IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
HUMAN

Human Journals
Research Article
April 2019 Vol.:15, Issue:1

TPGS Loaded Topical Nanoemulgel of Mefenamic Acid for the Treatment of Rheumatoid Arthritis

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ISSN 2349-7203
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Submission: 21 March 2019
Accepted: 26 March 2019
Published: 30 April 2019

Keywords: Mefenamic acid, rheumatoid arthritis, topical nanoemulgel, carbopol 940 (1% w/v), antioxidant activity, pharmacodynamic study

ABSTRACT

The present work aimed to develop TPGS loaded topical nanoemulgel of mefenamic acid for improving its efficacy, stability, and permeability for the treatment of Rheumatoid arthritis. A nanoemulsion was prepared using aqueous titration method composed of capryol 90:TPGS (1:1) as the oil phase, tween 60 as surfactant and transcutol P as cosurfactant and evaluated. Due to the low viscosity of nanoemulsion, nanoemulgel was formulated by incorporating carbopol 940 (1% w/v) as a gelling agent and evaluated. The results were expressed as mean ± standard deviation (S.D) and data was statistically analyzed using Graph Pad Instat 3, using a two-tailed paired t-test. Values at $p \leq 0.05$ were considered significant. The *in vitro* skin permeation study showed markedly low release (46.28%) of optimized nanoemulgel ($D1_{(1\%w/v)}$) for the better drug retention in skin layers. The nanoemulgel exhibited good antioxidant activity due to the presence of TPGS (having inherent antioxidant activity). The optimized nanoemulgel ($D1_{(1\%w/v)}$) shows the inhibition in inflammation (94.73%) and increased percent reaction time (92.91%), with excellent analgesic activity in the pharmacodynamic study. The stability studies (using HPLC method) indicated that only 0.030% of the drug was degraded from the optimized nanoemulgel in 3 months study period. The topical nanoemulgel of mefenamic acid was developed to a satisfactory level in terms of release of drug from it, optimum globule size, optimum viscosity, higher penetration, and better skin retention. The present study endorsed topical nanoemulgel of mefenamic acid to be a promising choice over conventional topical formulations for the treatment of rheumatoid arthritis.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease causing inflammation in the synovial membrane of joints with the migration of activated phagocytes and other leukocytes into synovial and periarticular tissue, especially in fingers, wrists, feet, and ankles resulting in painful deformity and immobility. About 1% of the whole world population is attacked by rheumatoid arthritis[1]. A recent study indicated that increased oxidative stress and/or defective antioxidant status also contributes to the pathology of rheumatoid arthritis. It is also reported that there is an increased state of oxidative stress in RA, in which the use of antioxidant supplementation in such patients is proposed[2].

Mefenamic acid, 2-[(2,3-dimethylphenyl)amino]benzoic acid[3] is an anti-inflammatory non-steroidal drug which belongs to the category of fenamates (anthranilic acid) and is widely used as an analgesic and antiphlogistic agent in the treatment of rheumatoid arthritis, osteoarthritis, and other joint disorders[4-6]. It is a COX-2 inhibitor which also antagonizes certain actions of prostaglandins which play an important role in pain and inflammation. It is classified as BCS class-II drug having high lipophilicity (logP 5.4) and poor solubility (0.04mg/ml)[7]. The oral and topical administration of NSAID's although considered safest amongst the various therapies for rheumatoid arthritis is associated with systemic side effects, ulceration and bleeding,[8] physical instability, leakage of entrapped drug,[9], etc.

In order to overcome the drawbacks associated with the other dosage forms and in order to improve the overall efficacy of the drug, nanoemulgel of mefenamic acid was envisaged as the aim of the present study. The formulation offers dual advantages of nanoemulsion as well as gel. The gels are a relatively newer class of dosage form created by entrapment of large amounts of aqueous or hydroalcoholic liquid in a network of colloidal solid particles, mainly consisting of inorganic substances, organic polymers of natural or synthetic origin[10]. They have a higher aqueous component that permits greater dissolution of drugs, and also there is easy migration of the drug through a vehicle that is essentially a liquid, compared with the ointment or cream base[11]. The nanoemulsion components of the gel will further improve the delivery of entrapped mefenamic acid due to its submicron size. The prepared nanoemulgel will thereby improve the overall efficacy of mefenamic acid in the improvement of rheumatoid arthritis. The formulation is also enriched with TPGS (Tocopheryl Polyethylene Glycol Succinate) which being a potent antioxidant would reduce oxidative stress and reduce the progression of a disease.

MATERIALS AND METHODS

Materials and Reagents

Mefenamic acid (MA) was provided by Jackson Laboratories Pvt Ltd. (Amritsar, Punjab). Carrageenan (Ranbaxy Lab Fine chemicals Ltd, New Delhi, India), Labrafac WL 1349, Labrafil M, Labrasol, Lauroglycol FCC, Transcutol P were procured from Gattefosse (Saint-Priest, Cedex, France). Tween 20, Tween 60, Tween 80 was provided from Central Drug Laboratories (New Delhi, India), HPLC grade water was purchased from Qualikems fine chem. Pvt. Ltd. All chemicals were of analytical grade.

Formulation development and optimization

Authentication of excipients

The authentication of procured oils (Capryol 90, oleic acid, soyabean oil, sunflower oil, fish oil, TPGS), surfactants (Tween 20, Tween 40, Tween 60, Labrasol, Span 80) and cosurfactants (Transcutol P, Glycerol, LabrafilM, Labrafac, PEG 600, Lauroglycol FCC) used for formation of nanoemulsions was checked by determine their refractive indices. The miscibility studies were conducted by taking one ml each of selected oils, surfactants and cosurfactants in the ratio (1:1) in 5 ml capacity stoppered vials separately and mixing using a vortex mixer. The mixture vials were kept at 25 ± 1.0 °C in an isothermal shaker for 72 hours to reach equilibrium. After 72 hours the vials were removed from the shaker and kept overnight to check the miscibility of surfactant and cosurfactant[12].

Screening of components

The solubility of mefenamic acid in different oils (capryol 90, oleic acid, clove oil, castor oil, fish oil, sunflower oil and TPGS), surfactants (Tween 20, Tween 40, Tween 60, Labrasol, Span 80) and cosurfactants (Transcutol P, Glycerol, Labrafil M, Labrafac, PEG 600, Lauroglycol FCC), an excess amount of drug was added to 1 ml of the selected oils, surfactants and cosurfactants separately in 5 ml stoppered vials to produce supersaturated solutions which were then mixed using vortex mixer and then kept for 72 hours at 25±1°C in isothermal shaker to reach equilibrium. After 72 hours, the equilibrated samples were removed from the shaker and centrifuged at 3000 rpm for 15 minutes. The supernatants were

taken and filtered through the 0.45µm membrane and analyzed by UV spectrophotometer at 286nm after suitable dilution, if necessary. The experiment was done in triplicate[13].

Spontaneous emulsification method

The aqueous phase titration method was employed for constructing the existing zone of nanoemulsion in pseudoternary phase diagrams representing three axes of the aqueous phase, oil, and mixture of surfactant and cosurfactant. To construct phase diagram, the screened surfactant was blended with cosurfactant in different Smix ratio (1:0, 1:1, 1:2, 1:3, 2:1, 3:1 and 4:1) with increasing concentration of surfactant to cosurfactant. The selected Smix ratio was further treated with oil at different ratios (1:9, 2:8, 3:7, 4:6, 1:2, 1:3, 1:3:5, 1:5, 1:6, 1:7 and 1:8) so that maximum ratio could be covered in order to obtain a precise phase diagram. In order to obtain a clear, isotropic and physical stable nanoemulsion with low viscosity, slow titration with water was done for each ratio of oil and Smix. The volume of distilled water was recorded. The nanoemulsion samples were left for 24 h to attain equilibrium and to screen metastable compositions[13]. Various placebo formulations were selected for drug loading (100 mg) in required quantities of oil (Capryol:TPGS::1:1), surfactant (Tween 60) and cosurfactant (Transcutol P). The prepared formulations were sonicated for 10 minutes at the amplitude of 80 and 2 min cycles using a probe sonicator to obtain clear and transparent liquid nanoemulsions[14]. The prepared nanoemulsions were placed in tightly closed glass vials and stored at ambient temperature. These drug-loaded formulations were subjected to physical stability tests [centrifugation stress (5000 rpm, 30 min), heating cooling stress (0 and 45ºC, eight cycles) and freeze-thawing stress (-21 and 25 ºC, ≥48 h)].

Thermodynamic stability studies

In order to find out the stable nanoemulsions and to discard the unstable nanoemulsions, the placebo nanoemulsions were subjected to the following thermodynamic studies.

1. **Centrifugation study**

The prepared nanoemulsions were subjected to centrifugation study at 3000 rpm for 30 mins at 25°C and observed for any physical instability, creaming or cracking. The formulations which did not show any physical instability were subjected to heating-cooling cycle[15].

2. **Heating cooling cycle**

The formulations which passed the centrifugation study were subjected to the heating-cooling cycle. This was carried out to study the effect of variation in temperature on the stability of nanoemulsion by subjecting the formulations to six cycles between refrigerator temperature 4°C and 40°C with storage at each temperature for not less than 48 hrs[16].

3. **Freeze-thaw cycle**

The formulations which passed the above two studies were then subjected to freeze-thaw cycle. It was performed for the accelerated stability testing of the nanoemulsion formulations. The formulations were subjected to -21°C and +25°C with storage at each temperature for not less than 48 hrs[16].

Characterization of Nanoemulsions

The drug-loaded nanoemulsions were evaluated for the following parameters:-

Viscosity

The viscosities of prepared nanoemulsion formulations were determined by using Brookfield Viscometer (Model DV-1 Prime) using spindle # 61 at 37± 0.5°C. The spindle speed was set at 60 rpm and a single run was performed at a temperature of 37± 0.5°C. The wait time for the operation was 2 mins. The viscosity of each sample was determined in triplicate and the standard deviation was calculated[17].

pH

The determination of pH is important for formulating topical preparations as too high or low pH results in undesirable effects leads to skin irritancy. It was determined by using digital pH meter at room temperature. The experiment was performed in triplicate and the standard deviation was calculated[12].

Drug content

A specific quantity (1 ml) of developed nanoemulsion was taken and dissolved in 50 ml of methanol. The volumetric flask containing nanoemulsion solution was shaken for 10-15 mins., in order to get complete solubility of the drug. This solution was filtered using a

Millipore filter (0.45µm) and 1 ml from this solution was taken and diluted to 100 ml with methanol. The absorbance was recorded by using UV-Visible spectrophotometer at λmax 286 nm using methanol as blank. The experiment was performed in triplicate and the standard deviation was calculated[18].

***In vitro* release profile of nanoemulsions by using dialysis membrane**

The *in vitro* release study was done by using a dialysis membrane mounted on Franz diffusion cell between the donor and receptor compartment. The release medium used for the study was 35 ml of acetate buffer pH 5.5: PEG 600 (6:4), thermo-stated at 37±1°C, and continuously stirred at 75 rpm throughout the experiment. One ml of the prepared drug-loaded nanoemulsion containing 10mg of the drug was placed in the donor compartment over the membrane. The whole assembly was kept on a magnetic stirrer and the solution was stirred continuously using a magnetic bead. The samples of 3 ml were withdrawn at time intervals of 1, 2, 3, 4, 5, 6, 7 and 8 hrs and replaced with equal amounts of fresh medium. The samples withdrawn were analyzed by using UV spectrophotometer (Shimadzu, 1800, Japan) at 286nm[19]. The experiments were done in triplicate.

***In vitro* skin permeation study and drug retention study of optimized formulation using excised rat skin**

1. *In vitro* skin permeation study using excised rat skin

a) Preparation of skin for permeation study

The *in vitro* skin permeation study of selected formulations was carried out in a Franz diffusion cell, using excised skin of Wistar rats. The hairs on the excised skin of rats were removed and underlying fat and subcutaneous tissues and dermis side were wiped with isopropyl alcohol (IPA) to remove adhering fat. The skin was washed with distilled water, wrapped in aluminum foil and stored at -20ºC till further use.

b) Stabilization of the skin

The skin was cut to appropriate size and mounted between the two half cells with stratum corneum facing the donor compartment. The receptor media consisting of phosphate buffer pH 7.4 containing 1% SLS was first sonicated for 30 minutes, to remove any dissolved gasses and then filled into the receptor compartment and magnetically stirred at 200 rpm for

proper mixing. The diffusion cell was thermostated at 37±0.5°C. The buffer solution was replaced after every half an hour to stabilize the skin. The skin was stabilized for 4-5 hours. After stabilization, the UV spectrum was taken and skin was considered stabilized when no UV absorption was visible.

c) Permeation studies

A section of cleaned skin (after being brought to room temperature), was cut and placed in the space between the donor and receptor compartment of the Franz diffusion cell, with the stratum corneum side facing the donor compartment and the dermal side facing the receptor compartment. The volume of the receptor medium was 35 ml of phosphate buffer pH 7.4 containing 1% SLS, thermostated at 37 ± 1 °C, which was continuously stirred at 75 rpm throughout the experiment. One ml of the prepared nanoemulsion (1% w/v) containing 10mg of the drug was placed in the donor compartment over the membrane. The whole assembly was kept on a magnetic stirrer and the solution was stirred continuously using a magnetic bead[20]. The samples of 3 ml were withdrawn at time intervals of 1, 2, 3, 4, 5, 6, 7 and 8 hrs and replaced with equal amounts of fresh medium. The withdrawn samples were analyzed by using UV spectrophotometer (Shimadzu, 1800, Japan) at 286nm[21].

d) Data Analysis

1. determination of flux and permeability coefficient

The cumulative amount of nanoemulsion permeated through the Wistar rat skin (Q, µg/cm^2) was plotted as a function of time (hrs). The drug flux (permeation rate) at the steady state (J_{ss}, µg/cm^2/h) was calculated from the slope and intercept of the straight line obtained by plotting the amount of mefenamic acid nanoemulsion permeated versus time in steady state condition. Permeability coefficient (k_p) was calculated by dividing the flux by initial drug concentration (C_o) in the donor portion of the cell.

2. Determination of drug retention in skin layers

The formulation remaining on the skin was removed, cleaned with cotton soaked in a 0.05% sodium lauryl sulfate and washed with distilled water. The skin sample was weighed, cut into small pieces and sonicated for 15 mins with methanol in order to extract the mefenamic acid.

The resulting solution was then centrifuged and filtered and then drug content (µg/cm^2) of skin was determined by U.V analysis using UV spectrophotometer.

Particle size and polydispersity index

Dynamic light scattering (DLS) otherwise called photon correlation spectroscopy (PCS) is used to analyze the fluctuations in the intensity of scattering by droplets/particles due to Brownian motion. Nanoemulsion droplet size, polydispersity and zeta potential can be assessed by PCS using a particle size analyzer. This instrument also measures the polydispersity index, which is a measure of the broadness of the size distribution derived from the cumulative analysis of dynamic light scattering. The polydispersity index indicates the quality or homogeneity of the dispersion. PCS gives z-average particle diameter[22].

Determination of zeta potential

Zeta potential is the measure of the magnitude of the electrostatic or charges repulsion or attraction between particles and, known to affect stability. Almost all particulate or macroscopic materials in contact with a liquid acquire an electronic charge on their surfaces[11]. Zeta potential of nanoemulsion formulation was determined using Zeta Sizer after suitable dilution with distilled water at 25^{0}C.

Transmission Electron Microscopy (TEM)

The morphology of the oil droplets in the nanoemulsion formulation was visualized with TEM analysis. TEM analysis was also significant in order to visualize any precipitation of the drug upon the addition of the aqueous phase. The nanoemulsion was diluted 100 times and a drop was applied to 300- mesh copper grid. The grid was left for one min. and it was inverted. A drop of phosphor-tungstic acid (PTA) was applied to the grid for 10 sec. Excess of PTA was removed by absorbing on a filter paper and the grid was analyzed using JEM-1000 transmission electron microscope (JEOL Ltd. Tokyo, Japan) operated at 1000 kV at 200 x ~1,200,000 x magnification[23].

Formulation and evaluation of topical drug loaded nanoemulgels

Formulation of topical nanoemulgels

The drug-loaded nanoemulsion was optimized based on the results of viscosity, drug content, *in vitro* drug release, *in vitro* skin permeation and drug retention study and used for incorporation of gelling agent for the formation of nanoemulgels. The topical nanoemulgels were formulated by adding a suitable gelling agent to the optimized nanoemulsion formulation as per the procedure is given below:-

a) Selection of a gelling agent

The very low viscosity often exhibited by nanoemulsion is inappropriate for topical use. In order to increase the viscosity of nanoemulsion, carbopol 971, carbopol 940, hydroxypropylmethylcellulose (HPMC 5-CPS) and sodium alginate were selected as gelling agents. For the preparation of nanoemulgel, initially, 1% w/v concentration of the polymers was slowly mixed with optimized nanoemulsion under stirring until the equilibrium was attained. The pH was stabilized by the addition of 0.1 ml of triethanolamine (TEA). The formed nanoemulgels were kept for 24 hrs and observed for transparency, homogeneity, grittiness and phase separation[24].

b) Optimization of the concentration of selected gelling agent

The concentration of the gelling agent which produced a clear, transparent and stable nanoemulgel formulation, free from grittiness was further optimized. Drug-loaded nanoemulgels containing different concentrations (0.5% w/v, 0.8%w/v, 1%w/v) of selected gelling agent were formulated and characterized.

c) Method of preparation

The preparation of nanoemulgels involved the addition of a gelling agent in the optimized nanoemulsion formulation. The carbopol 940 (1% w/v) was slowly mixed with optimized nanoemulsion under stirring until the equilibrium was attained. The pH was stabilized by the addition of 0.1 ml of triethanolamine (TEA). The formed nanoemulgels were kept for 24 hrs and observed for transparency, homogeneity, grittiness and phase separation[24].

Evaluation of topical drug loaded nanoemulgels containing different concentrations of selected gelling agent

The nanoemulgels produced using different concentrations of the gelling agent were characterized as follows:-

Physical characteristics of nanoemulgels

Physical characteristics of prepared nanoemulgels (0.5%w/v, 0.8%w/v and 1%w/v) were checked under room temperature for color, odor, appearance, etc[25].

Viscosity measurement

The viscosities of prepared nanoemulgel (0.5%w/v, 0.8%w/v and 1%w/v) formulations were determined by using Brookfield Viscometer using spindle # 61 at 37±0.5°C. The spindle speed was set at 60 rpm and a single run was performed at a temperature of 37±0.5°C. The wait time for the operation was 2 mins. Each sample was performed in triplicate and the standard deviation was calculated[17].

pH

Determination of pH is important for formulating topical preparations as too high or low pH results in undesirable effects leads to skin irritancy. pH of the prepared nanoemulgels formulations was determined by using digital pH meter at room temperature. Each sample was performed in triplicate and the standard deviation was calculated[26].

Spreadability

The spreadability of prepared nanoemulgels was determined 48 h after preparation by measuring the spreading diameter of nanoemulgel between the two glass plates after 1min. A weight of 1 gm of nanoemulgel was placed within a circle of diameter 1 cm premarked on glass plate over which a second glass plate was placed. The increase in diameter as a consequence of weights added leading to the spreading of the gel was noted[19].

Homogeneity and grittiness

Small quantities of formulated nanoemulgels were pressed between the thumb and the index finger. The consistency, homogeneity, and presence of any coarse particles in different

nanoemulgels were noted. For the evaluation of homogeneity and grittiness, a small quantity of the drug-loaded nanoemulgels (0.5%, 0.8%, and 1%) was rubbed on the skin of the back of the hand and observations recorded[11].

Drug content and content uniformity

The amount of drug contained in the prepared nanoemulgels (1g) of different concentrations (0.5%, 0.8%, and 1%) was determined by diluting the required amount of prepared formulations using methanol. The volumetric flasks each containing nanoemulgel solutions were shaken for 2 hrs on the mechanical shaker in order to get complete solubility of the drug. These solutions were filtered using Millipore filter (0.45µm) and 1 ml from these solutions were diluted to 10 ml with methanol. These mixtures were analyzed by UV spectrophotometer at 286 nm against methanol as blank[27].

For determination of content uniformity, samples were withdrawn from different parts of gels and analyzed as per the procedure is given above.

***In vitro* skin permeation and retention study of different nanoemulgels using excised rat skin**

1. *In vitro* skin permeation study through excised rat skin

The procedures adopted for the preparation and stabilization of skin is the same as per the permeation study of the nanoemulsion.

A section of skin (after being brought to room temperature), was cut and placed in the space between the donor and receptor compartment of the Franz diffusion cell, with the stratum corneum side facing the donor compartment and the dermal side facing the receptor compartment. The volume of the receptor medium was 35 ml of phosphate buffer pH 7.4 containing 1% SLS, thermostated at 37 ± 1 °C, which was continuously stirred at 75 rpm throughout the experiment. One gm of the prepared drug-loaded nanoemulgels containing 10mg of the drug was placed in the donor compartment over the membrane. The whole assembly was kept on a magnetic stirrer and the solution was stirred continuously using a magnetic bead[20]. The samples of 3 ml were withdrawn at time intervals of 1, 2, 3, 4, 5, 6, 7 and 8 hrs and replaced with equal amounts of fresh medium[21]. The samples were analyzed by using UV spectrophotometer (Shimadzu, 1800, Japan) at 286nm.

d) Data Analysis (determination of flux and permeability coefficient)

The cumulative amount of nanoemulgel permeated through the Wistar rat skin (Q, µg/cm^2) was plotted as a function of time (hrs). The drug flux (permeation rate) at the steady state (J_{ss}, µg/cm^2/h) was calculated from the slope and intercept of the straight line obtained by plotting the amount of mefenamic acid nanoemulsion permeated versus time in steady state condition. Permeability coefficient (K_p) was calculated by dividing the flux by initial drug concentration (C_o) in the donor portion of the cell.

2. Determination of drug retention in skin layers

The formulation remaining on the skin was removed, cleaned with cotton soaked in a 0.05% sodium lauryl sulfate and washed with distilled water. The skin was then weighed, cut into small pieces and sonicated for 15 mins with methanol in order to extract the mefenamic acid. The resulting solution was then centrifuged and filtered and then drug content (µg/cm^2) of skin was determined by UV analysis using UV spectrophotometer.

Antioxidant study

The normal white blood cell count in synovial fluid is less than 100 per milliliter, on average. In patients afflicted with osteoarthritis, this figure rises to approximately 800 per milliliter whereas in those with rheumatoid arthritis, this further increases significantly. The use of antioxidant vitamins (TPGS) in the formulation is also reported to be a possible means to reduce the pain and inflammatory events associated with rheumatoid arthritis[28]. The antioxidant activity of the prepared formulation was checked using:-

Reducing power assay

The reducing power assay was performed as per the procedure is given by Sharma et al. One milliliter of different concentrations (5.0 – 100 µg/ml) of ascorbic acid (as standard antioxidant) in distilled water and sample solutions were mixed with 2.5 ml of 0.2M phosphate buffer (pH 6.6) and 2.5 ml of 10% potassium ferricyanide [$K_3Fe(CN)_6$]. The mixtures were incubated at 50°C for 20 min and 2.5 ml of 10% trichloroacetic acid was added, which was then centrifuged at 3000rpm for 10 min. The upper of the solution (2.5ml) was separated; mixed with distilled water (2.5ml) and 0.1% ferric chloride (0.5ml). The absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated

more reducing power. Ascorbic acid was used as a reference standard and phosphate buffer (pH 6.6) was used as a blank solution[13].

***In vivo* studies**

The animal study was performed according to a protocol submitted and approved by the Institutional Animal Ethics Committee, Khalsa College of Pharmacy, Amritsar (IAEC/KCP/2015/012). The animals used for *in vivo* studies were adult Wistar albino rats of either sex (180-200g) kept under standard laboratory conditions, at temperature 25 ± 2°C and relative humidity 55 ± 5%. The animals were housed in properly cleaned cages, 6 per cage, with free access to standard laboratory diet and water.

1. Skin irritation study

Skin irritation study was carried out in healthy Wistar rats (180-220 g) of either sex. The animals were divided into three groups each comprising 6 animals maintained on standard animal feed and had free access to water. Before one day of starting the study, hairs were shaved from the back of rats and an area of 5 cm^2 was marked. The prepared mefenamic acid nanoemulgel and drug solution were applied for 7 days to animals in group II and group III respectively and the site was observed for any sensitivity and the reaction if any, was graded as 0, 1, 2, 3 for no reaction, slight patchy erythema, slight but confluent or moderate but patchy erythema and severe erythema with or without edema, respectively[11,20].

Histopathology study

After 7 days, the Wistar rats used for skin irritation study were sacrificed and the abdominal skin was removed and subjected to histopathological evaluation. Each specimen was stored in 10% formalin solution in phosphate buffer pH 7.4. The specimens were cut into sections vertically. Each section was dehydrated using ethanol embedded in paraffin wax for fixing and stained with hematoxylin and eosin. These samples were observed under a light microscope and compared with control samples for the presence of any irritation[24].

2. Pharmacodynamic study

a) Anti-inflammatory study

For the study, the animals were divided into five groups each comprising of 6 rats. For the conduct of the anti-inflammatory study, edema was induced in the left hind paw of the rats by subplantar injection of 1% (w/v) carrageenan. Placebo, nanoemulgel formulation and marketed formulation were applied 30 mins before carrageenan administration in group III, group IV and group V except for group I, which serve as control group. The group II serves as carrageenan induced animals group. The paw volume was measured at intervals of 1, 2, 3, 6, 12 and 24 hrs by mercury displacement method using plethysmograph[21,29,24]. The % inhibition of paw edema in the drug-treated group was compared with the control group and calculated according to the formulae [29] given by Lala and Awari:-

% Inhibition of drug = (% Edema$_{(Control)}$ – % Edema $_{(Formulation)}$ / % Edema $_{(Control)}$ × 100

% Edema = (Final volume of paw – Initial volume of paw / Initial volume of paw) × 10

The same animals were used for analgesic activity after one week washout period.

b) Analgesic study

The animals were divided into four groups each comprising 6 rats. For the conduct of the analgesic study, a hot-plate test was performed using an electronically controlled hot plate heated to 53℃ ± 0.1℃ with the cut-off time 30 seconds to avoid further tissues damage. Placebo formulation, optimized nanoemulgel, and marketed formulation were applied topically to the hind paw of the rat in group II (placebo formulation), group III (nanoemulgel formulation) and group IV (marketed formulation) except group I, which serve as control group. Thirty minutes after the drug administration, the gel remaining on the surface of the skin will be wiped off with a piece of cotton. Measurements of pain threshold for the treated animals were taken after 0.5, 1, 1.5, 2, 2.5 and 3 hours after the application of the gel. Latency to lift and licking a hind paw or attempted to jump from the apparatus was recorded for the control and drug-treated groups[30].

The percentage of analgesic activity [31] is calculated as per the procedure is given below by Rasheed and Kumar.

$$\% \text{ Analgesic activity} = [(T_2-T_1)/ (T_c-T_1)] \times 100$$

Where;

T_1 - the reaction time (s) before applying gel T_2 - the reaction time (s) after applying gel

To - cut off time in Sec.

Stability Study of the optimized nanoemulgel formulation by HPLC method as per ICH guidelines

The stability studies were carried out at 40±2°C/75±5%RH in the stability chamber for the optimized nanoemulgel for 3 months. The gel was stored at 40°C/75% RH in the collapsible tube for 3 months. The samples were withdrawn on 0 days and after a period of 1 month, 2 months and 3 months[27]. The samples were analyzed for pH, viscosity and drug content by using the HPLC method.

Statistical analysis

The results were expressed as mean ± standard deviation (S.D). The data obtained from various groups were statistically analyzed using Graph Pad Instat 3, using two-tailed paired t-test. Values at $p \leq 0.05$ were considered significant.

RESULTS

Formulation of nanoemulsion

Authentication of excipients

The authentication of procured oils, surfactants and cosurfactants was checked by determining their refractive indices. It is an important criterion to check out the purity of the excipients used in the formulation of nano-sized formulations. Table 1 (a-c) depicts refractive indices of the various excipients used in the formulation of nanoemulsion as their purity criteria.

Table 1 a: Refractive index of procured oils

Sr. No.	Oils	Refractive index ± SD (n=3)
1	Ajwain oil	1.484±0.012
2	Arachis oil	1.462±0.005
3	Capryol 90	1.432±0.002
4	Castor oil	1.474±0.002
5	Cinnamon oil	1.565±0.003
6	Clove oil	1.525±0.003
7	Corn oil	1.464±0.003
8	Fish oil	1.470±0.003
9	Isopropyl myristate	1.433±0.002
10	Oleic acid	1.454±0.003
11	Olive oil	1.467±0.002
12	Shark liver oil	1.461±0.002
13	Soyabean oil	1.475±0.002
14	Sunflower oil	1.466±0.002
15	TPGS	1.497±0.092

Table 1 b: Refractive index of procured surfactants

Sr. No.	Surfactants	Refractive index ± SD (n=3)
1	Labrasol	1.430±0.002
2	Tween 20	1.463±0.003
3	Tween 60	1.475±0.003
4	Tween 80	1.475±0.003

Table 1 c: Refractive index of procured cosurfactants

Sr. No.	Co-surfactants	Refractive index ± SD (n=3)
1	Glycerol	1.474±0.002
2	Labrafil M	1.425±0.001
3	Labrafac	1.47±0.001
4	Lauroglycol FCC	1.43±0.002
5	PEG 600	1.465±0.002
6	Transcutol P	1.428±0.002

Miscibility study

The miscibility study is an important parameter for the stability of nanoemulsion formulation. If the components of nanoemulsion become not miscible with each other, they will result in phase separation causing instability of the formulations. Capryol 90: Transcutol P Capryol 90: PEG 600, Labrasol: Transcutol P, Tween 60: Transcutol P, Tween 20: PEG 600, Tween 60: PEG 600, Span 80: Transcutol P was some of the miscible combinations used for the formulation of the stable nanoemulsion.

Screening of components

The solubility of excipients is the important criteria for the formation of stable nanoemulsion. Also, these should be selected carefully such that they are pharmaceutically acceptable for the preparation of nanoemulsion and fall under GRAS (Generally acceptable as Safe) category. The higher solubility of the drug in the oil phase is important for the nanoemulsion to maintain the drug in the solubilized form. The surfactants impart solubility to the formulation by preventing coalescence of the globules of the dispersed phase by forming a flexible film which is lipophilic in nature. The presence of cosurfactants which get adsorbed on the surface decreases the bending stress of interface and allows the interfacial and allows the interfacial film sufficient to take up different curvatures required to form nanoemulsion over a wide range of composition. The solubility of mefenamic acid in different components is given in Figure1 to Figure 3.

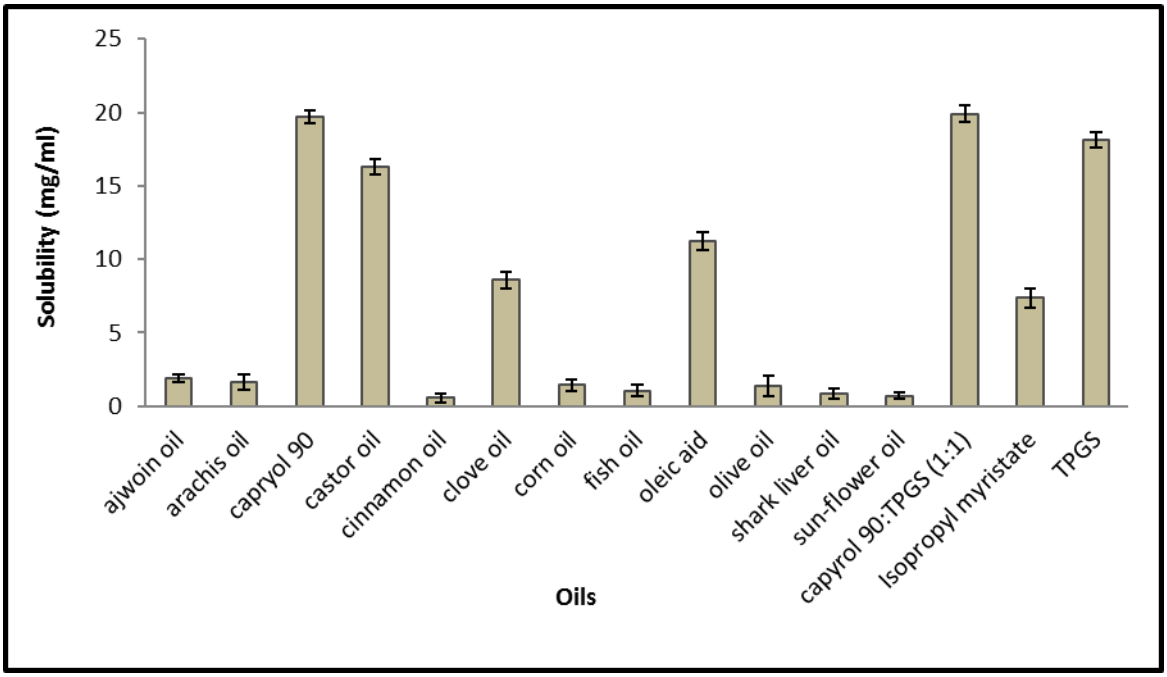


Figure 1: Solubility of mefenamic acid in various oils

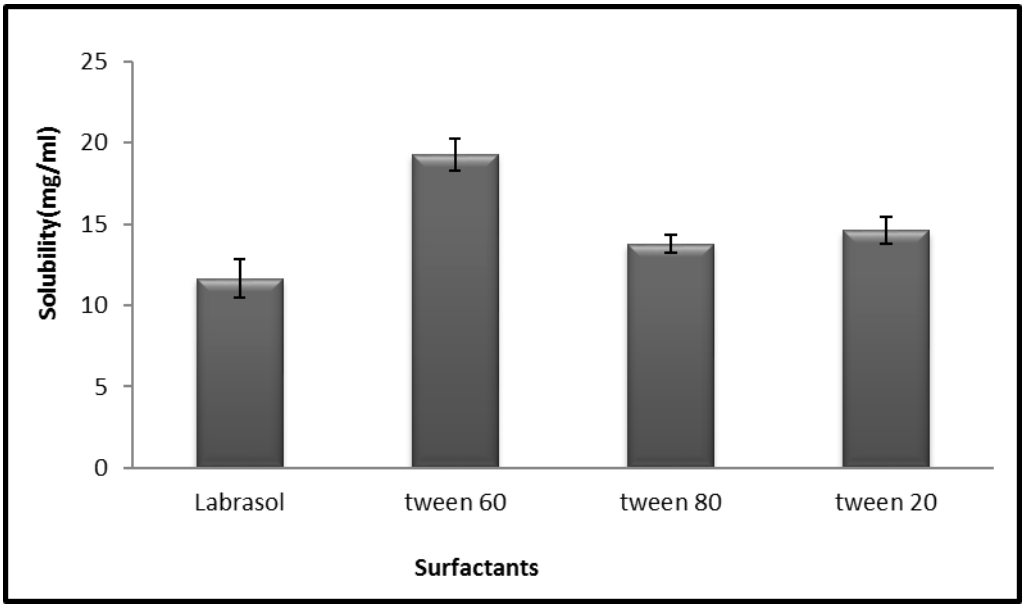


Figure 2: Solubility of mefenamic acid in various surfactants

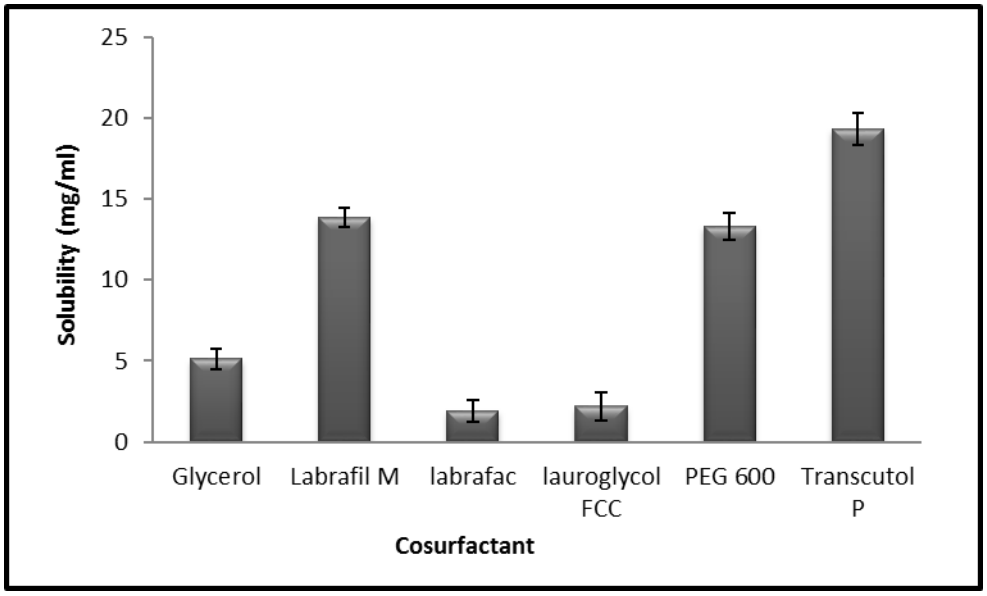


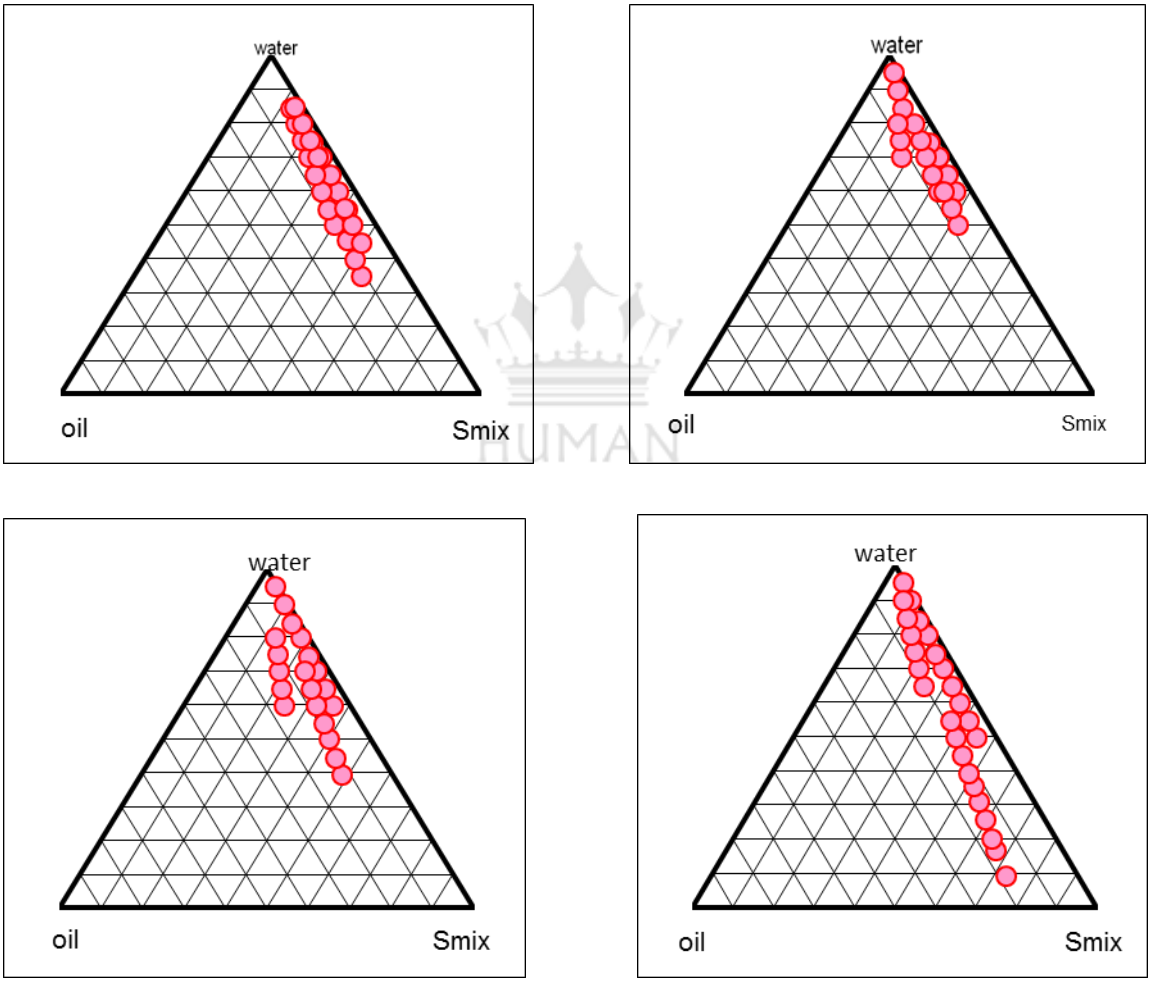
Figure 3: Solubility of mefenamic acid in various cosurfactants

On the basis of miscibility and solubility studies, Capryol 90: TPGS (1:1), Tween 60 and Transcutol P were selected as oil, surfactant, and cosurfactant respectively.

Spontaneous emulsification method

Phase behavior studies are essential for the study of surfactant systems and are performed by constructing phase diagrams that provide information on the boundaries of the different phases as a function of composition variables and temperatures, and, the more important structural organization can also be inferred. On the basis of solubility studies and miscibility studies, the mixture of 1:1 Capryol 90 and TPGS was selected as the oil phase, Tween 60 as a surfactant and Transcutol P as cosurfactant. For the construction of the existing zone of the nanoemulsion, pseudoternary phase diagrams were constructed using the aqueous phase titration method. For this, surfactant and cosurfactant (Smix) were mixed in different ratios (1:0, 1:1, 1:2, 1:3, 2:1, 3:1, 4:1). These Smix were mixed properly which were chosen in increasing concentration of surfactant to cosurfactant and vice versa for the formation of phase diagrams. For each Smix combination, oil:Smix ratios (1:9, 2:8, 3:7, 4:6, 1:2, 1:3, 1:3:5, 1:5, 1:6, 1:7, 1:8) were made in order to achieve maximum ratio which covered for the study of boundaries of phase formed precisely in the phase diagrams by using aqueous phase titration method. Slow aqueous titration was done for each weight ratio of oil and Smix under moderate stirring and visual observation was used for selection of transparent and easily flowable nanoemulsion[13]. The aqueous titration was continued until a clear, isotropic and

physically stable nanoemulsion with low viscosity was obtained. The volume of distilled water was then recorded. The samples were vigorously shaken by vortexing after each addition of titrating water and left for 24 h to attain equilibrium and to screen the metastable compositions. The physical state of nanoemulsion was marked on a pseudo-three-component phase diagram with one axis representing the aqueous phase, second representing the oil phase and third representing a mixture of surfactant and co-surfactant at a fixed weight ratio (Smix ratio). Pseudoternary phase diagrams were constructed using Capryol 90: TPGS (1:1), Tween 60, Transcutol P as oil, surfactant and co-surfactant respectively. Dotted area shows oil in water nanoemulsion region. The maximum nanoemulsion area was found in the Smix 3:1 ratio (Surfactant: Cosurfactant) as compared to rest Smix ratios are given in Figure 4.



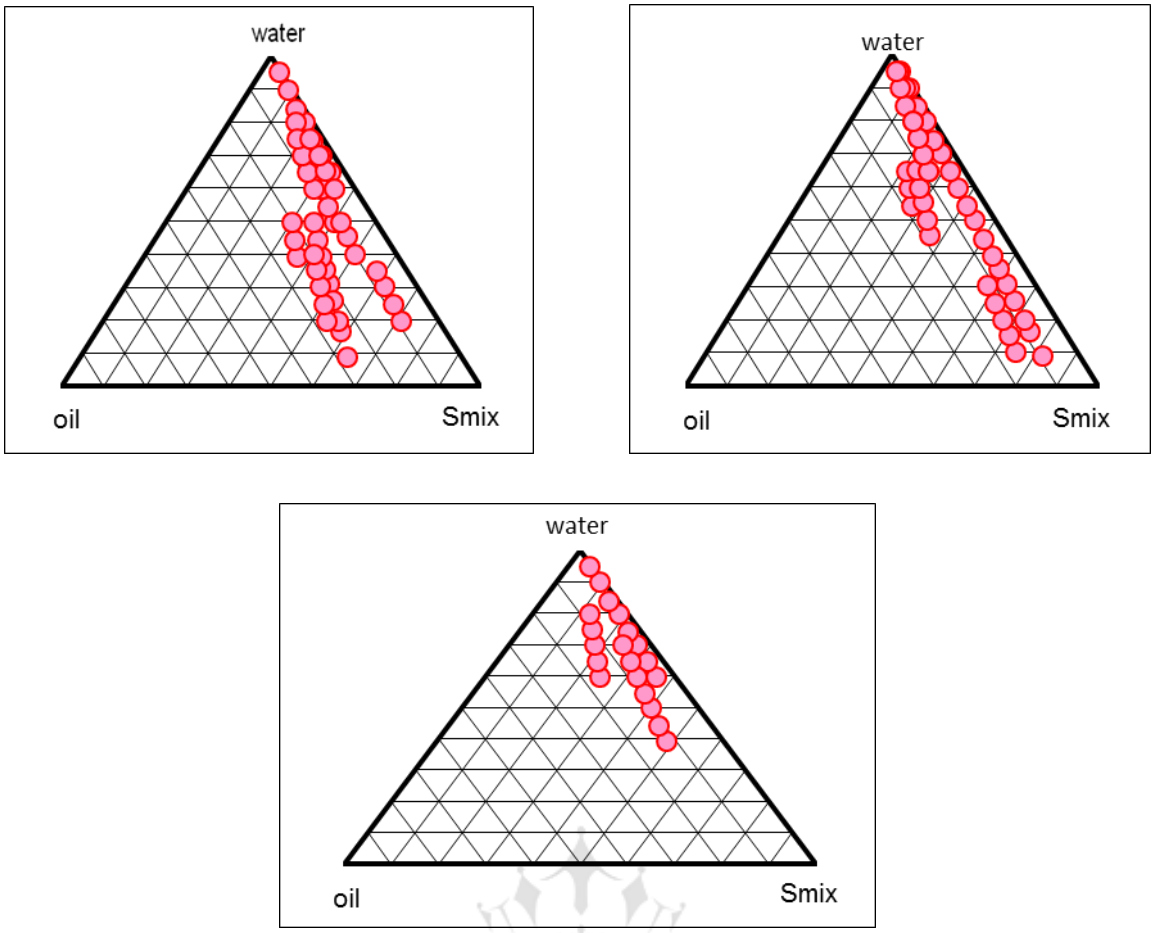


Figure 4: Pseudoternary phase diagram showing o/w nanoemulsion region for surfactant cosurfactant ratio of 1:0, 1:1, 1:2, 1:3, 2:1, 3:1 and 4:1 respectively

Method of preparation of nanoemulsions

From each pseudoternary phase diagram constructed, different placebo formulations were selected from nanoemulsion region so that drug could be incorporated in the oil phase. Only those formulations which had oil concentration that could dissolve dose of the drug had minimum surfactant concentration and water content was selected for further studies. The almost the entire range of nanoemulsion occurrence was covered. Nanoemulsions are thermodynamically stable systems and are formed at a particular concentration of oil, surfactant, and water with no phase separation, creaming or cracking. The various thermodynamic stability studies of placebo formulations of selected ratios (1:0, 1:1, 1:2, 2:1, 3:1 and 4:1) including heating-cooling cycle (to check stability of formulation at higher temperature), centrifugation (to check stability of formulation at low temperature) and freeze-thaw cycle stress tests (to check stability of formulation at higher shear) were done. During these studies, it was found that some formulations failed the test i.e. either they became

turbid and in some cases phase separation occurred. The reason may be a coalescence of oil droplets which may be due to due to Ostwald ripening. Another reason may be temperature quenching which leads to phase separation[32]. The formulations which passed the thermodynamic stability studies (Table 2) were further selected for drug loading in the nanoemulsion.

Table 2: The composition of placebo formulations which passed the thermodynamic stability studies

S. No.	Formulation Code	Oil (%v/v)	Smix (%v/v)	Water (%v/v)	Thermodynamic stability parameters	Inference
1	C1	5.00	45.00	50.00	✓	Pass
2	C4	6.25	43.75	50.00	✓	Pass
3	J1	0.48	4.29	95.24	✓	Pass
4	M2	9.09	36.36	54.55	✓	Pass
5	M5	8.33	41.67	50.00	✓	Pass
6	M6	7.14	42.56	50.00	✓	Pass
7	D1	5.00	45.00	50.00	✓	Pass
8	D2	10.00	40.00	50.00	✓	Pass
9	D4	22.22	33.33	44.44	✓	Pass
10	D5	16.67	33.33	50.00	✓	Pass
11	D8	8.33	41.67	50.00	✓	Pass
12	D10	6.25	43.75	50.00	✓	Pass
13	D11	5.00	40.00	55.00	✓	Pass
14	G4	14.81	29.63	55.56	✓	Pass
15	G5	13.70	41.10	45.21	✓	Pass
16	G6	11.11	38.89	50.00	✓	Pass
17	G9	5.56	44.44	50.00	✓	Pass

Formulation of drug loaded nanoemulsions

The Capryol 90: TPGS (1:1) was selected as the oil phase because of the maximum solubility of mefenamic acid in it. Tween 60 and Transcutol P were selected as Smix. This mixture was then titrated with distilled water. The aqueous titration method was employed for the formation of stable nanoemulsions and then subjected to physical stability studies. Some of the formulations failed the physical tests after loading of mefenamic acid and were discarded. The reason for this could be that there might have been the interaction of components of nanoemulsion with the drug molecules which led to solubility problems. The composition of stable drug loaded nanoemulsions after passing the thermodynamic stability studies is given in Table 3.

Table 3: Composition of stable drug loaded nanoemulsion formulations which passed the thermodynamic stability studies

Formulation Code	Capryol90: TPGS (ml)	Smix (ml)	Distilled water (ml)	Drug (mg)	A volume of nanoemulsion (ml)
M5	0.833	4.167	5	100	10
D1	0.5	4.5	5	100	10
G6	1.111	3.889	5	100	10

**Smix : Surfactant and Cosurfactant mixture of Tween 60 and Transcutol P*

Characterization of prepared nanoemulsion formulations

The prepared nanoemulsions were evaluated for its viscosity, pH and drug content. The viscosity studies are necessary for nanoemulsion to characterize the system physically and to control its physical stability. It was concluded that the viscosity of D1 formulation was optimum as given in Table 4. The optimum viscosity of topical nanoemulsion helps in its easy application on the skin. Determination of pH is important for formulating topical preparations as too high or low pH results in undesirable effects leads to skin irritancy. The pH of the nanoemulsion formulations was found in the range of 5-6, similar to the pH of the skin (Table 4). The topical preparation should have a pH similar to that of the skin to avoid irritation. The drug content was used to determine whether the drug is homogeneously dispersed in the throughout nanoemulsion. The D1 formulation showed the highest % drug content as compared to other formulations as given in Table 4.

Table 4: Evaluation parameters of prepared nanoemulsion formulations

S No.	Formulation Code	pH ± SD (n=3)	Viscosity (cP)±SD (n=3)	% Drug content ± SD (n=3)
1	M5	5.416 ± 0.518	26 ± 1.732	96.80 ± 0.012
2	D1	5.826 ± 0.519	21.66 ± 2.886	99.30 ± 0.019
3	G6	5.000 ± 0.707	30.66 ± 1.154	95.50 ± 0.013

**SD: Standard deviation*

***In vitro* release profile of nanoemulsions by using dialysis membrane**

The *in vitro* release studies were performed to compare the release of prepared mefenamic acid loaded nanoemulsion formulations (M5, D1, and G6) with mefenamic acid suspension. After performing *in vitro* release studies, it was found that formulation D1 showed best release characteristics as shown in Figure 5. It exhibited an *in vitro* release of 89.98%, which was significantly higher ($p \leq 0.05$) as compared to 72.48%, 63.70% and 30.59% for formulation M5, G6, and drug suspension respectively.

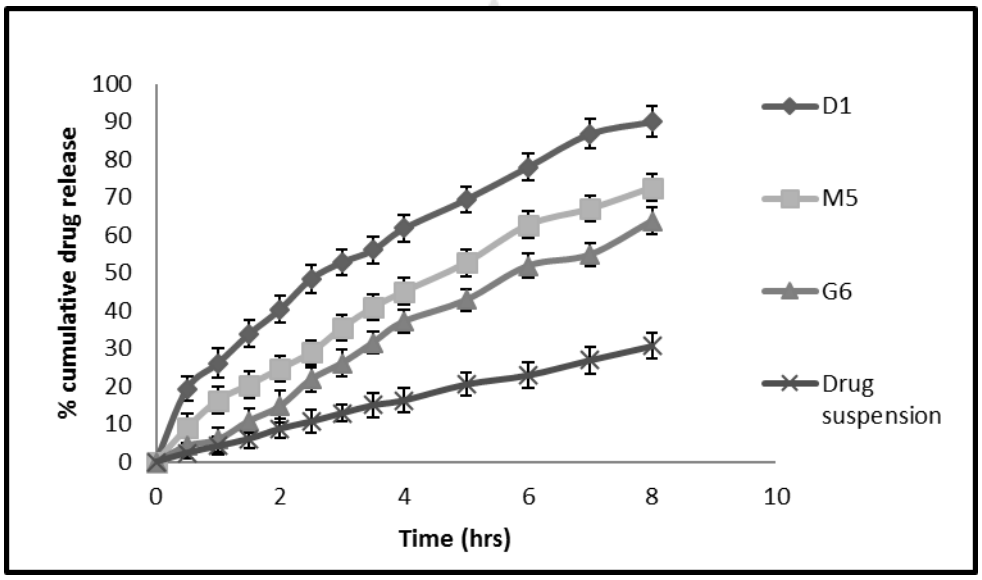


Figure 5: Comparison of *in vitro* release profile of selected nanoemulsion formulations with a drug suspension

On the basis of results of viscosity, pH, drug content and *in vitro* release studies, formulation D1 was selected as optimized formulation and was evaluated for assessment of release profile, *in vitro* skin permeation and drug retention study, particle size distribution and polydispersity index, zeta potential and transmission electron microscopy.

In vitro skin permeation study and drug retention study of optimized formulation using excised rat skin

The *in vitro* skin permeation of mefenamic acid from the optimized formulation (D1) was determined by using Franz diffusion cell as compared to the drug suspension. It was determined by calculating the cumulative amount of drug permeated through the skin. The flux and permeability of D1 formulation and drug suspension were also calculated. Figure 6 depicts the maximum release of mefenamic acid (77.30%) for the formulation D1 which was significantly higher ($p \leq 0.05$) than the mefenamic acid suspension (24.78%) after 8 h study. The flux (872.9µg/cm^2/h) and permeability coefficient (8.72×10^{-2}) of D1 nanoemulsion were significantly higher as compared to drug suspension which exhibits 305 µg/cm^2/h flux and 3.05×10^{-2} permeability coefficient which is given in Table 5.

Table 5: Determination of flux (J_{ss}) and permeability coefficient (k_p) of the optimized nanoemulsion (D1) and drug suspension

S No.	Formulations	Flux (µg/cm^2/h) ± SD (n=3)	Permeability coefficient (k_p) ± SD (n=3)
1	Nanoemulsion (D1)	872.9 ± 0.124	8.72×10^{-2} ±0.12
2	Drug suspension	305 ± 0.130	3.05×10^{-2} ±0.15

**SD: Standard deviation*

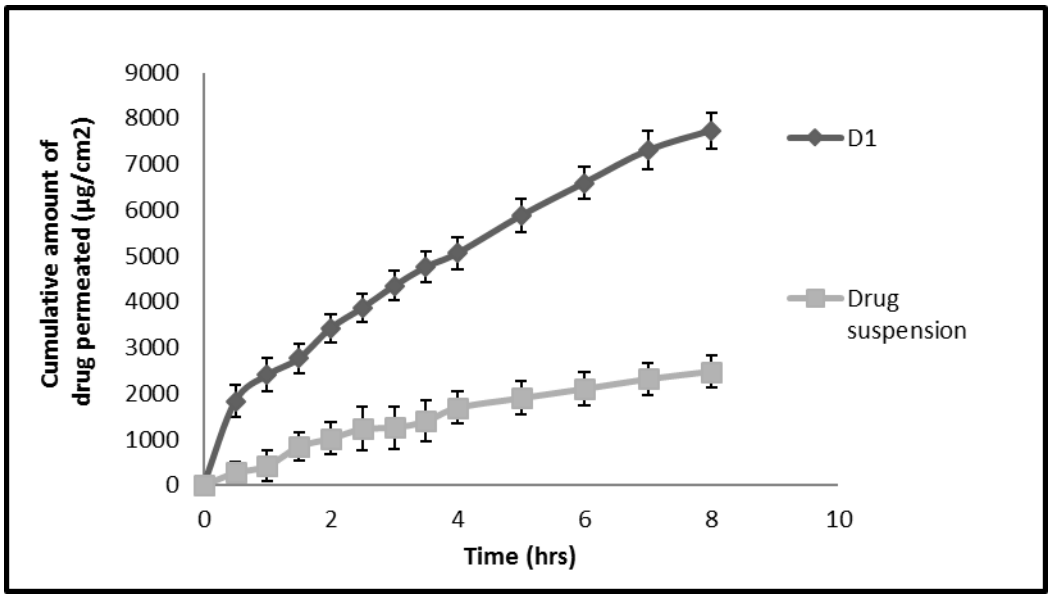


Figure 6: Cumulative amount of drug permeated versus time for optimized nanoemulsion formulation (D1) and drug suspension

Determination of drug retention in skin layers

The amount of drug retained in the skin was determined by drug retention study. Figure 7 and Figure 8 showed that more amount of mefenamic acid was localized into the skin layers after application of nanoemulgel formulation as compared to the drug suspension. More skin retention in case of nanoemulsion formulation (D1) was due to the greater extraction efficiency of skin lipids[33] as reported by Hussain *et al*.

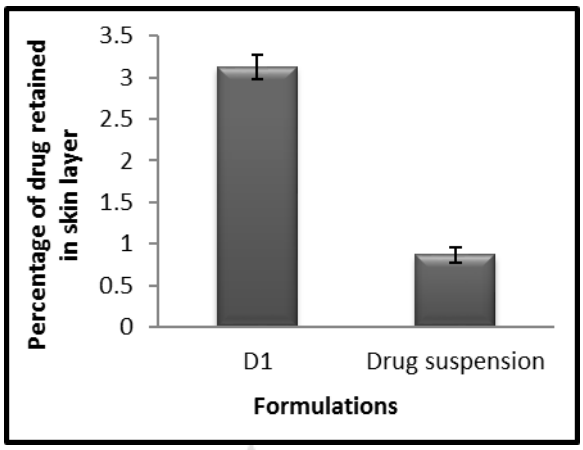


Figure 7: Comparison of formulations for drug retention in skin layers

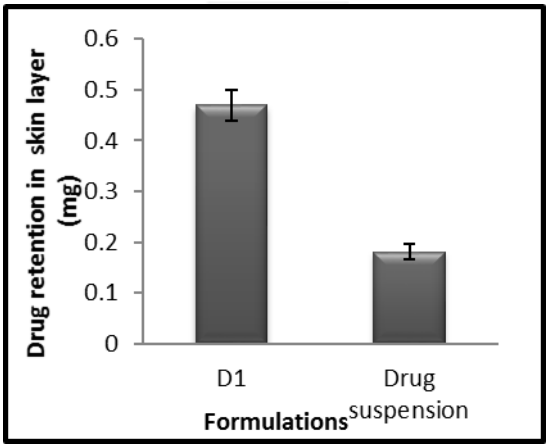


Figure 8: Comparison of formulations for percentage drug retained in skin layers

Particle size and polydispersity index

Average particle size (z-average) and polydispersity index (PDI) of the optimized nanoemulsion were determined and it is given in Figure 9. The low polydispersity index values (<1) indicated uniformity of droplet size and narrow size distribution of particles within the formulation[34].

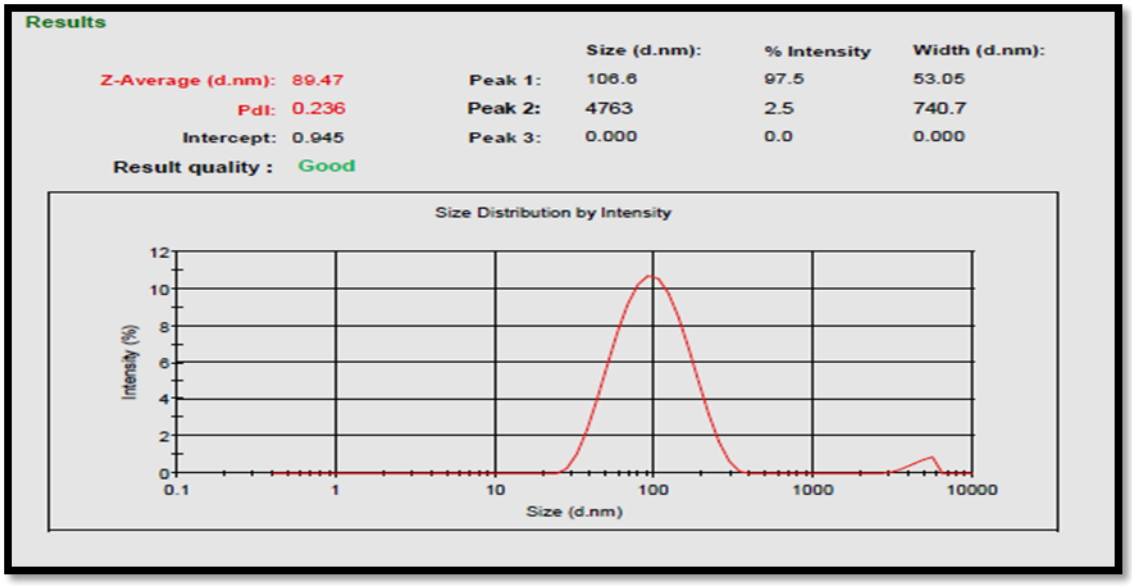


Figure 9: Average particle size and a polydispersity index of selected nanoemulsion formulation D1

Determination of zeta potential

The zeta potential of an optimized formulation (D1) was found to be -11.6, which indicated the stability of the nanoemulsion, as it was in the range of -30mV to +30mV that determines the stability of nanoemulsion due to the surface charges on the particles. Figure 10 shows the graph of the zeta potential of optimized formulation (D1).

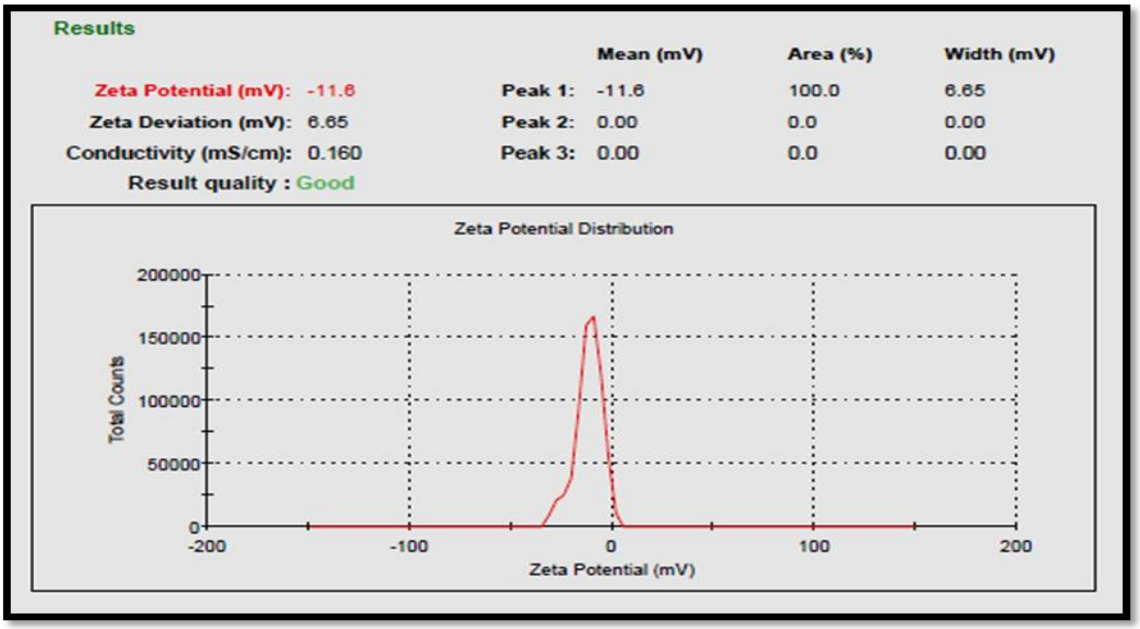


Figure 10: Graph of the zeta potential of the formulation (D1)

Transmission Electron Microscopy (TEM)

The morphology and size of the particle of nanoemulsion was determined by TEM micrograph. It showed that all the particles were spherical in shape and were less than 100 nm. Formulations having a particle size between 1-100 nm are considered as good nanoformulations. Figure 11 showed the transmission electron microscopic image of optimized nanoemulsion formulation (D1).

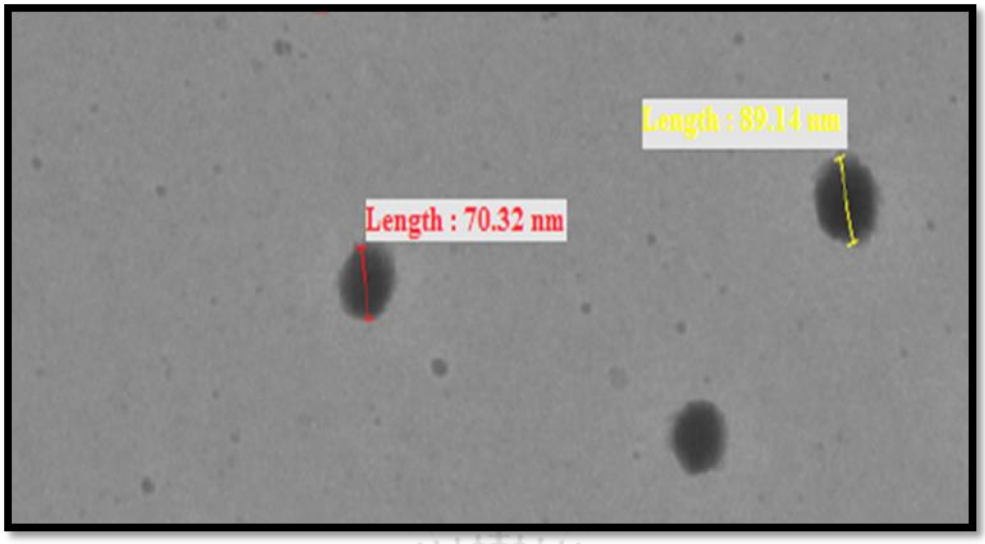


Figure 11: Transmission electron microscopic image of optimized nanoemulsion formulation (D1)

Figure 12 and Figure 13 depict stable nanoemulsion formulation (D1) and drug suspension respectively.

Figure 12: Stable drug loaded nanoemulsion formulation (D1)

Figure13: Mefenamic acid suspension

Formulation and evaluation of topical drug loaded nanoemulgels

The very low viscosity often exhibited by nanoemulsion is inappropriate for topical use[24]. Therefore, the nanoemulsion-based gel was formulated by incorporating appropriate gelling agent.

a) Selection of a gelling agent

Various polymers (carbopol 971, carbopol 940, HPMC 5-CPS, sodium alginate) of concentration 1%w/v were initially used for the preparation of nanoemulgels are given in Table 6. The nanoemulgels were formulated and checked for their physical appearance and the results are given in Table 6a.

Table 6: List of different polymers used for gel formulation

S No.	Polymers (gelling agent)	Concentration (w/v)	Observation
1	Carbopol 940	1%	The very clear and stable formulation
2	Carbopol 971	1%	The translucent but stable formulation
3	Hydroxypropylmethylcellulose (HPMC) 5-CPS	1%	Phase separation
4	Sodium alginate	1%	Grittiness and phase separation

On the basis of the above results, carbopol 940 was found to be most suitable for preparation of nanoemulgel formulation.

Table 6a: Physical characterization of nanoemulgels $D1_{(0.5\%w/v)}$, $D1_{(0.8\%w/v)}$ and $D1_{(1\%w/v)}$

Sr. No.	Property	$D1_{(0.5\%w/v)}$, Containing 0.5% Carbopol 940	$D1_{(0.8\%w/v)}$, Containing 0.8% Carbopol 940	$D1_{(1\%w/v)}$, Containing 1% Carbopol 940
1	Colour	Colorless	Colorless	Colorless
2	Appearance	Transparent	Transparent	Transparent
3	Odor	Odorless	Odorless	Odorless
4	After feel	Emollient	Emollient	Emollient
5	Type of Smear	Non-greasy	Non-greasy	Non-greasy
6	Removal	Easy	Easy	Easy
7	Phase Separation	Yes	No	No
8	Consistency	+	++	+++

(+) = Low (++) = Medium (+++) = Good

b) Optimization of concentration of selected gelling agent (Carbopol 940)

Nanoemulgels formulated using three different concentrations (w/v) of carbopol 940 (0.5%w/v, 0.8%w/v and 1%w/v) were used and subjected to various evaluation parameters.

Evaluation of topical drug loaded nanoemulgels containing different concentrations of selected gelling agent

Based on the physical characterization of nanoemulgels, $D1_{(1\%w/v)}$ exhibited desired consistency. The viscosities of prepared topical nanoemulgels formulations ($D1_{(0.5\%w/v)}$, $D1_{(0.8\%w/v)}$ and $D1_{(1\%w/v)}$) were determined by using Brookfield Viscometer. The $D1_{(1\%w/v)}$) showed good viscosity as compare to other nanomeulgel preparations. This leads to a better topical application to the skin. Determination of pH is important for formulating topical preparations as too high or low pH results in undesirable effects leads to skin irritancy. pH of the prepared nanoemulgels formulations was determined by using digital pH meter at room temperature. The pH value for all three nanoemulgel formulations was in favorable range for the topical application. The spreadability of the selected formulations was determined. $D1_{(1\%w/v)}$ nanoemulgel was found to have satisfactory spreadability which would lead to the easy application of gel on the skin. Also, the homogeneity and grittiness of the selected formulations were determined. It was found that there were no coarse particles present in all the nanoemulgels formulated and hence the prepared topical nanoemulgels were homogeneous. The results of the different parameters are given in Table 7.

Drug content and content uniformity

The drug content and content uniformity of the selected formulations were determined. There was no significant difference observed in the % drug content obtained after analyzing samples taken from different parts of the gels. It was concluded that the method used to disperse nanoemulsion in the gel base was satisfactory. The results also confirmed the uniformity of drug in the gels and results are given in Table 7.

Table 7: Evaluation parameters of prepared topical nanoemulgel formulations

Sr. No.	Formulation Code	pH ± SD (n=3)	Viscosity (cP)±SD (n=3)	Spreadability (gcm/sec) ± SD (n=3)	% Drug content ± SD (n=3)
1	$D1_{(0.5\%w/v)}$	5.626 ± 0.519	2251 ± 9.84	120.32 ± 0.21	92.50 ± 0.012
2	$D1_{(0.8\%w/v)}$	5.416 ± 0.518	2419 ± 0.06	123.55 ± 0.39	95.70 ± 0.019
3	$D1_{(1\%w/v)}$	5.521 ± 0.707	2440 ± 4.58	124.71 ± 0.11	99.70 ±0.013

**SD: Standard deviation*

***In vitro* skin permeation and retention study of different nanoemulgels using excised rat skin**

The *in vitro skin* permeation of mefenamic acid from the drug-loaded nanoemulgel formulations $D1_{(0.8\%w/v)}$, $D1_{(0.5\%w/v)}$ and $D1_{(1\%w/v)}$, was determined by using Franz diffusion cell as compared to drug suspension gel. It was determined by calculating the cumulative amount of drug permeated through the skin. The flux and permeability of $D1_{(0.8\%w/v)}$ nanoemulgel formulation and drug suspension gel were also calculated. On the basis of *in vitro* skin permeation results of various nanoemulgel formulations ($D1_{(0.5\%w/v)}$, $D1_{(0.8\%w/v)}$ and $D1_{(1\%w/v)}$), it was observed that as the concentration of gel increased from 0.5% to 1%, its rate of permeation through skin was decreased. The % Cumulative amount of drug permeated from ($D1_{(0.5\%w/v)}$, $D1_{(0.8\%w/v)}$ and $D1_{(1\%w/v)}$) was found to be 58.75%, 53.61% and 46.28% respectively as compared to drug suspension gel (18.99%). For effective topical delivery, the drug should not reach systemic circulation. Therefore, formulation $D1_{(1\%w/v)}$, which exhibit the least permeation, was chosen as the optimized nanoemulgel. Figure 14 shows the Comparison of *in vitro* skin permeation study of mefenamic acid nanoemulgel prepared with different carbopol 940 concentrations ($D1_{(0.5\%w/v)}$, $D1_{(0.8\%w/v)}$ and $D1_{(1\%w/v)}$) with drug suspension gel using excised rat skin.

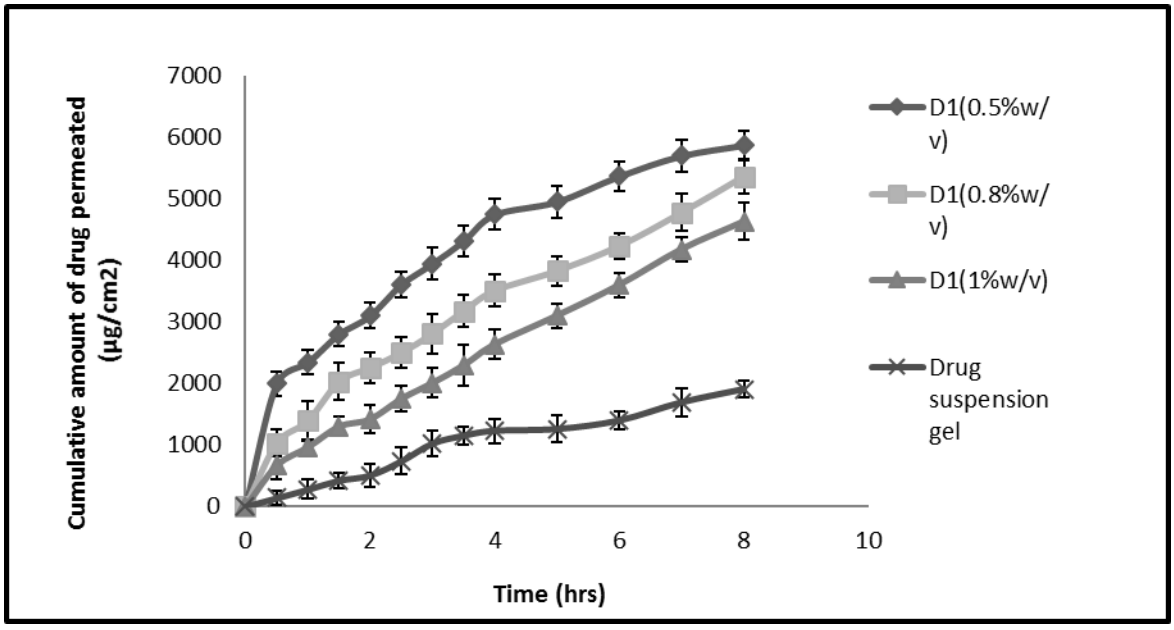


Figure 14: Comparison of *in vitro* skin permeation study of mefenamic acid nanoemulgel prepared with different carbopol 940 concentrations ($D1_{(0.5\%w/v)}$, $D1_{(0.8\%w/v)}$ and $D1_{(1\%w/v)}$) with drug suspension gel using excised rat skin

The flux was found to be significant higher ($p \leq 0.05$) in case of $D1_{(1\%w/v)}$ nanoemulgel (549.6µg/cm^2/h) as compared to $D1_{(0.5\%w/v)}$ nanoemulgel (531.6µg/cm^2/h), $D1_{(0.8\%w/v)}$ nanoemulgel (545.1µg/cm^2/h) and drug suspension gel (234.6 µg/cm^2/h) as shown in Table 8. Less permeation of the drug from $D1_{(1\%w/v)}$ nanoemulgel could be due to the greater retention of mefenamic acid nanoemulgel into skin layers and high viscosity[33] of the gel as reported by Hussain *et al*.

Table 8: Determination of flux (J_{ss}) and permeability coefficient (k_p) of prepared nanoemulgels ($D1_{(0.5\%w/v)}$, $D1_{(0.8\%w/v)}$ and $D1_{(1\%w/v)}$) and drug suspension gel

S. No.	Formulation Code	Flux(µg/cm^2/h) ± SD (n=3)	Permeability coefficient (k_p) ± SD (n=3)
1	$D1_{(0.5\%w/v)}$	531.6±0.125	5.31×10^{-2} ± 0.10
2	$D1_{(0.8\%w/v)}$	545.1±0.122	5.45×10^{-2} ± 0.12
3	$D1_{(1\%w/v)}$	549.6±0.131	5.49×10^{-2} ± 0.11
4	Drug Suspension gel	234.6±0.121	2.34×10^{-2} ± 0.13

**SD: Standard deviation*

Determination of drug retention in skin layers

The determination of drug retention (mg) and % drug retained in the skin were determined from the treated skin samples. From the results of drug retention study, it was concluded that a significantly higher ($p \leq 0.05$) drug retention (20.84%) was observed for nanoemulgel $D1_{(1\%w/v)}$ as compared to $D1_{(0.5\%w/v)}$ (8%) and $D1_{(0.8\%w/v)}$ (14.30%) and drug suspension gel (1.56%). The results are given in Figure 15 and Figure 16.

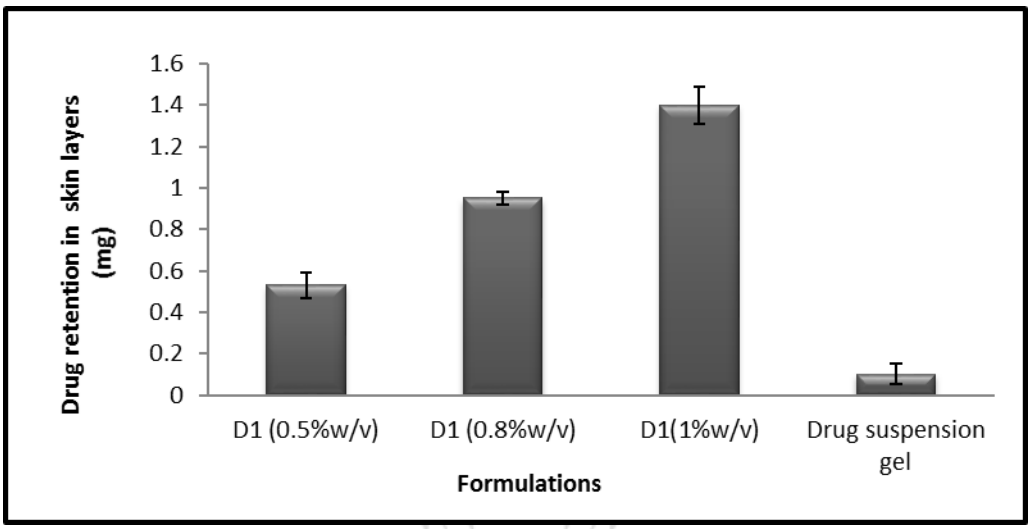


Figure 15: Comparison of formulations for drug retention in skin layers

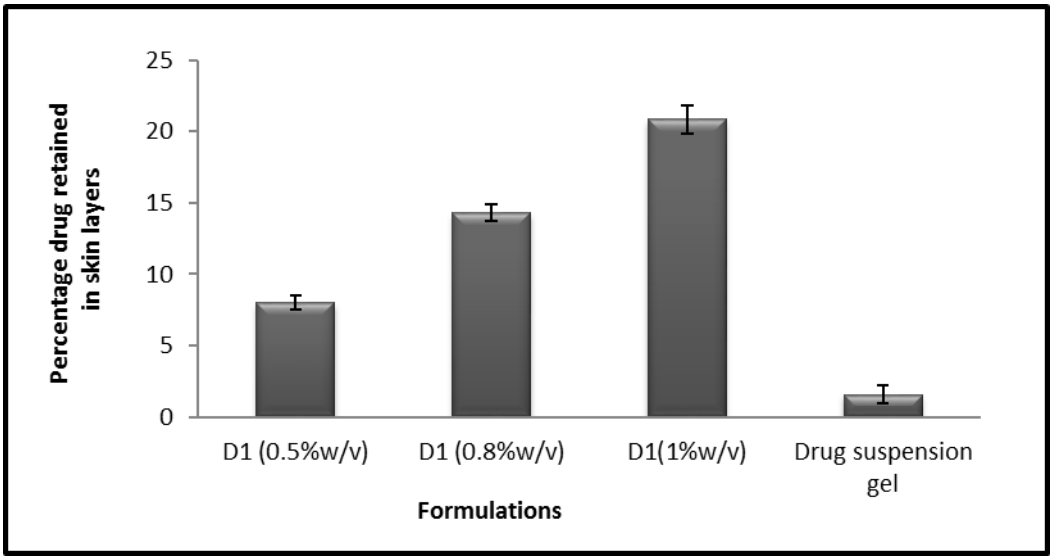


Figure 16: Comparison of formulations for percentage drug retained in skin layers

Based on the results of viscosity, pH, spreadability, homogeneity, drug content, *in vitro* skin permeation and drug deposition studies, nanoemulgel formulation $D1_{(1\%w/v)}$, was chosen as

the optimized formulation among those evaluated. Figure 17 depict the optimized nanoemulgel of mefenamic acid D1.

Figure 17: Optimized nanoemulgel of mefenamic acid D1

Antioxidant study

The reducing power capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The reducing ability of a compound generally depends on the presence of reductants, which exhibit antioxidative potential by breaking the free radical chain and donating a hydrogen atom. In this assay, the presence of reductant (i.e antioxidants) in the formulation caused the reduction of the Fe^{3+} ferricyanide complex to the ferrous form. Therefore the Fe^{2+} was monitored by measuring the formation of Perl's Prussian blue at 700 nm[13].

The results are shown in Figure 18. Based on the results, it was concluded that the reducing power of nanoemulgel formulation was in a concentration-dependent manner. At all concentrations, the reductive capability of the nanoemulgel formulation was similar to ascorbic acid. The nanoemulgel formulation showed more reducing power ability than the pure drug. It might be due to the effect of TPGS (having inherent antioxidant activity)[13].

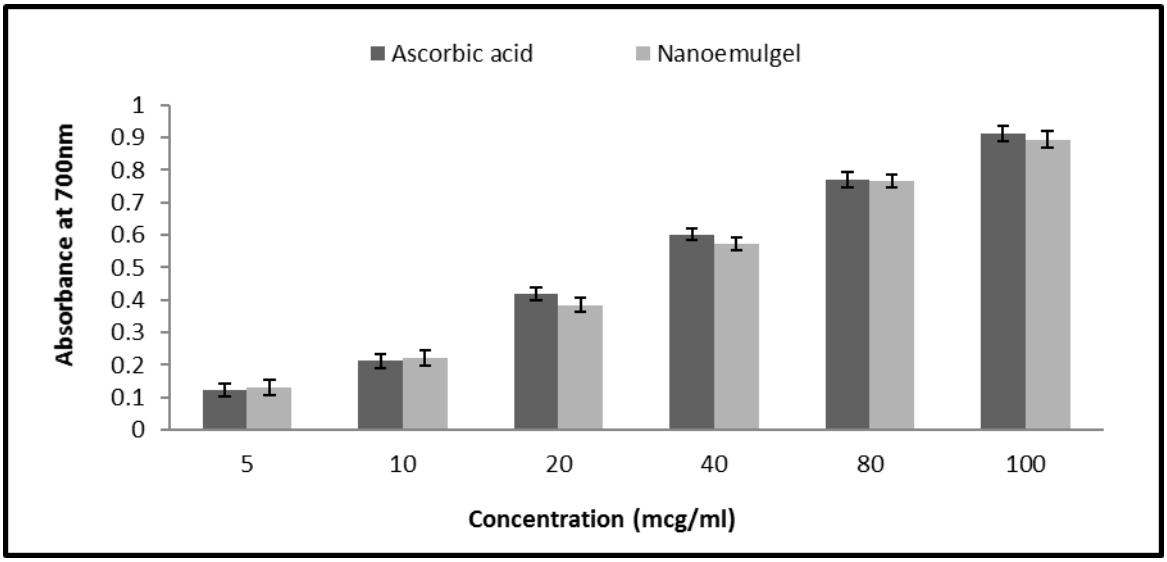


Figure 18: Reductive capabilities of Ascorbic acid and Optimized nanoemulgel formulation

These results suggested that nanoemulgel formulation had a remarkable potency to donate an electron to reactive free radicals converting them into more stable non reacting species and terminating the free radical chain reaction.

Therefore, the established antioxidant property of the formulation would be further useful in the treatment of rheumatoid arthritis because as per reports, a release of active O_2 species from neutrophils leads to extensive cell damage and necrosis[35].

***In vivo* studies**

Skin Irritation Study

Skin Irritation study was performed to check whether the drug-loaded formulation had an irritant effect on the skin. Then the histopathology of treated skin samples was done and it was found that after the 7 days application of nanoemulgel formulation, no erythema was found on the skin sample by comparing it with control and a plain drug suspension. Hence, the nanoemulgel formulation didn't produce any dermatological reaction and results in the good topical formulation. The results are given in Figure 19 to Figure 21.

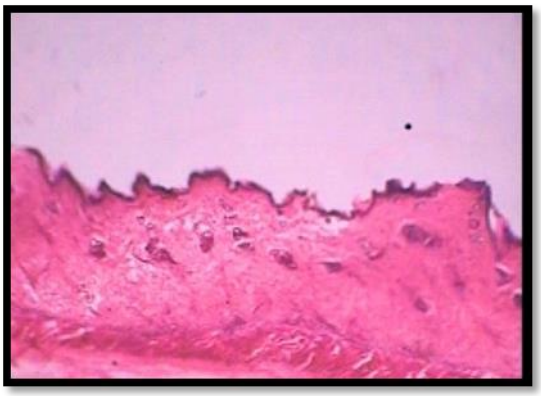


Figure 19: Histopathological evaluation of rat skin sample of control group animal

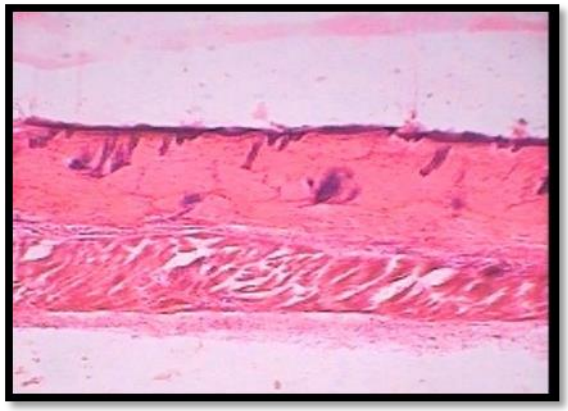


Figure 20: Histopathological evaluation of rat skin sample of nanoemulgel formulation

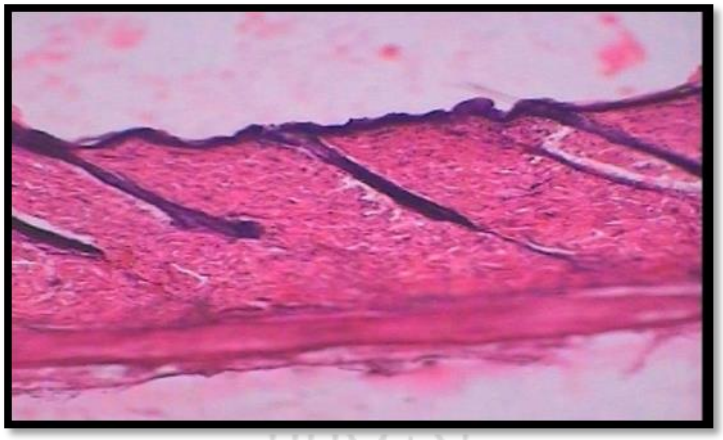


Figure 21: Histopathological evaluation of rat skin the sample of the plain drug suspension

Pharmacodynamic studies

a) Anti-inflammatory study

The anti-inflammatory action of various formulations was determined by using Plethysmograph after injecting subplantar injection of 0.1 ml of carrageenan solution (1% w/v) to the hind paws of the rats. A significant reduction ($p \leq 0.05$) in mean % edema was obtained in group III as compared to group II. The nanoemulgel formulation exhibited a significant reduction ($p \leq 0.05$) in mean % edema as compared to group II, III and V which is given in Figure 22. The mean % edema observed for group III administered with nanoemulgel was 2%, as compared to 38%, 22% and 6% observed in group Group II, III, and V respectively.

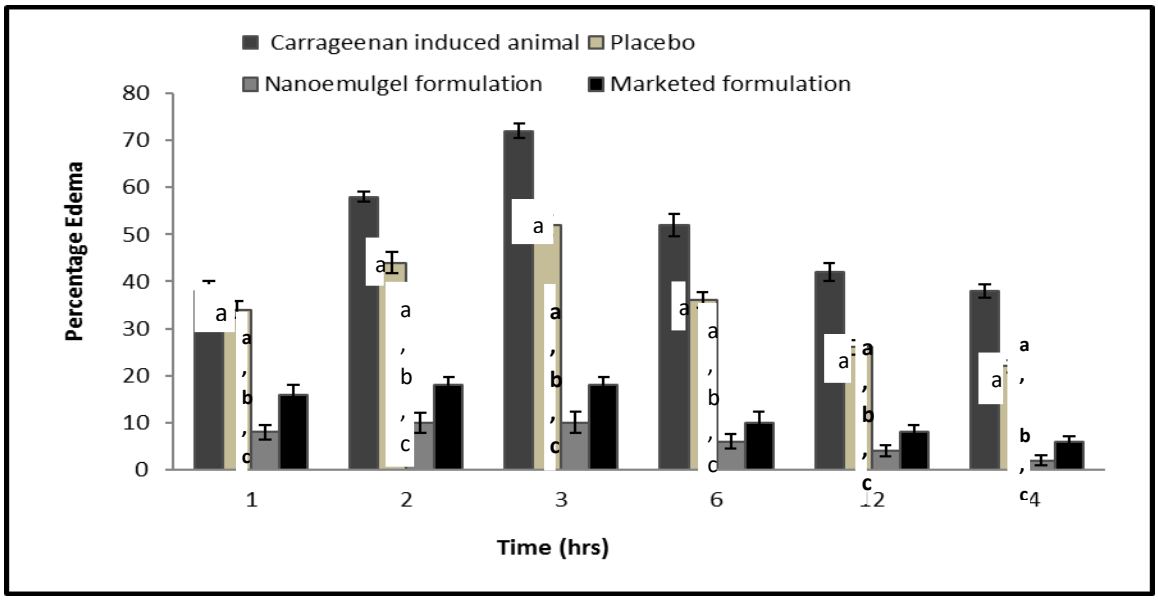


Figure 22: Effect of various formulations on mean % edema of rats (n=6) for 24 hrs. The values are expressed as mean ± SD ([a]p≤0.05 as compared to carrageenan control group; [b]p≤0.05, as compared to placebo formulation; [c]p≤0.05, as compared to marketed formulation)

In Figure 23, also the percent inhibition in edema in case of the nanoemulgel formulation was significantly higher (p≤0.05) (94.73%) as compared to marketed formulation (84.21%) and placebo formulation (42.10%) at the end of 24 hrs. So, from the above results, it was concluded that the mefenamic acid loaded nanoemulgel formulation showed excellent anti-inflammatory activity.

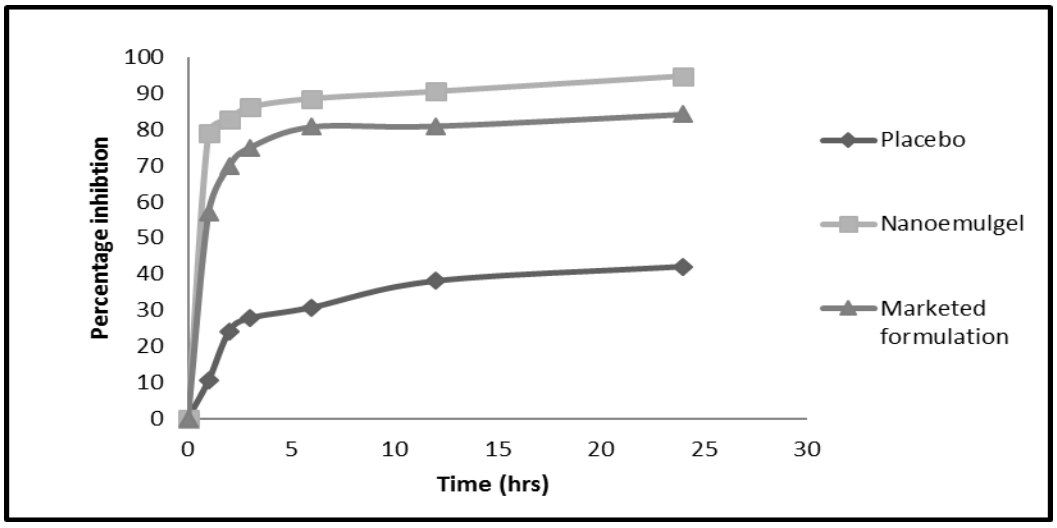


Figure 23: Effect of various formulations on % inhibition v/s time

b) Analgesic Study

The eddy's hot plate method was used to determine the analgesic effect of various formulations after placing the treated rats on the hot plate at the time interval of 3 hrs at the temperature of 53 ± 2ºC. The Figure 24 concluded that there was the significant increase in percentage retention time ($p \leq 0.05$) in the nanoemulgel formulation (92.91%) as compared to placebo (35.69%) and marketed formulation (80.58%) after 3 hours of study. It was concluded that nanoemulgel formulation exhibit excellent analgesic activity as compared to other formulations**.**

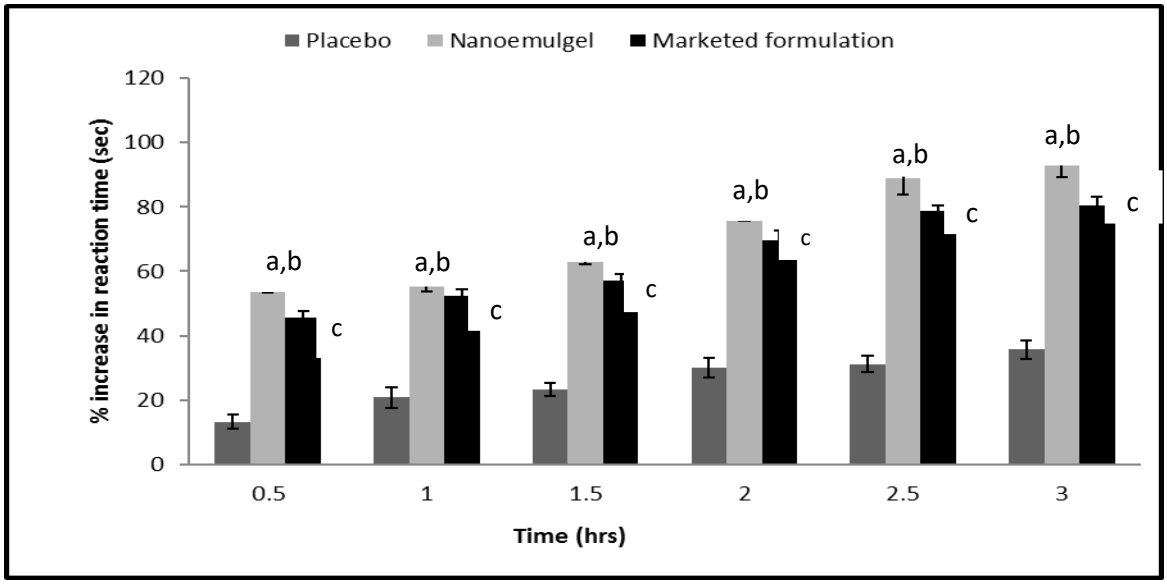


Figure 24: Effect of various formulations on % increase in retention time. The values are expressed as mean ± SD ([a]p≤0.05, as compared to placebo; [b]p≤0.05, as compared to Marketed formulation; [c]p≤0.05, compared to placebo formulation)

Stability Study of the optimized nanoemulgel formulation by HPLC method as per ICH guidelines

The stability studies of the optimized nanoemulgel formulation as per ICH guidelines were analyzed for pH, viscosity and drug content by using the HPLC method. Figure 25 concluded that no statistically significant ($p \leq 0.05$) difference was observed in viscosity, pH and drug content of the nanoemulgel formulation kept for stability studies over a period of 3 months. From the plot between log % drug remaining and time, it was found that only 0.030% of the drug degraded in 90 days (3 months).

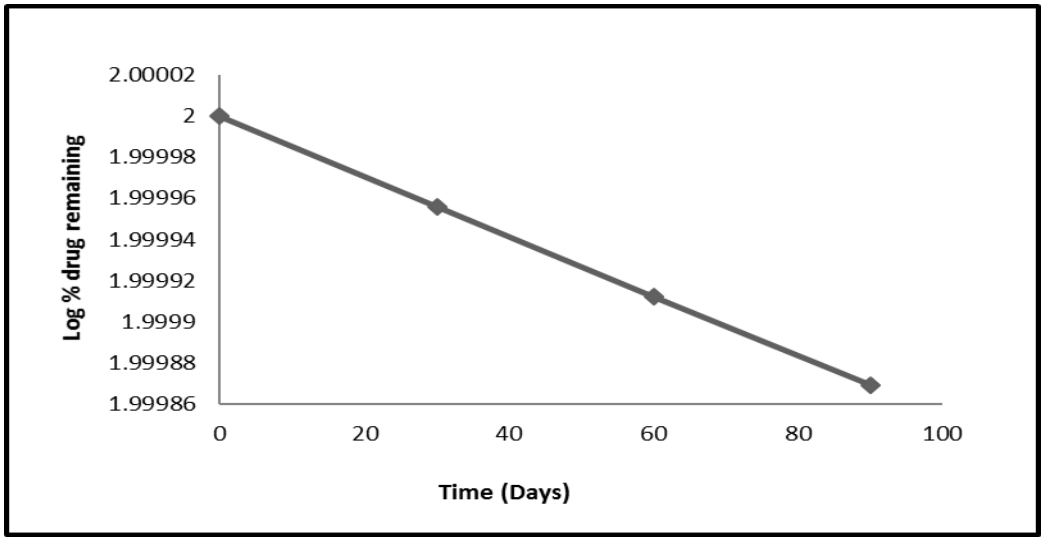


Figure 25: Graph of Log % drug remaining v/s Time (Days) for an optimized nanoemulgel formulation

DISCUSSION

Formulation and evaluation of nanoemulsions

In an attempt to enhance solubility and penetration that will be effective in inflammatory disorder in patients suffering from rheumatoid arthritis, TPGS loaded topical nanoemulgel of mefenamic acid was formulated. Nanoemulgels have emerged as one of the most interesting topical delivery systems as it has a dual release control system i.e. nanoemulsion and gel. With the gelling agent, it promotes better stability of nanoemulsion by reducing the surface and interfacial tension and also enhancing the viscosity of the aqueous phase for drug administration topically when compared with conventional topical gels. Drug delivered through nanoemulgel has better adhesion on the surface on the surface of the skin and high solubilizing capacity which leads to larger concentration gradient towards the skin hence influences better skin penetration[22]. The aqueous phase titration method was used for the development of nanoemulsion formulations by using probe sonicator. The solubility of the drug in the oil phase is an important criterion for the selection of oils for the ability of nanoemulsion to maintain the drug in the solubilized form which is greatly influenced by the solubility of the drug in the oil phase[36]. Usually, the oil which has the maximum solubilizing potential for a selected drug candidate is selected as an oily phase for the formulation of nanoemulsions. This helps to achieve maximum drug loading in the nanoemulsions[37]. Surfactants are used for stabilizing the systems. Three types of surfactant are anionic,

cationic, and nonionic. Nonionic surfactants are relatively less toxic than their ionic counterparts and typically have lower critical micelle concentration. Therefore, proper selection of surfactants becomes a crucial factor. Hydrophilic surfactant and cosurfactant are considered to lower the interfacial tension and to lower the necessary energy to form the nanoemulsions, therefore improving the stability[37]. Cosurfactants are added to obtain nanoemulsion systems at low surfactant concentration. Based on the results of solubility and miscibility studies, Capryol 90: TPGS (1:1) was selected as an oil phase along with Tween 60 and Transcutol-P as surfactant and cosurfactant respectively for the development of topical nanoemulgel. Pseudoternary phase diagrams were constructed using Capryol 90: TPGS(1:1), Tween 60 and Transcutol-P as oil, surfactant, and cosurfactant respectively. The maximum nanoemulsion area was found in the Smix 3:1 ratio (surfactant: cosurfactant) as compared to rest Smix ratios. Selected placebo formulations were passed through various thermodynamic stability studies and those formulations which failed the tests were discarded. The drug was loaded in stable formulations. The drug-loaded stable formulations were subjected to characterization parameters (viscosity, pH, refractive index, drug content, zeta potential, transmission electron microscopy, particle size and polydispersity index and *in vitro* release studies using dialysis membrane).

Among the different drug-loaded formulations evaluated, formulation D1, which exhibited satisfactory polydispersity index, viscosity, refractive index, percentage transmittance and maximum *in vitro* release of D1 (89.98%), was chosen as the optimized formulation for further studies. The drug release from mefenamic acid suspension was found to be (30.59%). From the comparative *in vitro* skin permeation studies of D1 formulation and the mefenamic acid suspension was done by using excised rat skin. It was concluded that maximum permeation of D1 formulation (77.30%) was detected, which was significantly higher ($p \leq 0.05$) than mefenamic acid drug suspension (24.78%) after 8 hrs of the study period.

Formulation and evaluation of topical nanoemulgels

Then, D1 nanoemulsion formulation was changed to gel by simply adding three different concentrations of carbopol 940 i.e. 0.5%, 0.8% and 1% to produce formulations D1 $_{0.5\%w/v}$, D1 $_{0.8\%w/v}$ and D1$_{1\%w/v}$. The nanoemulgel formulations D1$_{0.5\%w/v}$, D1 $_{0.8\%w/v}$ and D1$_{1\%w/v}$ were characterized for various parameters like physical evaluation, drug content and content uniformity, viscosity, homogeneity and grittiness, pH, spreadability and *in vitro* skin permeation and drug retention study using excised rat skin.

From the results of *in vitro* skin permeation of various nanoemulgel formulations $D1_{0.5\%w/v}$, $D1_{0.8\%w/v}$ and $D1_{1\%w/v}$, it was concluded that as the concentration of gel increased from 0.5% to 1%, its rate of permeation through the skin was decreased. The percent cumulative amount of drug permeated from nanoemulgel formulations $D1_{0.5\%w/v}$, $D1_{0.8\%w/v}$ and $D1_{1\%w/v}$ was found to be 58.75%, 53.61%, and 46.28% respectively as compared to drug suspension gel (18.99%). For effective topical delivery, the drug should not reach systemic circulation. Therefore, formulation $D1_{1\%}$ which exhibited the least permeation was chosen the optimized nanoemulgel. The flux was found to be significantly higher ($p \leq 0.05$) in case of $D1_{1\%\ w/v}$ (549.6 µg/cm^2/h) as compared to other preparations, $D1_{0.5\%\ w/v}$ (531.6 µg/cm^2/h), $D1_{0.8\%\ w/v}$ (545.15 µg/cm^2/h) and drug suspension gel (234.6 µg/cm^2/h).

From the results of drug retention study, it was concluded that a significantly higher ($p \leq 0.05$) drug retention (20.84%) was observed in case of nanoemulgel $D1_{1\%\ w/v}$ as compared to $D1_{0.5\%\ w/v}$ (8%), $D1_{0.8\%\ w/v}$ (14.30%) and drug suspension gel (1.56%).

Antioxidant study

The normal white blood cell count in synovial fluid is less than 100 per milliliter, on average. In patients afflicted with osteoarthritis, this figure rises to approximately 800 per milliliter, and in those with rheumatoid arthritis, it is significantly higher. White blood cells are known to contribute to pain and inflammation via several mechanisms, one of which involves the conversion of arachidonic acid to inflammatory leukotrienes (LTB-s4). This reaction is catalyzed by the 5-lipoxygenase enzyme within leukocytes. Evidence suggests that free radicals generated by white blood cells within the synovial fluid serve to increase the rate of synthesis of certain inflammatory mediators (e.g., LTB-4, PG-2, interleukin-1, tissue necrosis factor-alpha). As such, investigators have recently explored the use of antioxidant vitamins and minerals as a possible means to reduce the pain and inflammatory events associated with rheumatoid arthritis[38]. Therefore, the TPGS loaded nanoemulgel formulation was developed which has a remarkable potency to donate an electron to reactive free radicals, converting them into more stable non-reactive species and terminating the free radical chain reaction. Therefore, exhibit good antioxidant activity.

***In vivo* studies**

In the skin irritation study, the nanoemulgel and drug suspension were applied on shaved skin for 7 days. From the histopathology study of skin samples, it was found that after the 7

days application of nanoemulgel formulation, no erythema was found on the skin sample by comparing it with control and a plain drug suspension. Therefore the nanoemulgel formulation didn't produce any dermatological reaction which results in a good topical formulation.

Pharmacodynamic study

Anti-inflammatory study

Rheumatoid arthritis (RA) is characterized by persistent synovial tissue inflammation and hyperplasia "swelling". Therefore the anti-inflammatory activity was conducted on rats by applying different formulations and it was measured by using Plethysmograph after administration of carrageenan solution 0.1 ml (1%w/v) through sub-plantar route. The results of the anti-inflammatory study confirmed, statistically significant ($p \leq 0.05$) inhibition in inflammation after application of nanoemulgel $D1_{1\%\ w/v}$ (94.73%), as compared to placebo (42.10%) and marketed gel (84.73%) after 24 hrs of the study period.

Analgesic study

In rheumatoid arthritis, patients also feel pain along with inflammation in the joints. Therefore, many analgesic agents are employed in order to reduce the pain in the joints. Therefore mefenamic acid loaded topical nanoemulgel was applied to reduce the pain. The analgesic activity was measured by using Eddy's hot plate method. it was concluded that there was a significant increase in percentage retention time ($p \leq 0.05$) in the nanoemulgel formulation (92.91%) as compared to placebo (35.69%) and marketed formulation (80.58%) after 3 hours of study. Therefore, nanoemulgel formulation exhibits excellent analgesic activity as compared to other formulations**.**

Stability studies

The stability studies were performed by employing HPLC method as per ICH guidelines. The topical nanoemulgel shows no statistically significant ($p \leq 0.05$) difference in its viscosity, pH and drug content over a period of 3 months. From the plot between log % drug remaining and time, it was found that only 0.030% of the drug degraded in 90 days (3 months).

CONCLUSION

In conclusion, topical nanoemulgel of mefenamic acid was developed to a satisfactory level in terms of release of drug from it, optimum globule size, minimum polydispersity index, optimum viscosity, lower surfactant concentration, higher penetration, and better skin retention. The present study endorsed topical nanoemulgel of mefenamic acid to be a promising choice over conventional topical formulations for the treatment of rheumatoid arthritis.

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