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Preparation, characterization, and *in vitro* antimicrobial assessment of nanocarrier based formulation of nadifloxacin for acne treatment

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The objective of the present study was to develop a nanocarrier based formulation of nadifloxacin and to investigate its *in vitro* antimicrobial effect against *Propionibacterium acnes.* Nanocarrier based microemulsion formulations were prepared by aqueous titration method, using oleic acid as oil phase, Tween-80 as surfactant and ethanol as co-surfactant in different ratios. This procedure yielded monodisperse microemulsions exhibiting a mean droplet size in the range of 95–560 nm. This range of particle size is good to treat follicle related disorders like *acne vulgaris* because the size of follicles is in the range of 50–100 μm. Furthermore the optimized formulations were characterized for surface morphology by transmission electron microscopy and refractive index. The permeation studies were carried out using rat skin mounted in Franz diffusion cells. Flux of the optimised formulation was 2.24 times that of control. The diameter of inhibition zone of the microemulsion was found good but smaller than that of a clindamycin disc because of the higher therapeutic efficacy of clindamycin against *P. acnes.* The results indicated that the developed microemulsion shows promising results against *P. acnes* bacteria and may be a good approach for acne treatment.

1. Introduction

Propionibacterium acnes (obligate-anaerobic bacteria) and *Staphylococcus epidermidis* (aerobic bacteria) bacteria are among the common microflora of the skin which under favourable conditions start multiplication and trigger an inflammation in acne (Kumar et al. 2008; Nenoff et al. 2004). *P. acnes* acts as an immunostimulator which can produce a variety of biologically active molecules and enzymes such as lipases, proteases, hyaluronidase, and chemotactic factors which are involved in the development of inflammatory acne (Chomnawang et al. 2007; Jeremy et al. 2003). Apart from this, it has also been suggested that *P. acnes* secretes peptidoglycon which can stimulate the production of the proinflammatory cytokinin such as interleukin (IL-1, IL-8) and tumor necrosis factor-alpha (TNF-α) by human monocytic cell lines which lead to inflammation (Gollnick and Schramm 1998).

The antibacterial activity of nadifloxacin against methicillin–susceptible *Staphylococcus aureus* (MSSA), methicillin–resistant *S. aureus* (MRSA), *S. epidermidis* and *P. acnes* bacteria was assessed and no test organism became resistant to nadifloxacin. The minimum inhibitory concentration (MIC90) values of nadifloxacin was 0.20 μg/ml and 0.78 μg/ml against *P. acnes* and *S. epidermidis,* respectively, while MIC50 values were 0.20 μg/ml for *P. acnes* and 0.05 μg/ml for *S. epidermidis* (Nenoff et al. 2004; Swanson 2003). **N**adifloxacin is available in market in conventional dosage forms like cream for treatment of skin infections which has the major limitation of having poor penetration through the pilosebaceous follicle. Thus, an effective concentration of drug is not sustained for a longer period of time. Carrier based delivery by microemulsion is a novel drug delivery approach used for follicular targeting to treat acne. The microemulsion which consists of oil, surfactant, co-surfactant and water can transport drugs to the target site and maintain a higher drug concentration than the conventional dosage forms like ointment, gel and cream. As a result the therapeutic effect of a drug loaded in a microemulsion can be enhanced severalfold as compared to a conventional dosage form (Date et al. 2006). The objective of the present investigation was to develop and evaluate the antimicrobial effect of a microemulsion formulation against *P. acnes* bacteria.

2. Investigations, results and discussion

2.1. Preparation and characterization of microemulsion

For the formulation of microemulsion oleic acid was selected as an oil phase due to the good solubility of nadifloxacin (12.56 ± 0.54 mg/ml) and it also acts as a penetration enhancer, as it increases the fluidity of the intercellular lipid barriers in the stratum corneum by forming separate domains which interfere with the continuity of the multilamellar stratum corneum and induces highly permeable pathways there (Hadgraft 2001). Tween 80 was used as a surfactant because of its non ionic nature and good miscibility with oleic acid. Since Tween 80 is a non ionic surfactant, it is less toxic and is known to be less affected by pH and change in ionic strength. Cosurfactants are added in a microemulsion systems to reduce the concentration of surfactant by forming a flexible film at the interfacial layer, reduce the

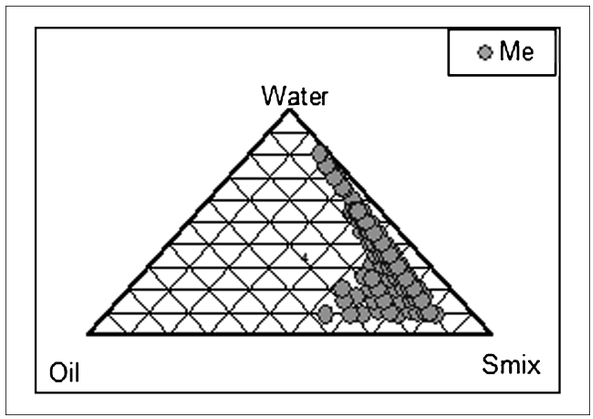

Fig. 1: Pseudoternary phase diagram among oil, water and S_{mix} (Tween 80: ethanol)

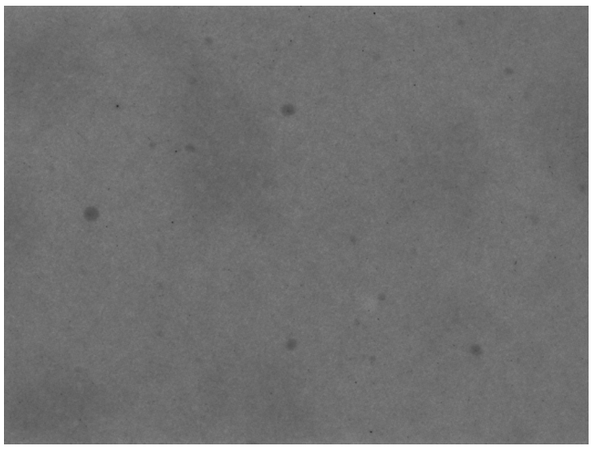

Fig. 2: TEM analysis of microemulsion formulation A

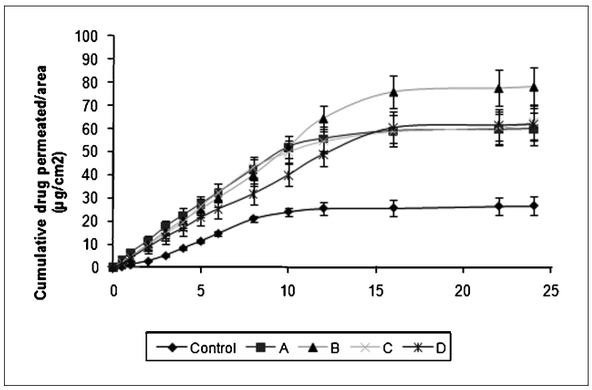

Fig. 3: *In vitro* skin permeation study of nadifloxacin from microemulsion and control

interfacial tension and increase the fluidity of the interface (Tenjarla 1999). We have used ethanol as a cosurfactant because it also acts as a penetration enhancer for topical delivery and increases the miscibility of the aqueous and oily phase due to its partitioning between these phases. The microemulsion formulations were selected from the pseudoternary phase diagram (Fig. 1) and in order to remove metastable formulation, these were subjected to a stress stability test. The chemical and physical stability of the microemulsion was studied by visual observation of clarity and phase separation. During physical stability testing some formulations became turbid and in some phase separation occurred. One reason of this instability of the microemulsion may be the Ostwald ripening in which molecules move as a monomer and coalescence of small droplets takes place, resulting in the formation of large droplets by diffusion processes driven by the gain in surface free energy. Another reason may be that when temperature quench occurs during stress stability study instability of microemulsion occurs due to separation of oil phase and droplet distribution of smaller size is favored by the change in curvature free energy (Wennerstrom and Olsson 2009). These formulations were thermodynamically stable because energy required is less which also confirms the longer shelf life of the developed microemulsion. The optimized formulations were prepared by dissolving nadifloxacin in oleic acid, S_{mix} was added and finally water was added for the formation of translucent microemulsion. The selected formulations which had different composition of oleic acid, Tween 80, ethanol and distilled water are given in Table 1. The particle size of the selected microemulsion formulations were in the range of 95–560 nm. The average particle size of the formulation A was 97 nm (Fig. 2). The oil globules were in the nano range and spherical in shape observed by transmission electron microscopy (Fig. 2). The mean values of refractive index of drug loaded and placebo formulations were found in the range of 1.401 to 1.412. Furthermore the values of the refractive indexes of placebo were not significantly different from those of preparations containing the drug ($p \geq 0.05$). It was concluded that the microemulsion formulations were not only physically but also chemically stable and remained isotropic in nature, thus having no interactions between microemulsion components and drug.

Table 1: Composition of stable microemulsion formulation after drug loading

Microemulsion	Oleic acid (% w/w)	Tween 80 (% w/w)	Ethanol (% w/w)	Distilled water (% w/w)	Nadifloxacin (mg)
A	13	17	34	36	10
B	12	28	28	32	10
C	14	17	34	35	10
D	15	16.66	33.33	35	10

2.2. In vitro skin permeation study

In vitro skin permeation studies were performed to compare the permeation of nadifloxacin from four different microemulsion formulations and suspension. *In vitro* skin permeation profile of all microemulsions was significantly different from that of nadifloxacin suspension (control) as shown in Fig. 3 and Table 2. The permeability parameters of formulation A were significantly different from other formulations. The high permeation rate of the microemulsion might be attributed to several factors. Firstly, the high concentration of nadifloxacin released from the microemulsion resulted in a high concentration gradient, which might be

Table 2: *In vitro* skin permeation study parameter for nadifloxacin

Formulation code	Flux ($\mu g/cm^2/h$)	Permeability constant (k_p)	Drug disposition in skin ($\mu g/mg$)	Enhancement ratio (Er)
Control	3.06	0.244×10^{-2}	0.19	—
A	6.86	0.686×10^{-2}	0.51	2.24
B	4.74	0.474×10^{-2}	0.38	1.54
C	3.92	0.392×10^{-2}	0.26	1.28
D	4.57	0.457×10^{-2}	0.34	1.49

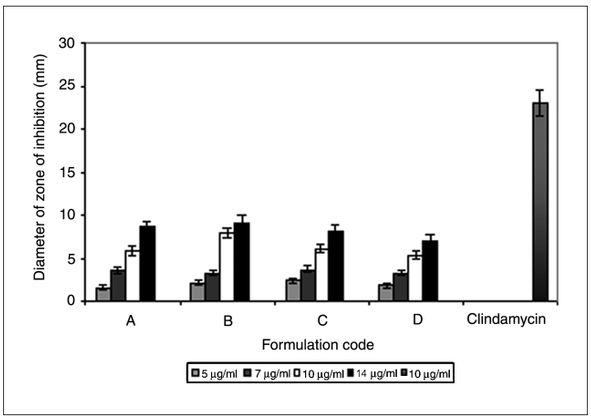

Fig. 4: Effect of nadifloxacin concentration in microemulsion and clindamycin disc on the diameter of zone of inhibition of *P. acnes* bacteria

the main permeation mechanism of nadifloxacin into the skin. The microemulsion could act as drug reservoir where drug is released from the inner phase to outer phase and then further into the skin (Peltola et al. 2003). Secondly, due to the small droplet size, droplets settle down and come in close contact with the skin and a large amount of inner oleic acid in microemulsion might have penetrated into the skin. Oleic acid and ethanol are strong permeation enhancers. Drug disposition in the skin was found to be highest for formulation A while minimum for control indicating microemulsion is a good method for accumulation of nadifloxacin for longer period of time.

2.3. Antimicrobial assay

To elucidate the antibacterial activity of nadifloxacin microemulsion formulation against *P. acnes*, *in vitro* antibacterial activity was determined by a disc diffusion method. Clindamycin disc was employed as a positive control. The drug had good antibacterial activity against *P. acnes* bacterial cells, although its activity was less than that of clindamycin. The inhibitory zones obtained indicated the antibacterial activity of the microemulsion formulation of nadifloxacin against *P. acnes*. Log nadifloxacin concentration in the range of 0.6989–1.146 (nadifloxacin concentration 5–14 µg/ml in formulation) were plotted against average diameters of the zone of inhibition (Fig. 4). Since nadifloxacin was loaded in the oil phase of microemulsion, it is anticipated that the delivery might have occurred through mass transport of drug from inside the microemulsion droplets through the aqueous phase to the membrane of the *P. acnes* bacteria, depending on the droplet size of microemulsion (McClements 2005). The drug release from these formulations showed almost linear release (Table 3) of drug except formulation B indicating burst release of drug and also having a larger inhibitory zone compared to other formulation. The burst release of nadifloxacin might be attributed to the least amount of oil present in the formulation which encapsulated the drug in the oil phase. The formulations A and D produced a linear response having an R^2 value greater than 0.98 indicating a linear release of drug but formulation A had more diameter of zone of inhibition.

Table 3: Linearity of nadifloxacin release from microemulsion

Formulation Code	*Diameter of zone of inhibition (mm)	R^2
A	8.6 ± 0.68	0.9853
B	9.2 ± 0.76	0.9021
C	8.2 ± 0.67	0.9676
D	7.1 ± 0.64	0.9801

* N = 3

2.4. Short term stability study

To study the effect of storage temperature on particle size and drug content, the microemulsion formulations were exposed to different temperatures (4 °C, 25 °C and 40 °C) for 90 days. A minute increase in particle size was observed under 4 °C and 25 °C storage conditions. Nevertheless, a significant growth in average particle size was observed at high temperature (40 °C). The average particle size at 40 °C was increased from 97 nm to 165 nm in 90 days where as 105.21 nm and 109 nm at 4 °C and 25 °C respectively. The results strongly support the protecting ability of Tween 80 at moderate temperature. It was also observed that the particle aggregation process accelerated with increased temperature. This might have occurred due to increased input energy of particle microemulsion resulting in increased kinetic energy of the particles which favour the collision among the particles. There was no significant difference in the nadifloxacin content under different storage conditions. This indicates the protecting ability of microemulsion for the nadifloxacin.

In present study, different microemulsion formulations of nadifloxacin were developed, characterized, and evaluated for *in vitro* skin permeation and antimicrobial activity against *P. acnes* bacteria. The *in vitro* permeation studies showed that the flux of the optimized microemulsion formulation was 2.24 times that of control. The nano range of particle size was good to treat follicle related disorders like acne vulgaris due to follicle sizes in the range of 50–100 µm. It was concluded that nadifloxacin was released from the microemulsion and inhibited the growth of bacteria in a dose dependent manner. In comparison to other formulations, formulation A produced a more promising result, so this microemulsion can be a good approach for acne treatment.

3. Experimental

3.1. Materials

Nadifloxacin was received as a gift sample from Wochardt Laboratory Limited (Aurangabad, India). Oleic acid, isopropyl myristate, ethanol and Tween 80 were purchased from CDH (Mumbai, India). Diethyleneglycol monoethyl ether (Transcutol P®), propylene glycol monocaprylate (Capryol® 90), propylene glycol laurate (Lauroglycol® FCC) and oleoyl macrogol-6 glycerides (Labrafil® M 1944 CS) were obtained as kind gift samples from Colorcon-Asia Pvt. Ltd. (Goa, India). Microbiological media used for anaerobic studies was purchased from Hi Media (Mumbai, India). All other chemicals and reagents were of analytical reagent (AR) grade.

3.2. Preparation and characterization of microemulsion

Microemulsion was prepared by aqueous titration method (spontaneous emulsification method), using oleic acid as an oil phase, Tween-80 as a surfactant and ethanol as a co-surfactant. Pseudo ternary phase diagram was constructed among oil phase, S_{mix} (mixture of surfactant and co-surfactant) and distilled water. The ternary phase diagram showing a maximum area of microemulsion was selected for formulation optimization. Different formulations selected from a phase diagram were investigated for a physical stability test like centrifugation, heating cooling and freeze thaw cycle. The average size of the droplets in the microemulsion was determined by photon correlation spectroscopy (Nano ZS90, Malvern Instrument, U.K.). The measurements were performed using a He-Ne laser at 633 nm with an Avalanche photo diode detector. Light scattering was monitored at 25 °C at a 90° angle. Morphology and structure of the microemulsion were studied using a Morgagni 268D electron microscope (FEI Company, Netherlands) operating at 70 kV capable of point-to-point resolution. In order to perform transmission electron microscopy (TEM) observations, a drop of the microemulsion was suitably diluted with water and applied on a carbon-coated grid, then treated with a drop of 2% phosphotungstic acid and left for 60 s. The coated

grid was dried and then taken on a slide and covered with a cover slip and observed under the microscope. Refractive indices of placebo and drug-loaded formulations were determined using an Abbes type refractometer (Nirmal International, Delhi, India).

3.3. In vitro skin permeation studies

In vitro skin permeation of nadifloxacin from selected microemulsions and aqueous suspension of nadifloxacin was performed on Franz diffusion cell with an effective diffusional area of 1.13 cm^2 and 10 ml of receiver chamber capacity using rat abdominal skin as permeation membrane. The full thickness rat skin was excised from the abdominal region and hair was removed with the help of an electric clipper. The skin was prepared properly and stored in the deep freezer at −21 °C until further use. On the day of experiment the skin was brought to room temperature and mounted between donor and receiver compartment of the Franz diffusion cell where the stratum corneum side faced the donor compartment and the dermal side faced the receiver compartment. Initially the donor compartment was empty and the receiver chamber was filled with phosphate buffer saline (PBS) pH 7.4 and temperature was maintained at 37 ± 1 °C using a re-circulating water bath. The receiver fluid was stirred with a magnetic rotor at a speed of 200 rpm. After complete stabilization of the skin, 1 ml of microemulsion formulation containing 1 mg of drug or nadifloxacin suspension containing 1 mg nadifloxacin was placed into each donor compartment. Samples were withdrawn at regular intervals (0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 16, 22 and 24 h), filtered through a 0.45 membrane filter and drug content was analysed by UV spectrophotometer at the wavelength of 290 nm.
The cumulative amount of nadifloxacin permeated through the unit area of skin (μg/cm^2) was plotted as a function of time (t) for each formulation. Rate of drug permeation at steady state (Jss) was determined from the slope of the linear portion of graph plotted between cumulative drug permeated and time. Permeability coefficient (Kp) was calculated by dividing Jss with initial concentration of drug in donor cell (Co) according to the following equation

$$Kp = \frac{Jss}{Co} \tag{2}$$

Enhancement ratio (Er) was calculated by dividing the Jss of respective formulation with Jss of control formulation by using the equation:

$$Er = \frac{Jss \text{ of formulation}}{Jss \text{ of control}}$$

At the end of the test, the formulation remaining on the skin was removed, cleaned with cotton soaked in a 0.05% sodium lauryl sulphate and washed with distilled water. The skin was then weighed, cut into small pieces and sonicated for 15 min with methanol in order to extract the nadifloxacin content. The resulting solution was then centrifuged and their drug content (μg/mg of skin) was determined by a UV spectrophotometer.

3.4. In vitro antimicrobial study

For evaluation of optimized microemulsion formulations, *in vitro* antimicrobial studies were performed against the test organism *P. acnes* for antibacterial activity. The microbiological assay was based on the comparison of inhibition of growth of bacteria by a measured concentration of nadifloxacin microemulsion to be examined with that produced by a known concentration of standard preparation of antibiotic having known activity.

3.4.1. Test samples and standard sample

Optimized microemulsion formulations were used as test sample for the assessment of antimicrobial activity which was based on the measurement of zone of inhibition. The zones of inhibition were measured at four concentration levels, i.e., 5, 7, 10 and 14 μg/ml for different microemulsion formulations. A clindamycin disc (10 μg/ml) was used as a standard sample.

3.4.2. Preparation of Brain Heart Infusion (BHI) agar

BHI agar (dehydrated media 52 g, yeast extract 5 g, proteose peptone 5 g, hemin 10 ml, L-lysine 0.5 to 1 g and agar 5 g) was dissolved in 1 l of distilled water. The solution was boiled and the pH of the clear solution was adjusted to 7.4. It was autoclaved at 15 lbs for 15 min after filtration and was cooled to 50 to 60 °C. human blood 5–10% was added, 20 ml of this media was poured in petri plates and the plates were stored in a refrigerator at 4 °C.

3.4.3. Test organisms

Propionibacterium acnes (MTCC No. 1951) was isolated between 2006 and 2007 from patients suffering from severe acne in AIIMS hospital, New Delhi (India). Bacteria were maintained in enriched growth medium with 50% glycerol at –80 °C.

3.4.4. Preparation of heat killed bacteria

P. acnes were cultured in BHI broth with 1% glucose for 72 h at 37 °C in an anaerobic chamber consisting of 10% CO_2, 10% H_2 and 80% N_2 atmosphere. The log-phase bacterial culture was harvested, washed three times with phosphate buffer saline (PBS), and incubated at 80 °C for 30 min to kill the bacteria. The heat-killed *P. acnes* were stored at −80 °C until use.

3.4.5. Determination of antimicrobial activity (antimicrobial assay)

The method followed for the *in vitro* antimicrobial study was disc diffusion method. The discs were prepared from sterile whatman paper III under sterile conditions. A previously liquefied and sterilized 20 ml BHIA medium was poured into plastic petri plates of 100 mm size and kept for solidifying under sterile conditions. One loop full of *P. acnes* was streaked on 20 ml BHIA media and four discs were transferred into plates. The required volumes of microemulsion having different concentrations of nadifloxacin were poured into the disc aseptically. One standard clindamycin disc (10 μg/ml) was also transferred into the petri plate for comparing the zone of inhibition with microemulsion formulation. These petri plates were incubated in the anaerobic jar (Hi-Media) for 48 h at 35 ± 1 °C. Gas packs containing citric acid, sodium carbonate and sodium borohydride were used to maintain and check the anaerobiosis, where citric acid releases carbon dioxide and sodium borohydride releases hydrogen when they come in contact with oxygen. An indicator strip of methylene blue, when introduced into the jar, changes in colour from white to blue in the absence of anaerobiosis. After 48 h the zone of inhibition was measured for different test formulations and the standard formulation.

3.5. Stability at different temperatures

In order to investigate the effect of storage conditions on the drug content and particle size a short term stability study was performed. The optimized formulation was stored at 4 °C (refrigerator), 25 °C (room temperature) and 40 °C (temperature regulated oven) for a period of 90 days. Samples were withdrawn after 90 days for particle size and drug content measurement.

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