FORMULATION, CHARACTERIZATION AND IN-VIVO ASSESSMENT OF TOPICAL NANOEMULSION OF BETAMETHASONE VALERATE FOR PSORIASIS AND DERMATOSE

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ABSTRACT

The aim of the current work was the formulation, characterization and \textit{in vivo} study of betamethasone valerate nanoemulsion based on the induction of contact dermatitis in rats using a dispersion of nickel sulfate in solid vaseline at 5\%, carragenenn induce inflammation and their irritation study. Nanoemulsions were prepared by aqueous phase titration method, using sefsol, tween 20, transcutol P, and distilled water as the oil phase, surfactant, co surfactant and aqueous phase, respectively. Selected formulations were subjected to physical stability studies and consequently \textit{in vitro} skin permeation studies. A surface study of optimized formulation was done by transmission electron microscopy. \textit{In vivo} anti-inflammatory, \textit{In vivo} irritation study and \textit{In vivo} nickel induced contact dermatitis activity were done. The droplet size of nanoemulsion ranged from 150 to 200 nm. The optimized formulation exhibited viscosity 26.95 ± 1.71mP, refractive index 1.431, pH 6.5, and conductivity 10^{-4} \text{scm}^{-1}. The optimized nanoemulsion was converted into hydro gel using carbopol 934. Drug deposition in skin was found to be 58.46 \text{μg/cm}^2. \textit{In vivo} anti-inflammatory activity indicated 84.2\% and 45.05\% inhibition of inflammation in case of developed nanoemulsion gel (A5) and marketed cream, respectively. The irritation score was found to be 1.83 which indicates our optimized nanoemulsion was not cause any irritation. Result of nickel induced dermatis demonstrate that the nanoemulsion formulation (A5) gel did not appear to stimulate an inflammatory or immune response using the contact dermatitis model.

Keywords: Anti-inflammatory effects, Betamethasone Valerate, Nanoemulsion, Transmission Electron Microscopy, Irritation Study.

INTRODUCTION

Psoriasis is a common dermatological condition affecting 2\% of the population. It is a chronic (prolonged) inflammation of the skin characterized by erythematous scaly plaques. Corticosteroid such as betamethasone valerate (BV) has been used extensively in topical therapy for the treatment of mild to moderate psoriasis and different dermatoses.\textsuperscript{[1-3]} Betamethasone valerate (BV) is a glucocorticoid receptor agonist which possesses immunosuppressive, anti-inflammatory, and anti-proliferative effects. It exerts its action by inhibition of phospholipase A\textsubscript{2} which leads to the inhibition of
synthesis of arachidonic acid and controls the biosynthesis of prostaglandins and leukotrienes. Its various dosage forms such as ointment, cream, lotion, and foam are in use for the treatment of mild to moderate psoriasis and dermatoses. However, the clinical use of BV has some practical disadvantages such as poor permeability through skin which reduces its therapeutic effectiveness at the target site. The main limitation lies in the barrier function of the skin, which is considered one of the most impermeable epithelia of the human body to exogenous substances. Therefore, the major challenges for a topical formulation are to provide a sufficient increase in drug penetration into the skin, without inducing any significant irreversible alteration to the skin barrier function.

There has been increased interest during recent years in the use of topical vehicle systems that could modify drug permeation through the skin. Many of the dermal vehicles contain chemical enhancers and solvents to achieve these goals. But use of these chemical enhancers may be harmful, especially in chronic application, as many of them are irritants. Therefore, it is desirable to develop a topical vehicle system that does not require the use of chemical enhancers to facilitate drug permeation through the skin. One of the most promising techniques for enhancement of skin permeation of drugs is nanoemulsion. Nanoemulsions offer several significant advantages including low skin irritation, powerful permeation ability, and high drug-loading capacity for topical delivery when compared with the other carriers such as liposome or solid lipid nanoparticles. Topical corticosteroid is most commonly used drug to treat different type of skin diseases. Their clinical effectiveness in the treatment of psoriasis and atopic dermatitis is related to their vasoconstrictive, anti-inflammatory, immune suppressive, and antiproliferative effects.

However, the use of topical glucocorticoid is limited due to their adverse effects, such as skin atrophy, steroid acne, hypopigmentation, allergic contact dermatitis and their poor absorption through skin. Currently, researches have been focused mainly on the development of strategies to improve the benefit-risk ratio of glucocorticosteroids, increase their absorption and should not cause any histopathological change in the structure of skin. In the recent year nanotechnology emerges (specillay nanoemulsion) excellent tool for delivering poorly water soluble drug to the deeper layer of skin with a view to decreasing the adverse effects via decrease in dose. In this method reduced the particle size of its sub micron level upto 10-200nm to improving the absorption and therapeutic concentration of the drug in the target tissue, allowing reproducible and long-term release of the drug at the target site, reduces the frequency of drug administration, and improve its pharmacokinetic profile. BV is a lipophilic corticosteroid used for the treatment of skin disorders such as dermatoses and psoriasis.

Therefore O/W nanoemulsion is prepared in which drug present in disperse phase of oil. In this system high amount of surfactant and co surfactant is used which has irritation potential so, formulator needs to be study irritation potential before application. Moreover, showed that the incorporation of Clobetasol Propionate (CP) into lecithin/chitosan nanoparticles induced an accumulation of CP especially in the epidermis without any significant permeation across pig ear skin. This disease occurs due to delayed hypersensitivity reaction, mediated by T lymphocyte to an antigen protein or a hapten linked to a protein. Notable among the mediators able to modulate the actions of lymphocytes are adenine nucleosides and nucleotides, in particular, extracellular ATP, which is able to regulate the cell–cell interactions and is important in the processes of cell activation, differentiation, development, proliferation, and death, as well as effector lymphocyte response.

Extracellular nucleotides are messengers that modulate the exocrine and endocrine systems, the vasculature and haemostatic mechanisms, and musculoskeletal, immune and inflammatory cells. These nucleotides represent an important means of modulating the activity of lymphocytes. Adenine nucleotides (ATP, ADP, and AMP) and their nucleoside derivative, adenosine, are important signaling molecules that mediate diverse biological and pathological processes. NTPDase (ecto-nucleoside triphosphate diphosphohydrolase; CD39) is an integral membrane protein that metabolizes extracellular ATP and ADP to AMP. It affects the cell proliferation and apoptosis via its modulation of ATP levels in the pericellular fluid. Taking all these considerations into account, the formulation, characterization and in vivo protocol was based on the induction of contact dermatitis in rats using a dispersion of nickel sulfate in solid Vaseline at 5%, carragennsen induce inflammation and their irritation study.

**MATERIALS AND METHODS**

**Materials:** BV was obtained as a gift sample from Ranbaxy Research Laboratory (Gurgaon, India). Isopropyl Alcohol, and oleic acid were purchased from S.D. Fine chemicals (New Delhi). Propylene
glycol mono caprylic ester (Sefsol 218®) was obtained as a gift sample from Nikko Chemicals Co., Ltd, Japan. PEG 400, Tween 80, Tween 20, and ethanol were purchased from Merck (Merck, India). Caprylo caproyl macrogol-6 glycerides (Labrasol), diethyleneglycol monoethyl ether (Transcutol P), and Plurol Oleique were obtained as a kind gift samples from Gattefosse (Mumbai, India). All other chemicals used were of analytical grade.

**Screening of excipients:** The most important criterion for screening of components is the solubility of poorly soluble drug in oils, surfactants, and co-surfactants.

**Screening of oil for microemulsion:** To find out the suitable oil that can also provide excellent skin permeation rate of BV, the solubility of BV was determined in different oils, viz. oleic acid, isopropyl myristate, sfsol oil, castor oil, labrafal, sfsol, and oleic acid. One milliliter of different oils was taken in small vials and excess amount of the drug was added. The vials were tightly stoppered and were continuously stirred for 72 h at 25°C, and after 72 h, samples were centrifuged at 5000 rpm for 20 min. The supernatant was separated, filtered through a 0.45-μm membrane filter, and after appropriate dilution with methanol, solubility of drug in different oils was determined by UV method at 254 nm.

**Screening of surfactant and co-surfactant for microemulsion:** To find out the suitable surfactant and co surfactant, the solubility of BV was determined in various surfactants including labrasol, Tween 80, Tween 20, and a combination of Tween 80: labrasol. The solubility of BD was also checked in co-surfactants including isopropyl alcohol, transcutol P, PEG 400, and ethanol.

**Phase studies:** On the basis of solubility studies Tween 20 and Ethanol were chosen as surfactant and co-surfactant, respectively. Distilled water was used as an aqueous phase. For the determination of existence zone of nanoemulsion, pseudoternary phase diagrams were constructed using water titration method (spontaneous emulsification method). Surfactant and co-surfactant (Smax) were mixed in different weight ratios (1:0, 1:1, 1:2, 2:1, and 1:3). These Smax were chosen in increasing concentration of co-surfactant with respect to surfactant. For each phase diagram, oil and specific Smax were mixed well in different ratios. Sixteen different combinations of oil and Smax (1:9, 1:8, 1:7, 1:6, 1:5 1:4, 1:3, 1:2, 4:6, 5:5, 6:4, 7:3, 8:2, and 9:1) were made so that maximum ratio could be covered for the study to delineate the boundaries of the phases formed precisely in the phase diagrams. Slow titration with aqueous phase was done for each weight ratio of oil and Smax under moderate stirring, and visual observation was used for transparent and easily flowable nanoemulsion. Gels were claimed for those clear and highly viscous mixtures that did not show a change in the meniscus after being tilted to an angle of 90°. The physical state of nanoemulsion was marked on a pseudo three component phase diagram with one axis representing the aqueous phase, second representing oil, and the third representing a mixture of surfactant and co-surfactant at fixed weight ratio (Smax ratio).

**Selection of formulations:** From the pseudo ternary phase diagrams showing maximum nanoemulsion area, a number of nanoemulsions with different formulas were selected covering the entire range of nanoemulsion occurrence in the phase diagrams with minimum surfactant and maximum water concentration. 0.1 % w/w of BV, which was kept constant in all the selected formulations, was added to the oil phase during the formulation of nanoemulsions. Selected formulations were subjected to various physical stability tests.

**Physical stability studies:** To overcome the problem of metastable formulations, physical thermodynamic stability tests were performed. The selected formulations were subjected to centrifugation at 5000 rpm for 30 min. The formulations that did not show any phase separations were taken for the heating and cooling cycle. Six cycles between refrigerator temperature (4 °C) and (45 °C) with storage at each temperature of not less than 48 h were carried out. Those formulations that were found stable were subjected to a freeze-thaw cycle test. Three such cycles were done for the formulations between -20 °C and +25 °C for 24 h. After 24 h the nanoemulsions were removed and kept at room temperature. The physically stable nanoemulsions returned to their original form within 2-3 min.

**Characterization of nanoemulsions**

**In vitro skin permeation studies:** In vitro skin permeation studies were performed on a fabricated Franz diffusion cell with an effective diffusional area of 7.24 cm² and 5 mL of receiver chamber capacity using rat abdominal skin. The full-thickness rat skin was excised from the abdominal region, and hair was removed with an electric clipper. The subcutaneous tissue was removed surgically, and the dermis side was wiped with isopropyl alcohol to remove adhering fat. The cleaned skin was washed with distilled water and stored in the deep freezer at -21 °C until
Further use. The skin was brought to room temperature and mounted between the 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 (pH 7.4). The receiver fluid was stirred with a magnetic rotor at a speed of 100 rpm, and the assembled apparatus was placed in the oven and the temperature was maintained at 37 ± 1 °C. All the receiver fluid was replaced every 30 min to stabilize the skin. It was found that the receiver fluid showed negligible absorbance after 4.5 h and beyond indicating complete stabilization of the skin. After complete stabilization of the skin, 1 ml of nanoemulsion formulation (0.1 gram/100 ml of BV) was placed into each donor compartment and sealed with paraffin film to provide occlusive conditions. Samples were withdrawn at regular intervals (0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 16, 20, and 24 h), filtered through a 0.45-membrane filter, and analyzed for drug content by UV spectrophotometer at λmax of 254 nm.

Permeation and distribution data analysis: The cumulative amount of BV permeated through the albino rat skin (Q, µg/cm²) was plotted as a function of time (t, h) for each formulation. The permeation rate (flux) at the steady state (J, µg/cm²/h) were calculated. Respectively, Permeability coefficient (Kp) was calculated by dividing the flux by initial drug concentration (C₀) in the donor portion of cell as given below: 

\[ K_p = \frac{J}{C_0} \]

Estimation of drug content in skin: At the end in vitro permeation 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 cut into small pieces and sonicated for 15 minutes with methanol in order to extract the BV content. The resulting solution was then centrifuged and their drug content (µg/cm² of skin) was determined by UV analysis.

Particle size and polydispersity index: The average size and polydispersity index of the nanoemulsion droplets were determined by photon correlation spectroscopy (Nano ZS90, Malvern Instrument, Worcestershire, UK) at 633 nm which is based on the principle of dynamic light scattering. The measurements were performed using a He-Ne laser at 633 nm by using Avalanche photo diode detector. Light scattering was monitored at 25°C at a 90° angle. Droplet size distribution studies were performed at refractive index of 1.40 because the refractive index for all formulation was in this range. The viscosity and dielectric constant of the medium were set at 4.55 mPas and 79.4, respectively. Zeta potential was determined by using second-generation PALS (Phase Analysis Light Scattering), called M3PALS which measures the particle velocity.

Viscosity, refractive index, conductivity, and pH: Viscosity of nanoemulsion was determined by using Brookfield DV III ultra V6.0 RV cone and plate rheometer (Brookfield Engineering Laboratories, Middleboro, MA, USA). Refractive index was determined for different nanoemulsion formulations by using Abbe's refractometer (Nirmal International, Delhi, India) at 25°C in triplicate. The pH was determined for the optimized nanoemulsion by using a calibrated digital pH meter (Metaller Toledo MP 220, Greifensee, Switzerland) in triplicate at room temperature. Conductivity was measured by using a digital thermo conductivity meter (1152, Emcee Electronics, Venice, FL, USA) and current flow was observed.

Surface morphology by transmission electron microscopy: Morphology and structure of the nanoemulsion were studied using Morgagni 268D transmission electron microscopy (TEM) (FEI, Netherlands) operating at 70 kV and capable of point-to-point resolution. Combination of bright field imaging at increasing magnification and diffraction modes were used to reveal the form and size of nanoemulsion droplets. In order to perform the TEM observations, a drop of nanoemulsion was applied on carbon-coated grid with 2% phosphotungstic acid and was left for 30 s. The dried coated grid was taken on a slide and covered with a cover slip. The slide was observed under the electron microscope.

Preparation of nanoemulsion gel: Optimized Nanoemulsions gel was prepared by dispersing 1% w/w of Carbopol-940 in sufficient quantity of distilled water. This dispersion was kept in dark for 24 h for complete swelling of Carbopol-940. 0.1 gram of BV was dissolved in 20% w/w of Sefsol-218. BV solution was added slowly to Carbopol-940 dispersion. 0.5% w/w of triethanolamine (TEA) was added in this mixture to neutralize Carbopol-940. Then 40% w/w mixture of Tween 20 and Transcutol P (1:2) were added slowly. Then remaining quantity of distilled water was added to get the final preparation 100% w/w. After that pH was measured by above mention method.

Dermatitis induction by 5% nickel sulfate: Contact dermatitis was induced by 5% nickel sulfate in solid
Vaseline according to the protocol described by authors, Animals were divided into four groups (n = 8). After tricotomization, all groups received sensitization with nickel sulfate in the abdomen, except the first group which received only solid Vaseline and remained under the same environmental and feeding conditions as the other groups, this being the control group (C). The induction of dermatitis was performed 6 days after sensitization by the application of 5% nickel sulfate in solid Vaseline (5 applications with intervals of 72 h) in each ear after tricotomization. The first group received only solid Vaseline and these rats were euthanized 72 h after the last application of the sensitization agent. The second group was induced to allergic contact dermatitis, which was not managed, and the rats were euthanized 72 h after the last application of nickel sulfate, being the positive control (D). The third group received the topical administration in each ear of the optimized nanoemulsion (A5) gel and fourth group received marketed cream. Dose of application for nanoemulsion and marketed formulation was equal to 1 microgram of BV. Group 3 & 4 were treated daily with 0.5 ml of the drug loaded optimized nanoemulsion gel (A5) and marketed cream for 5 days on days 1, 3, and 5. All formulation was applied uniformly throughout the ear tissue with massage in order to obtain a better drug penetration. After the completion of each treatment, the animals were euthanized and the blood was collected by cardiac puncture to determine the NTPDase activity. NTPDase activity of lymphocytes mononuclear leukocytes were isolated from rat blood collected with EDTA and separated using Ficoll-Hypaque density gradients as described by Boyum. After the isolation of mononuclear cells, NTPDase activity was determined by colorimetric assay. All samples were run in duplicate or triplicate, and specific activity is reported as nmol Pi released/min/mg protein. Protein was measured by the Coomassie Blue method using bovine serum albumin as the standard as described by Bradford.

**In vivo anti-inflammatory study:** The protocol to carry out in vivo anti-inflammatory efficacy studies was approved by the SBS College of Pharmacy, Patiala, Amritsar, Punjab; India. The committee’s guidelines were followed for the studies. The anti-inflammatory and sustaining action of the optimized formulation was evaluated by the carrageenan induced hind paw edema method by using digital plethysmometer (Ugo Basile, Italy) in Wistar rats of either sex weighing 180–200 g. A left hind paw of each rat was marked, just below tibiotarsal junction, so that every time the paw was dipped up to the fixed mark to ensure constant paw volume. Animals were randomly divided into 3 groups (control, optimized nanoemulsion gel (A5) and marketed formulations) each containing six rats. Formulations were applied on the dorsal area of 9 cm² gently with the help of micropore adhesive, 0.5 h before carrageenan injection. No formulation was applied to the control. Acute inflammation was produced by injecting 0.1 mL of 1% (w/v) carrageenan suspension in the subplantar region of the left hind paw 0.5 h after treatment with drug. The paw volume was measured at 0.5, 1, 2, 3, 4, 6, 8, 12, 16, 20, and 24 h. The amount of paw swelling was determined for 24 h and expressed as percent edema relative to the initial hind paw volume. Percent inhibition of edema was calculated for optimized formulation and marketed cream with respect to control group using the following formula:

\[
\% \text{Inhibition} = \frac{\% \text{Edema (Control)} - \% \text{Edema (Formulation)}}{\% \text{Edema (Control)}} \times 100
\]

**In vivo Skin irritation test:** Although all the materials used for preparation of nanoemulsion were falled under generally regarded as safe (GRAS) category. Concentration of all materials is very critical issue for this formulation. Large amount of surfactants is usually irritant to the skin. Therefore skin irritation test was performed to confirm concentration of materials used for nanoemulsion preparation is safe. Van-Abbe et al. mentioned that a value between 0 and 9 indicates that the applied formulation is generally not irritant to human skin. Skin irritation test was performed on optimized nanoemulsion gel (A5) and marketed cream on Wistar rats weighing 180–200 g. Wistar rats were divided into 2 groups: optimized nanoemulsion gel (A5) and Marketed cream were applied to each group, containing 6 rats. The animals were kept under standard laboratory conditions, temperature at 25 ± 1°C and relative humidity (55 ± 5%). The animals were housed in polypropylene cages, six per cage, with free access to standard laboratory diet and water as mention above. A single dose of 10 μl of optimized nanoemulsion gel (A5) and marketed cream were applied to the left ear of the rat and the right ear as a control. The development of erythema was monitored for 14 days using the reported method.

**RESULTS AND DISCUSSION**

**Criteria for excipient selection**

The excipients selected were needed to be pharmaceutically acceptable, nonirritating, and nonsensitizing to the skin and to fall into the GRAS
Screening of excipients and oil
Drug loading per formulation is a very critical factor in the development of nanoemulsion systems for drugs, which is dependent on the drug solubility in oil phase. Nonionic surfactants are widely used in topical formulations as solubilizing agents, but some recent results indicate that they may affect the skin barrier function. Among the various surfactants evaluated, the maximum solubility of BV was found in Tween 20 [Figure 1] so it was selected as surfactant. The maximum solubility of BV in co-surfactant was found with Transcutol P [Figure 1]. The maximum solubility of BV in oil was found with Sefsol [Figure 1].

Transcutol P was selected as co-surfactant because it is very good penetration enhancer and solubilizing agent. The penetration enhancement of lipophilic drugs by alcohols is due to the higher solubility of the drug substance in the lipophilic area of the stratum corneum because of the presence of alcoholic enhancers. The effect of alcohols on the phase behavior of nonionic nanoemulsion depends on the number of carbons of alcohol. The presence of alcohol overcomes the need for any additional input of energy. These properties make the components useful as vehicles for drug delivery. Alcohols can influence the formation of nanoemulsion by both interfacial and bulk effects. So for the development of pseudo ternary phase diagram Sefsol oil was selected as the oil phase, Tween 20 as surfactant, Transcutol P as co-surfactant, and distilled water as aqueous phase.

Phase studies
The relationship between the phase behavior of a mixture and its composition can be captured with the aid of a phase diagram [Figure 2]. The aim of the construction of pseudo ternary phase diagram was to find out the existence range of nanoemulsion. Care was taken to ensure that observations are not made on metastable system. Pseudo ternary phase diagrams were constructed separately for each $S_{max}$ ratio for getting o/w nanoemulsion regions. The area of nanoemulsion isotropic region changed slightly as the ratio of surfactant in $S_{max}$ was increased. In the phase diagrams, the existence of large or small nanoemulsion region depends on the capability of the particular $S_{max}$ to solubilize the oil phase. The extent of solubilization results in a greater area with the formation of more clear and homogenous solution. The construction of pseudoternary phase diagrams was started using surfactant, i.e., Tween 20 alone (1:0). It was found that the region of nanoemulsion existence was very less and most of the region was composed of emulsions.

Therefore, with surfactant Tween 20, co-surfactant Transcutol P was also incorporated in the ratio 1:1 and it was found that region of nanoemulsion existence increased greatly. Increase in the concentration of co-surfactant to (1:2) resulted in even larger area of nanoemulsion existence, along with some emulsion, gels, or nanoemulsion gels area. Even when increase the ratio of surfactant leads to decrease the nanoemulsion region due to less flexible interfacial film between oil and water. Increasing co-surfactant concentration further from 1:2 to 1:3, and 1:4 resulted in the reduction of the nanoemulsion existence area and more area was composed of emulsion and gels. The existence of nanoemulsion region whether large or small depends on the capability of that particular surfactant or surfactant mixture to solubilize the oil phase. The extent of solubilization results in a greater area with more of clear, homogenous solution. It was seen that when the surfactant (Tween 20) was used alone, oil phase was solubilized to a lesser extent implying that surfactant alone was not able to reduce the interfacial tension of the oil droplets to sufficiently low level and thus was not able to reduce the free energy of the system to ultra low level desired to produce nanoemulsions. When a co-surfactant was added, the interfacial tension was reduced to much lower level and very small free energy was achieved which helped in larger nanoemulsion area existence in phase diagram. With a further increase in co-surfactant from 1:1 to 1:2, a further drop in interfacial tension and free energy was achieved resulting in maximum area of nanoemulsion formation. With a further increase in co-surfactant concentration (1:3), the interfacial tension of interfacial film increased when compared with above, and more of gel area and less of nanoemulsion area was observed. When increase in surfactant concentration with respect to co-surfactant...
in $S_{\text{mix}}$ again more gel area and less nanoemulsion area observed due to increase in interfacial tension of interfacial film. On the basis of above phase diagram study only $S_{\text{mix}}$ [1:2] ratio was considered for further studies and all the formulations were taken from $S_{\text{mix}}$ [1:2] ratio pseudo ternary phase diagram [Figure 2].

**Selection of formulation from phase diagram**
From the study it is suggested that large amount of surfactant causes skin irritation and toxicity-related problem, and therefore it is preferable to use the minimum amount of surfactant and co surfactant in the formulation. The surfactant concentration should be selected so that it gives the minimum flux and maximum drug deposition in dipper layer of skin which is an important criterion for topical application but its level should not be toxic to cause any irritation to the skin. This is usually not obtained with formulations that contain the highest amount of surfactant because high surfactant concentration decreases the thermodynamic activity of the drug in the vehicle, and the affinity of the drug to the vehicle becomes greater. Different proportion of oil was taken just to obtain desire quantity of drug dose from phase diagram [Table 1].

**Physical stability studies**
Nanoemulsions are considered to be thermodynamically stable systems that are formed at a particular concentration of oil, surfactant, and water, with no phase separation, creaming, or cracking. Selected formulations from phase diagram were subjected to different stress stability testing like heating cooling cycle, centrifugation, and freeze-thaw cycle. During physical stability testing, some formulations became turbid and in some phase separation occurred. One reason of this instability in nanoemulsions may be due to 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. The other reason may be that when temperature quench occurs during stress stability study, instability of nanoemulsion occurs due to separation of oil phase and droplet distribution of smaller size is favored by the change in curvature free energy. Only those formulations, which showed no phase separation, creaming, cracking, coalescence, and phase inversion during stress stability tests, were selected for further studies [Table 1].

**Characterization of nanoemulsions**
The formulations that passed physical stability test were evaluated for droplet size, polydispersity index, viscosity, pH, and refractive index.

In vitro skin permeation studies
The permeation ability of various BV loaded nanoemulsions was evaluated using the in vitro permeation experiments. A steady increase of BV in the receptor chamber with time was observed. The permeation profiles of nanoemulsions were in accordance with the Fick’s diffusion equation. On the basis of permeation studies, it was found that the nanoemulsion A5 consisting of 20 % oil phase, 44 % ($S_{\text{mix}}$ 1:2 ) and 36% distilled water exhibited 47.16 of cumulative amount of drug permeated ($\mu g/cm^2$) with the flux of 0.218 ($\mu g/cm^2/h$) after 24 h [Figure 3]. Optimized nanoemulsion A5 gel was prepared according to given procedure and its composition was listed in [Table 2] and pH was found to be 5.9. When optimized nanoemulsion gel (A5) compare with marketed cream it exhibited 45.75 of cumulative amount of drug permeated ($\mu g/cm^2$) with the flux of 0.417 ($\mu g/cm^2/h$) after 24 h [Figure 3]. The amount of drug deposited ($\mu g/cm^2$) and flux ($\mu g/cm^2/h$) of optimized nanoemulsion gel (A5) and marketed cream were given in [Table 3]. It was found that optimized nanoemulsion gel A5 has high amount of drug deposited in skin with minimum flux value which was expected for topical drug delivery.

**Particle size and polydispersity index**
The average size and polydispersity index of the nanoemulsion droplets were determined by photon correlation spectroscopy (Nano ZS90, Malvern Instrument, Worcestershire, UK). The droplets size of all nanoemulsions ranged from 150 to 200 nm. The polydispersity index showed that all the nanoemulsions had narrow size distribution. The average particle size and polydispersity index of the nanoemulsion formulation A5 were found to be 155.08 nm. (Figure 3) and 0.210 respectively, indicating micro range of droplets with minimum variation in particle size.

**Refractive index, pH, conductivity, and viscosity of nanoemulsion**
Viscosity of the nanoemulsion (A5) formulation was very low (26.95 ± 1.71mP) as expected for o/w emulsion [Table 4].The low viscosity may be due to presence of low amount of $S_{\text{mix}}$ (1:2) also the low concentration of oil. Refractive index is the net value of the components of nanoemulsion and indicates isotropic nature of formulation. Refractive index of nanoemulsion was determined using an Abbes type refractometer (Nirmal International, New Delhi, India) at 25 ± 0.5 °C. The mean value of the refractive index for the formulation A5 was found to be 1.431. The specific conductivity of nanoemulsion A5 was found to be $10^4$ s cm$^{-1}$. The apparent pH of
the formulation was measured by pH meter (AccumetAB 15, Fisher scientific, USA) in triplicate at 25 ± 1°C and found to be around 6.5 [Table 4].

**Surface morphology of particle**

The TEM studies were carried out to get more insight about the morphology of the nanoemulsion systems. From the results of TEM it was concluded that the particles of optimized formulation were spherical in shape and finely distributed with micron size range between 150 and 200 nm [Figure 6].

**Contact dermatitis**

The in vivo NTPDase activity of lymphocytes after each treatment is shown in [Figure 7]. A significant increase in NTPDase activity was observed in lymphocytes of the group treated with BV- loaded nanoemulsion, in relation to ATP and ADP [Figure. 7A and 7B, respectively] compared to all other groups (p < 0.01). The ADP and the ATP hydrolysis values for the group treated with BV optimized loaded nanoemulsion (A5) gel and the control groups were not statistically different. The higher NTPDase activity may be associated with the high levels of extracellular ATP resulting from the inflammatory process, which occurs in cases of allergic contact dermatitis. During the dermatitis, these high levels of ATP would have an affinity for P2X7 purinergic receptors, leading to a Th1 pattern of immune response with the production of inflammatory cytokines. The NTPDase would act by decreasing the levels of ATP, which in low concentration would bind to the P2Y receptors, reversing the pattern of immune response to Th2 with the release of anti-inflammatory cytokines. Thus, it is possible that the increased hydrolysis of adenine nucleotides also leads to an increase in the extracellular adenosine concentration, which has immunosuppressive and anti-inflammatory effects. Adenosine plays a central and direct role in the regulation of inflammatory responses and in limiting inflammatory tissue destruction. [50] In this context, the higher NTPDase activity in the treatment with the BV-loaded nanoemulsion (A5) gel, at intervals of 48 h, could be related to the higher anti-inflammatory effect in comparison with marketed cream. Therefore, the best result observed for the BV loaded nanoemulsion (A5) gel at 0.1%, even after a longer interval of time, may be related to the slower BV release as shown in [Figure 7] and its accumulation in the hair follicles as previously demonstrated [51] for substances in the form of nanosize. This result demonstrates that the optimized nanoemulsion formulation gel (A5), which did not appear to stimulate an inflammatory or immune response using the contact dermatitis model.

**Anti-inflammatory studies**

The anti-inflammatory effects of optimized nanoemulsion and marketed cream were compared with the control. The anti-inflammatory activity of optimized nanoemulsion was evaluated using the carrageenan-induced hind paw edema method using digital Plethysmometer. The rat's left footpad became edematous soon after injection of carrageenan and reached its peak at 12 h (84.45 %). The optimized nanoemulsion inhibited edema ($P < 0.05$) 84.2 % up to 12 h. Marketed cream inhibited the edema 45.05 % up to 12 h. Based on the anti-inflammatory studies, it can be concluded that BV optimized nanoemulsion showed maximum inhibition of edema than the marketed cream [Figure 8].

**Skin irritation test**

The mean values of skin irritation score for BV loaded nanoemulsion gel (A5), and marketed cream were found to be $1.83 \pm 1.16$ and $1.33 \pm 0.816$ respectively [Table 5]. From these results of 14 days test it was concluded that optimized nanoemulsion was safe to be used as topical drug delivery system. It clearly indicated that nanoemulsion has more skin irritation potential due to high amount of surfactant in comparison to the marketed cream. Marketed cream has lowest irritation potential due to less amount of surfactant. Overall all the formulation have low irritation score hence it is safe for human use.

**CONCLUSION**

On the basis of in vivo studies the developed nanoemulsion (A5) exhibited low irritation score, good anti-inflammatory action and high amount of drug deposition in skin. It can be recommended for human use after performing safety studies on human volunteers.
Table 1: Selection of formulation and physical stability studies of drug loaded formulations
Oil used: Sefsol oil, Surfactant used: Tween 20, Co-surfactant used: Transcutol P, External phase: Distilled water

<table>
<thead>
<tr>
<th>S\textsubscript{mix} Ratio</th>
<th>Formulation code</th>
<th>Oil (S\textsubscript{mix})</th>
<th>Water (S\textsubscript{mix})</th>
<th>H/C</th>
<th>Cent.</th>
<th>Freeze</th>
<th>Result</th>
<th>Drug Conc. (BV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A1</td>
<td>10</td>
<td>30</td>
<td>60</td>
<td>X</td>
<td>X</td>
<td>√</td>
<td>Failed</td>
</tr>
<tr>
<td>1:2</td>
<td>A2</td>
<td>10</td>
<td>34</td>
<td>56</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>Passed</td>
</tr>
<tr>
<td></td>
<td>A3</td>
<td>15</td>
<td>37</td>
<td>48</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>Passed</td>
</tr>
<tr>
<td></td>
<td>A4</td>
<td>15</td>
<td>40</td>
<td>45</td>
<td>√</td>
<td>X</td>
<td>√</td>
<td>Failed</td>
</tr>
<tr>
<td></td>
<td>A5</td>
<td>20</td>
<td>44</td>
<td>36</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>Passed</td>
</tr>
<tr>
<td></td>
<td>A6</td>
<td>25</td>
<td>50</td>
<td>25</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>Passed</td>
</tr>
</tbody>
</table>

H/C = Heating and Cooling Cycle, Cent. = Centrifuge

Table 2: Compositions of nanoemulsion (A5) and nanoemulsion gel (A5)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Nanoemulsion (A5)</th>
<th>Nanoemulsion gel (A5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXB (% w/w)</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Carbopol-940 (% w/w)</td>
<td>-</td>
<td>1.0</td>
</tr>
<tr>
<td>Sefsol 218 (%w/w)</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Tween 20 : Transcutol-P (% w/w) (1:2)</td>
<td>44</td>
<td>44</td>
</tr>
<tr>
<td>Triethanolamine (% w/w)</td>
<td>-</td>
<td>0.5</td>
</tr>
<tr>
<td>Distilled water to (% w/w)</td>
<td>100.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Table 3: Amount of drug deposited (μg/cm\(^2\)) and flux (μg/cm\(^2\)/h) of optimized nanoemulsion gel (A5) and marketed cream.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Cumulative amount of drug permeated (μg/cm(^2))</th>
<th>Flux (μg/cm(^2)/h)</th>
<th>Amount of drug deposited in skin (μg/cm(^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimized nanoemulsion Gel (A5)</td>
<td>45.75</td>
<td>0.417</td>
<td>58.46</td>
</tr>
<tr>
<td>Marketed cream</td>
<td>38.43094</td>
<td>0.631</td>
<td>42.95</td>
</tr>
</tbody>
</table>

Table 4: Parameters for the nanoemulsion formulation A5

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Parameters</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Particle size (nm)</td>
<td>155.08</td>
</tr>
<tr>
<td>2.</td>
<td>Refractive index</td>
<td>1.431</td>
</tr>
<tr>
<td>3.</td>
<td>pH ±SD (n=3)</td>
<td>6.5 ± 0.011</td>
</tr>
<tr>
<td>4.</td>
<td>Viscosity (mP) ±SD (n=3)</td>
<td>26.95 ± 1.71</td>
</tr>
<tr>
<td>5.</td>
<td>Conductivity (s cm(^{-1}))</td>
<td>(10^{-4})</td>
</tr>
</tbody>
</table>
Table 5: Skin irritation score of the optimized nanoemulsion gel (A5) and marketed cream

<table>
<thead>
<tr>
<th>S. No</th>
<th>Group</th>
<th>Score after (days) 1</th>
<th>Score after (days) 2</th>
<th>Score after (days) 3</th>
<th>Score after (days) 4</th>
<th>Score after (days) 7</th>
<th>Score after (days) 14</th>
<th>Mean score±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Nanoemulsion gel (A5)</td>
<td>2</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>1.83±1.16</td>
</tr>
<tr>
<td>2.</td>
<td>Marketed cream</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>1.33±0.816</td>
</tr>
</tbody>
</table>

Figure 1: Solubility of BV in different oil, surfactants and co-surfactants.

Figure 2: Pseudoternary phase diagram indicating o/w nanoemulsion region using Sefsol oil, Tween 20 (surfactant), and Transcutol P (co-surfactant). $S_{mix} 1:2$. 
Figure 3: *In vitro* permeation studies of different nanoemulsions.

Figure 4: *In vitro* permeation studies of optimized nanoemulsion gel (A5) and marketed cream.

Figure 5: Droplet size and size distribution of nanoemulsion formulation (A5).
Figure 6: TEM photograph of particle size of nanoemulsion (A5) drug loaded (BV).

Figure 7: ATP (7A) and ADP (7B) hydrolysis in lymphocytes obtained from the control group (C), contact dermatitis group (D), groups with dermatitis treated with Drug loaded nanoemulsion gel (A5) and Marketed cream. Data were analyzed statistically by one-way ANOVA followed by the Tukey–Kramer test (1A) and Kruskal–Wallis Test (1B), p < 0.05.
Figure 8: Comparison of anti-inflammatory activity of nanoemulsion gel (A5) with marketed cream.

REFERENCES