 10 g of the formulation was transferred to a glass vessel and the spindle of viscometer was placed in the formulation. Later viscosity was measured by running the spindle at various revolutions such as 0.5, 1, 2, 3, 4, 5, 10, 20, 30, 40, 50, and 100 rpm with increasing and decreasing rpm and average value was determined.[18]

Table 1: Composition of acitretin loaded proniosomal gels using 2^3 factorial design

Batch	*Acitretin (mg)*	*Cholesterol*	*Soya Lecithin*	*Tween 80 : Span 60 (1:1)*
APG1	1	+	+	+
APG2	1	+	+	-
APG3	1	+	-	+
APG4	1	+	-	-
APG5	1	-	+	+
APG6	1	-	+	-
APG7	1	-	-	+
APG8	1	-	-	-

Spontaneity analysis

The stipulated quantity of proniosomal gel (20 mg) was placed in a beaker container and was smeared uniformly on the walls of a beaker. Then, add 2 mL of normal saline solution to the beaker wall and keep aside for approximately 20 minutes. Then, the vesicles (niosomes) number was counted by Neubauer's chamber by placing a drop of the solution.[20]

Entrapment efficiency

The upper clear liquid was evaluated at 357 nm spectrophotometrically to determine free drug concentration." With The upper clear liquid was evaluated by High Performance Liquid Chromatography (HPLC) to determine free drug concentration.

Entrapment efficiency (%) = [(Conc^n of total drug - Conc^n of free drug)/ Conc^n of total drug] ×100

Vesicle particle size (PS) and zeta potential (ZP) measurement

The niosomes PS and ZP of the optimized CPG3 was determined with the help of Litesizer 500 Zeta Potential Analyzer at 25°C. First of all take 100 mg of CPG3 and disperse it in 10 mL of phosphate buffer with mechanical shaking for 2 minutes.[21]

Animal Studies

Animals

Nulliparous Swiss albino mice and Sprague Dawley rats were used for this experimental examination. Standard laboratory conditions was provided to the experimental Animals for improve the results we provide 12 hours light-dark cycle, an atmosphere maintained under a humidity of 50 ± 5%, and room temperature (25 ± 2°C). Normal water *ad libitum* and normal pelletized feed diet is provided to animals (Amrut rodent Feed, Pune). The study was approved by the Animal Ethics Committee, TRANS-GENICA SERVICES PVT. LTD. Nagardeola, Tal- Pachora Dist- Jalgaon, Maharashtra, India. (Approval No. 1277/PO/RcBt/S/09/CPCSEA/TRS/PT/023/000)

Imiquimod induced psoriatic model

To assess the anti-psoriatic activity of APG3 against Imiquimod-induced psoriasis. We used Nulliparous Swiss albino mice (n = 6). Prior to study, mice were shaved from the backside. The experimental animals were divided as follows.

Group I: normal control.

Group 2: Psoriatic control

Group 3: Treatment 1 group /Plain proniosomal gel group (PPG)

Group 4: Treatment 2 group/ Acitretin proniosomal group

Group 5: Standard group/ salicylic acid treated group

Treatment of APG and PPG was initiated from 7th day to end of the study, PASI score was noted.

None (0), mild (1), moderate (2), and severe (3) and very severe (4).

At the end of the study (14th day), all the animals were sacrificed and further investigations like Biochemical, Proinflammatory cytokines & histopathology were performed.[22-25]

Skin irritation studies (Primary dermal test)

Animals were divided into two groups and study was performed to examine the potentiality of APG3. The skin of animals were shaved and APG3 along with 0.8% v/v formalin was applied on left of shaved back skin while right back side was considered as the control. The animals were specially examined for erythema, and edema for 30 minutes and later on signs of erythema and edema was checked.[25-27]

Assessment of pro-inflammatory cytokines

cytokines like TNF-α as well as IL-6 were evaluated by using the skin homogenate sample with the help of using ELISA sandwich and finally the concentration was estimated by using standard curve graph.[28]

Assessment of antioxidant parameters in skin

Antioxidants like superoxide dismutase (SOD), reduced GSH, and malondialdehyde (MDA) was estimated with the help of kits which are commercially available in the market.[28,29]

Histology

At the end of the experimental protocol all animals were sacrificed and the shaved back skin sample of mice was collected, stored in natural phosphate buffer and formalin. Followed by the sample was fixed, dehydrated, and finally fixed into paraffin. Finally, skin section of 5 mm thickness were taken by using a microtome. Later on, sections were stained with hematoxylin and eosin. Furthermore, those sections were examined under the light microscope.[29]

Statistical analysis

The study was analysed by one-way and two-way ANOVA using software Graph Pad Prism 6.0, USA. Analysis is represented as mean ± SEM for TWO separate experiments with *p-value* < 0.001.

RESULTS AND DISCUSSION

Preparation of APG

The purpose of this study was to develop acitretin loaded proniosomal gel by applying 2^3 (two level three variable) factorial design. Non-ionic surfactants span 60 and tween 80 in 1:1 ratio, cholesterol and soya lecithin were taken as three variables in the composition with two levels high and low on the basis of pre-formulation studies. Coacervation-phase preparation method is a simple and time-saving method and can be performed with the help of simple laboratory equipment. Proniosomal gel was prepared by mixing all alcohol-soluble ingredients in methanol and water-soluble ingredients in the aqueous phase. Non-ionic surfactants Span 60 and Tween 80 in 1:1 ratio can form vesicles with good stability. Cholesterol was incorporated as a membrane stabilizer in proniosomal gel. It is responsible for the conversion of micelles formed by surfactants into vesicles. In addition, cholesterol also imparts rigidity to the membrane of vesicles by decreasing the leakage probability of the entrapped drug. Lecithin is added as a membrane stabilizer and permeation enhancer due to its high phase transition temperature.

Evaluation of APG

Viscosity

In gel formulation, viscosity plays an important role because it is responsible for the spreadability, extrudability and release of the drug from the applied preparation. If gels are highly viscous it decreases the drug release by retaining the drug in the gel base and opposite to that if the viscosity of gel is lower it increases the gel spreadability and release rate of the incorporated drug. The viscosity of the prepared APG formulations was found from 8596 ± 0.97 to 9940 ± 1.03 cps (Table 2).

Spontaneity analysis

A number of niosomes formed after hydration APG can be called as rate of spontaneity. The results of rate of the spontaneity of APG is excellent and presented results ranging from 7 ± 1.45 to 13 ± 0.86 (Table 2).

Entrapment efficacy

For the optimization of formulations, the entrapment efficiency of a drug is one of the essential paradigms. The more the drug entrapped in the vesicles more amount of drug will diffuse at the site of application. The results of drug entrapment efficiency of APG presented satisfactory to good entrapment efficiency ranging from 81.44 ± 0.25 to 94.91 ± 0.51 as shown in Table 2.

Vesicle particle size (PS) and zeta potential (ZP) measurement

The optimized batch APG3 was characterized for PS and ZP. The graph of zeta sizer showed that the niosomes ranging from approximately 100 to 600 nm can be seen on the graph with average hydrodynamic diameter of 345.7 nm. The polydispersibility index was found to be 0.159. Zeta potential of the optimized batch APG3 was found to be -2.3 mV as shown in Figure 1.

Effect of APG3 on Body weight in Imiquimod induced Psoriatic mice

In current study, we found that there is a continuous reduction in the body weight of animals in psoriatic group while the animals treated with APG3 shows significant restoration of body weight as compared with psoriatic groups ($P < 0.001$) shown in Figure 2.

Effect of APG3 on oxidative stress in Imiquimod induced psoriatic mice

The level of antioxidants like GSH, SOD, Catalase were found to be decreased in psoriatic group while the animals treated with APG3 shows significant elevation in the level of those antioxidants as compared to psoriatic group ($p < 0.001$). Also the level of MDA is elevated in psoriatic animals and significantly reduced after treatment of APG3 as shown in Figure 3.

Table 2: Viscosity, rate of spontaneity and rntrapment efficiency determination of different batches

Batch	*Viscosity (cps)*	*Rate of spontaneity*	*Entrapment Efficiency*
APG 1	9940 ± 1.03	11 ± 1.27	89.46 ± 0.24
APG 2	9124 ± 1.31	10 ± 1.31	90.25 ± 0.75
APG 3	9710 ± 1.24	13 ± 0.86	94.91 ± 0.51
APG 4	8596 ± 0.97	9 ± 1.10	90.63 ± 0.78
APG 5	9785 ± 1.28	10 ± 1.87	87.34 ± 0.65
APG6	9715 ± 1.27	10 ± 1.63	85.42 ± 0.98
APG7	9321 ± 1.23	8 ± 1.90	86.34 ± 0.75
APG8	8796 ± 0.95	7 ± 1.45	85.44 ± 0.25

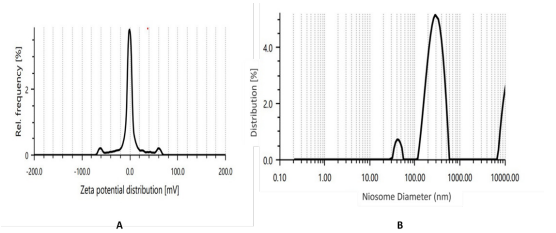

Figure 1: A- Zeta potential distribution, B- Niosomes size distribution – Intensity

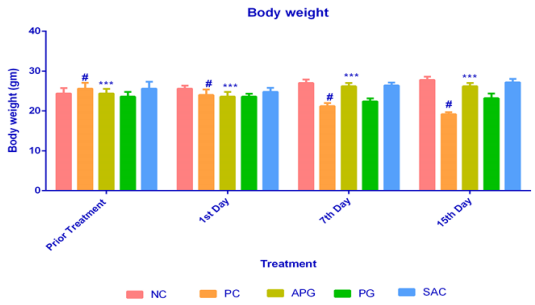

Figure 2: Effect of APG3 on Body weight in Imiquimod induced psoriatic mice

Effect of APG3 on proinflammatory cytokines in Imiquimod induced psoriatic mice

Cytokines estimation was assessed by using ELISA sandwich assay. In animals in psoriatic group it is observed that there is significant elevation of TNF- α as well as IL-6. That is normalized by the animals treated with APG3. Figure 4 indicates that APG3 have the potentiality to overcome Psoriasis ($p > 0.001$) as shown in Figure 4.

Effect of APG3 on PASI in Imiquimod induced psoriatic rat

PASI score was analyzed in animals from 1st day to 7th day of the study. Remarkable elevation in PASI score was observed in psoriatic animals. Figure 5A shows that rats treated with APG3 indicate a reduction in PASI score from 1st day to the end of study.

Effect of APG3 on primary dermal irritation (skin irritation) in Imiquimod induced psoriatic rat

Skin irritation index was analysed in animals from the 1st day to 7th day. It was found that in psoriatic animals there was an

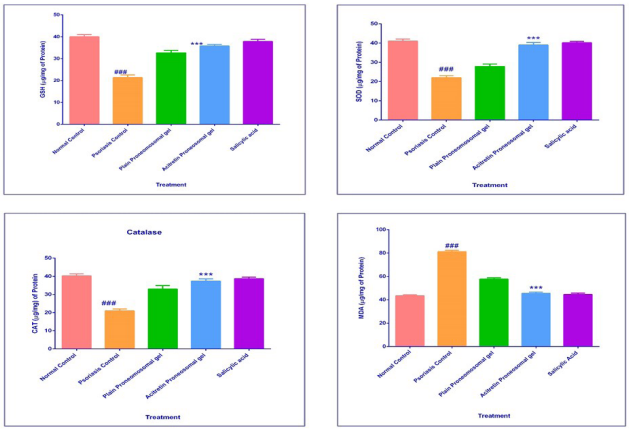

Figure 3: Effect of APG3 on oxidative stress in Imiquimod induced psoriatic mice

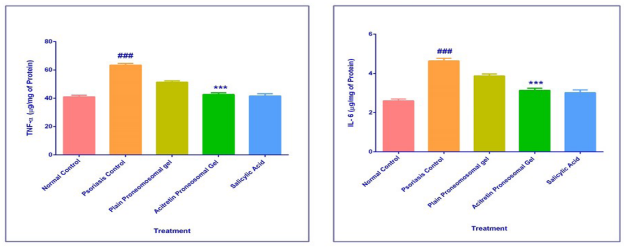

Figure 4: Effect of APG3 on proinflammatory cytokines in Imiquimod induced psoriatic mice

elevation in skin irritation score. Figure 5B displays APG3 treated animals have a consistent reduction in skin irritation score from the 1st day to 7th day.

Effect of APG3 on histopathology of the inflamed skin of mice in Imiquimod induced psoriatic mice

The psoriatic control group showed abnormal skin cells appearance and architecture. It was also observed that the infiltration of the skin cells with edema was increased. Figure 6 shows the mice treated with APG3 showed recovery with normal skin cells appearance and architecture as observed by a reduction in spaces along with infiltration of skin cells as shown in Figure 6.

The formulation variables can significantly influence the characteristics and the performance of the prepared formulation. The prepared proniosomal formulations were optimized on the basis of its viscosity, rate of spontaneity and entrapment efficiency.[30] Initially different grades of non-ionic surfactants like tween and span were screened. Based on preformulation studies and its results the selected surfactants i.e., tween 80 and span 60 (1:1), lecithin and cholesterol were varied thus at high levels and low levels.[31] At last, the drug concentration was optimized. Several shreds of evidence suggested that viscosity was higher with higher concentrations of cholesterol and surfactants, tween 80 and span 60 contraries to that viscosity decreased at lower concentrations of cholesterol and surfactants.[32-34] Entrapment efficiency (EE %) of the developed proniosomal gels with two surfactants tween 80 and span 60 in ratio of 1:1 revealed the highest EE % of value 94.91 ± 0.51 and lowest EE % of 81.44 ± 0.25. On

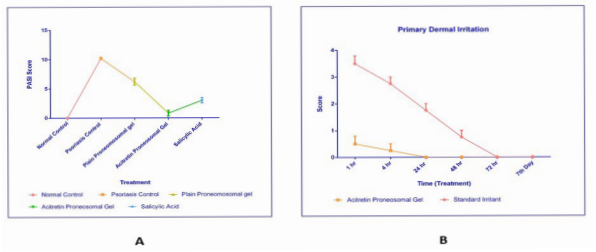

Figure 5: A - Effect of APG3 on PASI in Imiquimod induced Psoriatic Rat, B -Effect of APG3 on Primary dermal irritation (Skin irritation) in Imiquimod induced Psoriatic Rat

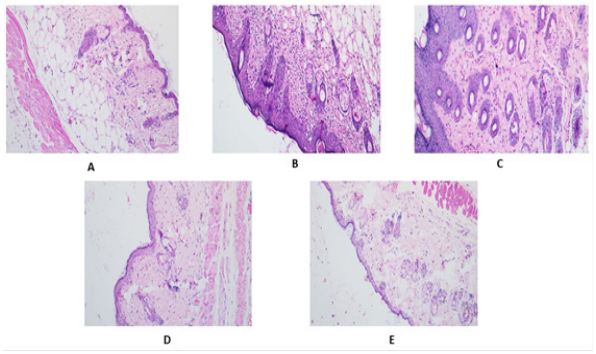

Figure 6: Effect APG3 on histopathology of the inflamed skin of mice in Imiquimod induced Psoriatic mice. A. Normal Control; B. Psoriasis Control; C. Plain Gel; D. APG3; E. Salicylic acid. Scale bar =100 µ

the basis of earlier reported literatures higher concentrations of cholesterol and surfactants tween 80 and span 60 the rate drug entrapment was higher whereas at lower concentration it was decreased.[32] Additionally, the psoriasis is characterized by the release several cytokines such as interferon- γ, TNF-α and numbers of interleukins involved in the inflammation are followed by infiltration of immune cells into the skin and finally hyperkeratosis.[35-37] *In-vivo* antipsoriatic activity was evaluated on basis of PASI scorings along with primary dermal irritation study. Higher degree of erythema, thickening of the back skin and scales were significantly seen in psoriatic group whereas APG3 treated group showed a remarkable recovery. From the first day to end of the study (14th day), it was observed that APG3 showed a remarkable reduction in skin thickness and PASI score. Furthermore, from 7th to 14th day, the APG3 found to have a considerable protective effect. Also, in histopathological screening it was found that the IMQ induced parakeratosis, acanthosis, epidermal cuticle and perivascular infiltration of inflammatory cells were normalized by the treatment of APG 3. In summary, acitretin-loaded proniosomal gel presented excellent pharmacological effect against IMQ-induced psoriasis. This finding suggests that acitretin-loaded proniosomal gel has the potentiality as a therapeutic agent to overcome psoriasis.

CONCLUSION

In this present research work, we developed acitretin-loaded proniosomal gel for the management of psoriasis. Characterization and evaluation revealed about the stability of the formulation. The prepared acitretin proniosomal gel has characteristic nano size and shows significant fall in PASI scores after treatment in Imiquimod-induced psoriasis mice; therefore, acitretin proniosomal gel can be used for effective treatment of psoriasis.

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