Research Article

Development and Evaluation of Nanoemulsion gel for Transdermal Delivery of Ketoprofen

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ABSTRACT

To enhance the solubility and permeability of poorly water soluble ketoprofen, nanoemulsion gel was formulated for the treatment of rheumatoid arthritis. Among the oils, surfactants and co-surfactants CAPTEX 200, tween 80 and PEG 400 were selected as they showed maximum solubility to ketoprofen. The pseudo ternary phase-diagrams was constructed to find optimal concentration. The prepared nanoemulsions were subjected trough thermodynamic stability testing, scanning electron microscopy (SEM), zeta-potential, pH, viscosity and diffusion studies. The optimized formulation of nanoemusion H-2 have zeta potential -16.3mV with particle size below 100 nm, pH (7.2), & diffusion studies showing 90.84% drug release after 24 hours. The optimized formulation was incorporated into Carbopol 940 to form nanogel and evaluated for viscosity, pH, in-vitro permeation studies, skin irritation test and anti-inflammatory activity. The viscosity of nanoemulgel was found to be 2050 mPaS, pH (7.5) & it will not produce any local irritation to the skin. The in-vitro skin permeations profile of optimized formulation H2 showed a significant increase ($p < 0.05$) in inhibition after 24h was compared with marketed ketoprofen gel and nanoemulsion gel. The significant increase in permeability ratio (Kp), flux (Jss) and enhancement ratio (Er) was observed. The results suggested that nanoemulsion gels are potential vehicles for improved transdermal delivery of ketoprofen.

Keywords: Nanogel, scanning electron microscopy, zeta-potential, diffusion studies, skin irritation test, anti-inflammatory activity.

INTRODUCTION

Nanoemulsions are colloidal dispersions composed of an oil phase, aqueous phase, surfactant and co-surfactant at appropriate ratios. Unlike coarse emulsions micronized with external energy, nanoemulsions are based on low interfacial tension. This is achieved by adding a co-surfactant, which leads to spontaneous formation of a thermodynamically stable nanoemulsion. The droplet size in the dispersed phase is very small, usually below 10-200 nm in diameter, which makes the nanoemulsions transparent liquids[1,2]. In principle, nanoemulsions can be used to deliver drugs to the patients via several routes, but the topical application of nanoemulsions has gained increasing interest. The three main factors determining the transdermal permeation of drugs are the mobility of drug in the vehicle, release of drug from the vehicle, and permeation of drug into the skin. Nanoemulsions improve the transdermal delivery of several drugs over the conventional topical preparations such as emulsions and gels. Mobility of drugs in nanoemulsions is more facile as compared to the nanoemulsion with gel former which will increase its viscosity and further decrease the permeation in the skin. The superior transdermal flux from nanoemulsions has been shown to be mainly due to their high solubilisation potential for lipophilic and hydrophilic drugs. This generates an increased thermodynamic activity towards the skin[3].It was found that Nanoemulsions could be a very good carrier for topical delivery of highly lipophilic drugs. Ketoprofen is a non-steroidal anti-inflammatory drug (NSAID) which has been extensively used in treatment of rheumatism. Although ketoprofen is highly permeable through stomach, its poor water solubility (log partition co-efficient is 3.11) limits its entry into systemic circulation[4]. During gastric emptying, ketoprofen enters the small intestine where it can't permeate through the membrane despite being solubilised. Moreover, it is associated with oral side effect including gastric irritation when administered orally. Therefore, an eventual need has emerged to develop a transdermal dosage form of ketoprofen to minimize the oral side effect. One of the most promising techniques for enhancement of transdermal drug delivery is formulation of nanoemulsion gel. The main aim of this study is to develop and evaluate ketoprofen loaded nanoemulsion gel for treatment of arthritis and osteoarthritis to overcome the troubles associated with its oral delivery.

MATERIALS AND METHODS

Materials

Ketoprofen was a gift sample from BMR Enterprise, Hyderabad, India. Captex 200, Triacetin, IPM and Oleic acid and carbopol 934P were purchased from BMR Enterprise, Hyderabad. Tween80, Propylene Glycol, Polyethylene Glycol, Triethanolamine and Ethanol were purchased from SD Fine Chemicals Ltd. Mumbai. All other chemicals and solvents used were of analytical grade.

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Screening of oils

Oil is used in the preparation of nanoemulsion. Hence, to select the best oil the solubility of various oils in ketoprofen was done. The solubility ketoprofen in various oils were determined by adding an excess amount of drug in 5ml of selected oils (CAPTEX 200, Triacetin, IPM and Oleic acid) in 15mL capacity stoppered vials and mixed well. The vials containing the mixtures were kept at 37±1.0°C in a rotary shaker for 72 hours to reach equilibrium. After the sample were centrifuged at 3000 rpm for 15 minutes in a rotary shaker. The supernatant was filtered and concentration of ketoprofen was determined using UV spectrophotometer at 257 nm.[5]

Screening of surfactant

Surfactant and co-surfactant are used in the preparation to reduce interfacial tension between oil and water phase. Hence, to select the best surfactant the emulsification ability of the surfactants was screened. The solubility ketoprofen in various surfactants were determined by adding an excess amount of drug in 5ml of selected surfactant (Tween 80, Tween 20 and Span 20) in 15mL capacity stoppered vials and mixed well. The vials containing the mixtures were kept at 37±1.0°C in a rotary shaker for 72 hours to reach equilibrium. After the sample were centrifuged at 3000 rpm for 15 minutes in a rotary shaker. The supernatant was filtered and the concentration of ketoprofen was determined using UV spectrophotometer at 257 nm.[6]

Screening of Co-surfactants

Co surfactants are the excipients added to the emulsion for further stabilizing the interfacial film and prevent coalescence of the droplets. The solubility ketoprofen in various co-surfactants were determined by adding an excess amount of drug in 5ml of selected co-surfactant (PEG 400, PEG 200 and PG) in 15mL capacity stoppered vials and mixed well. The vials containing the mixtures were kept at 37±1.0°C in a rotary shaker for 72 hours to reach equilibrium. After the sample were centrifuged at 3000 rpm for 15 minutes in a rotary shaker. The supernatant was filtered and the concentration of ketoprofen was determined using UV spectrophotometer at 257 nm.[7]

Pseudoternary diagram

On the basis of the solubility studies, a combination of CAPTEX 200 was selected as the oil phase. Tween-80 and PEG 400 were selected as surfactant and co-surfactant, respectively. The distilled water was used as an aqueous phase. Pseudo ternary phase diagrams were constructed using titration technique[8]. Captex 200 was used as oil phase. Smix was composed of Tween 80 as surfactant and PEG 400 as co-surfactant. Four weight ratios (1: 1, 1:2, 2:1 and 3: 1) of Tween 80 to PEG 400 were optimized to determine the optimum ratio which can result in maximum nanoemulsion existence area. The ratios of Captex 200 to surfactant and co surfactant mixture were varied as 1 : 9, 2 : 8, 3 : 7, 4 : 6, 5 : 5, 6 : 4, 7 : 3, 8 : 2, and 9 : 1 % w/w. After water added drop wise to each oil-Smix mixture under gentle agitation to identity nanoemulsion region until mixture become clear at certain point. From the end point, compositions of the titrated samples, the mass percent (weight ratio) compositions of the Captex 200, surfactant and co-surfactant mixture (Smix) and water were calculated and plotted on triangular coordinates to construct the pseudo ternary phase diagrams using CHEMIX ternary diagram software.[9]

Preparation of Nanoemulsion from Phase Diagrams

Formulations were selected from the Nanoemulsion region of the constructed phase diagram to incorporate drug into the oil phase. The formulation was chosen with the criteria of maximum oil being emulsified with the minimum amount of Smix. Appropriate quantities of oil (i.e Captex 200), surfactant (Tween 80) and co-surfactant (PEG 400) were weighted and mixed well. Smix ratio i.e surfactant: co-surfactant is selected according to the best phase diagram ratio which shows maximum emulsification area. The drug was accurately weighted to represent 2.5% w/w of the weight of the formulation was added to the rest formulation and then an appropriate amount of water was added to the mixture in drop wise manner. The droplet size is further reduced by using Sonication method. The nanoemulsion containing ketoprofen was obtained by using ultrasonic homogenizer. All nanoemulsion was stored at ambient temperature for further studies.[10]

Characterization and Evaluation of Nanoemulsion

Different characterization parameters for Nanoemulsion include scanning electron microscopy, pH, viscosity, zeta potential, refractive index, *in-vitro* diffusion studies, & thermodynamic stability studies. The surface charge of the Nanoemulsion droplets has a marked effect on the stability of the emulsion system and Nanoemulsion droplets were in the size below 100nm[11]

Thermodynamic Stability Studies

To overcome the problem regarding the thermodynamic stability, stability study were performed, which are as follows [12]

Heating Cooling Cycle

Heating and cooling cycle were done in refrigerator ranging the temperature between 4°C and 45°C for 48 hours. The formulations which were stable at these temperatures were subjected to centrifugation test.

Centrifugation

Centrifugation study for the selected formulations was done at 3500 rpm for 30 min. Formulations that did not show any phase separation were taken for the freeze thaw stress test.

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Freeze Thaw Cycle

Freeze thaw cycles were carried out between a temperature - 21°C and +25°C where the formulation was stored for not less than 48 hours at each temperature. Those formulations, which passed these thermodynamic stress tests, were selected for further study.

Zeta potential analysis

Zeta potential for nanoemulsion was determined using Zetasizer HSA 3000 (Malvern Instrument Ltd., UK). Samples were placed in clear disposable zeta cells and results were recorded. Before putting the fresh sample, cuvettes were washed with the methanol and rinsed using the sample to be measured before each experiment.[13]

Polydispersity index

The mean droplet size and polydispersity index were calculated from intensity, volume and bimodal distribution assuming spherical particles. A small droplet sizes are very much prerequisite for drug delivery as the oil droplets tend to fuse with the skin thus providing a channel for drug delivery. So, to find the mean particle size polydispersity index (PI) is measured.[14]

Viscosity determination

Brookfield DVE viscometer (Brookfield Engineering Laboratories, Inc, Middleboro, MA) was used for the determination of viscosity of the formulations. Analysis of sample without dilution was done using spindle no. 63 at 30 r.p.m. [15]

Refractive index

The refractive index of placebo formulation and drug loaded formulations was determined using an Abbe-type refractometer (Macro Scientific Works, Delhi, India). [16]

pH

The apparent pH of the formulation was measured by using digital pH meter which is standardized previously.[17]

***In-vitro* diffusion studies**

In vitro diffusion studies were performed on a modified Keshary Chien (K.C) diffusion cell with an effective diffusional area of 4.76 cm^2 and 35 mL of receiver chamber capacity, using cellophane paper. The cellophane paper was brought and mounted between the donor and receiver compartments of the Keshary-Chien diffusion cell. Initially, the donor compartment was empty and the receiver chamber was filled with phosphate buffer of 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 a hot air oven where the temperature was maintained at 37 ± 1°C. 5 mL nanoemulsion formulation was placed into the donor compartment and sealed with paraffin film to provide occlusive conditions. Samples were withdrawn at regular intervals (1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 18 and 24 h), filtered through 0.45-mm membrane filter, diluted with PBS and analyzed for drug content by UV-Visible spectrophotometer at 257 nm.[18]

Scanning Electron Microscopy (SEM)

Morphology and structure of the Nanoemulsion were studied using Scanning electron microscopy. It was used to reveal the form and size of Nanoemulsion droplets. Observations was performed as, a drop of the Nanoemulsion was directly deposited on the holey film grid and observed after drying.[19]

Selection of optimized formulation

Formulation H-2 showed the highest drug release compared to other nanoemulsion formulations. Thus, formulation H-2 was converted into nanoemulsion gel formulation (NH-2) by adding 1% (*w/w*) Carbopol-940. The skin permeation profile of nanoemulsion formulation was compared with nanoemulsion gel and normal marketed gel (fastum) using one-way analysis of variance (ANOVA). [20]

Preparation of optimized formulated ketoprofen gel

Carbomer 940 was selected as gel matrix base. Carbomer 940 was swollen in little water for 24 h and a high viscous solution was obtained, and then optimized nanoemulsion was slowly added to the viscous solution of carbomer 940 under magnetic stirring. The pH values were subsequently regulated to 6–9 by using triethanolamine and nanoemulgel was obtained.[21]

Characterization and Evaluation of optimized Nanoemulsion gel

Different characterization parameters evaluated for Nanoemulsion gel include viscosity, pH, *in-vitro* skin permeation studies, skin irritation test and anti-inflammatory activity.[22]

Viscosity Determination

Brookfield DVE viscometer (Brookfield Engineering Laboratories, Inc, Middleboro, MA) was used for the determination of viscosity of the formulations. Analysis of sample without dilution was done using spindle no. 63 at 30 r.p.m. [23]

pH

The apparent pH of the formulation was measured by using digital pH meter which is standardized previously.[24]

***In vitro* skin permeation studies between optimized nanoemulsion, optimized nanoemulgel and marketed gel**

In-vitro skin permeation studies were performed on a Keshary Chien (K.C)-diffusion cell with an effective diffusional area of 4.76 cm^2 and 35 mL of receiver chamber capacity, using rat abdominal skin. The full thickness of rat skin was excised from the abdominal region and hairs were removed with an electric clipper.

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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 at –21 °C until further use. The skin was brought to room temperature and mounted between the donor and receiver compartments of Keshary Chien (K.C)-diffusion cell where the stratum corneum side was facing the donor compartment and the dermal side was facing the receiver compartment. Initially, the donor compartment was empty and the receiver chamber was filled with phosphate buffer saline (PBS) pH 7.4. The receiver fluid was stirred with a magnetic rotor at a speed of 100 rpm. After complete stabilization of the skin, 5 mL nanoemulsion formulation and 5g of gel was placed into the donor compartment and sealed with paraffin film to provide occlusive conditions. Samples were withdrawn at regular intervals (1, 2, 3, 4, 5, 6, 8, 10, 12, 14 and 24 h) filtered through 0.45mm membrane filter and analyzed for drug content by using UV-Visible spectroscopy at 257nm.[25]

Permeation data analysis

The permeation profiles were constructed by plotting the cumulative amount of ketoprofen gel permeates per unit dialysis membrane area (g/cm2) versus time. Linear regression analysis was used to calculate the steady state flux (Jss, g/cm2/hr) by using the slope of the plot. The following equation was used to determine the permeability co-efficient (Kp) of the drug through the stratum corneum.[26]

$$Kp = Jss/c$$

Where, C is the initial concentration of the drug in the donor compartment. The penetration enhancing effect was calculated in terms of enhancement ratio (ER) by using the following equation.

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

Skin irritancy test

Skin irritancy test was done on male rats, weighing 190–200 g. The animals were kept under standard laboratory conditions, temperature (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 (Lipton Feed, India) and water ad libitum. A small amount of nanoemulsion gel was applied to the left ear of the mice, with the right ear as a control and observed for any sensitivity and reaction if any was graded as. [27]

***In-vivo* studies**

Approval to carry out in vivo studies was obtained from the Institutional Animal Ethics Committee, bearing approval no. MRCP/CPCSEA/IAEC/2017-18/Pceu/02 and their guidelines were followed throughout the studies. The anti-inflammatory and sustaining actions of the optimized formulations were evaluated by the carrageenean-induced hind paw edema method developed by Winter et al. in Wistar rats. Young male Wistar rats, weighing 180–220 g, were randomly divided into 3 groups: control, nanogel and marketed gel, each 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 (Lipton Feed) and water ad libitum. The abdominal region of the rats was shaved 12h before starting the experiments, except in the control group. Nanoemulsion gel and marketed gel were applied on the shaved abdominal region of all animals (except in the control group) half an hour before subplanter injection of carrageenean into right paws. Paw edema was induced by injecting 0.1 mL of 1% (w/w) homogeneous suspension of carrageenean in distilled water. The paw volume was measured at 1, 2, 3, 6, 12 and 24 h after injection using a plethysmometer. The amount of paw swelling was determined from time to time and expressed as percent edema relative to the initial hind paw volume. Percent inhibition of edema produced by each formulation treated group was calculated against the respective control group. Results of anti-inflammatory activity were compared using the following formulae. [28]

$$\text{Edema Rate (E\%)} = Vt – Vo/Vo *100$$

$$\text{Inhibition Rate (I\%)} = Ec – Et/Ec *100$$

Where,

Vo is the mean paw volume before CFA injection (ml)

Vt is the mean paw volume after CFA injection (ml)

Ec is the edema rate of control group

Et is the edema rate of the treated group.

RESULTS AND DISCUSION

The physicochemical properties of ketoprofen suggest that it has good potential for topical drug delivery. The important criteria for selection of excipients for Nanoemulsion formulation and development was that components were to be pharmaceutically acceptable, non-irritating, and non-sensitizing to the skin and to fall into the GRAS (generally regarded as safe) category. Higher solubility of the drug in the oil phase was another important criterion; as it would help the Nanoemulsion to maintain the drug in solubilize form. Safety was a major determining factor in choosing a surfactant, as a large amount of surfactants may cause skin irritation. Non-ionic surfactants are less toxic than ionic surfactants. An important criterion for selection of the surfactants was that the required hydrophilic lipophilic balance (HLB) value to form the o/w Nanoemulsion be greater than 10. The right blend of low and high HLB surfactants leads to the formation of a stable Nanoemulsion formulation. In this study, we selected Tween 80 as a surfactant with an HLB value of 15. The co-surfactant selected for the study was PEG 400, which has an HLB value of 15. Ketoprofen is a highly lipophilic drug is BCS class II drug its properties suggest that it has good potential for transdermal drug delivery. Therefore, in the present study different

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Nanoemulsions were prepared for transdermal delivery of ketoprofen.

Solubility of ketoprofen

The maximum solubility of ketoprofen was found in CAPTEX 200 (45.29±0.202mg/ml) as compared to other oils. High drug solubility was found in Tween 80 (38.14 ± 0.14mg/ml) and PEG 400 was selected as co-surfactant as it forms stable Nanoemulsion, also acts as permeation enhancer. Therefore, Tween 80 and PEG 400 were selected as surfactant and co-surfactant i.e Smix respectively, for the phase study.

Table 1: Solubility of ketoprofen in oils

Oil	Concentration of drug (mg/ml)
Triacetin	35.6 ± 0.10
IPM	12.33 ± 0.09
Captex 200	45.29 ± 0.20
Oleic acid	28.72 ± 0.59

Mean ± SD, n=3

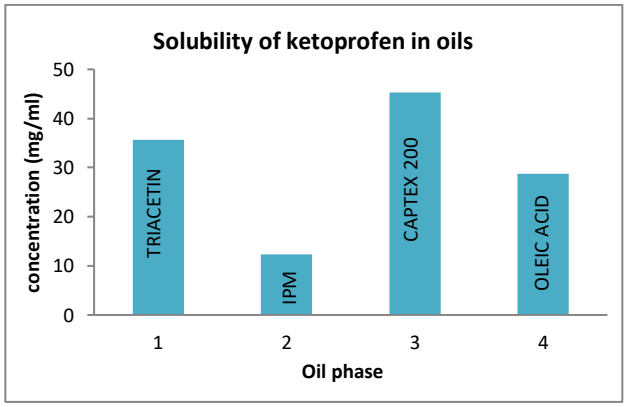

Figure 1: Solubility of ketoprofen in oils

Table 2: Solubility of ketoprofen in surfactant

Surfactant	Concentration of drug (mg/ml)
Tween 20	32.3 ± 0.10
Span 20	10.2 ± 0.115
Tween 80	48.5 ± 0.14

Mean ± SD, n=3

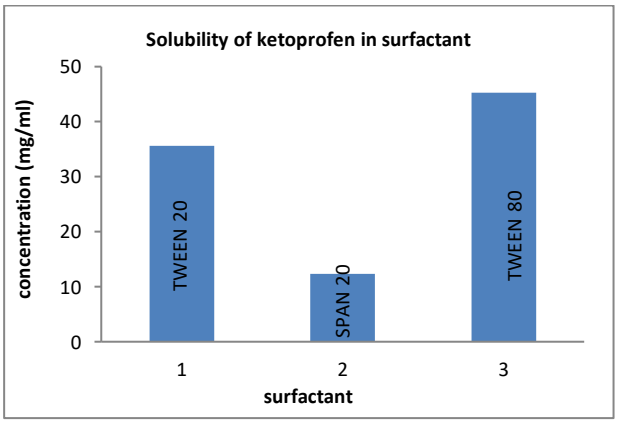

Figure 2: Solubility of ketoprofen in surfactants

Table 3: Solubility of ketoprofen in co-surfactant

Co-Surfactant	Concentration of drug (mg/ml)
PEG 400	43.56 ± 0.15
PEG 200	40.82 ± 0.132
PG	35.49 ± 0.12

Mean ± SD, n=3

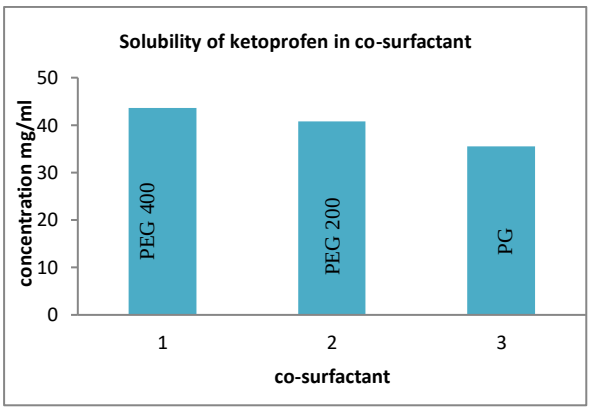

Figure 3: Solubility of ketoprofen in co-surfactant

Pseudo-ternary phase diagram

The relationship between the phase behaviour of a mixture and its composition can be captured with the aid of a phase diagram. Pseudoternary phase diagrams were constructed separately for each Smix ratio, so that o/w Nanoemulsion regions could be identified and Nanoemulsion formulations could be optimized. Pseudoternary phase diagrams were constructed separately for each Smix ratio as shown in the figure 4.5 which represents Smix 1:1, 1:2, 2:1 and 3:1 respectively. It was observed in 1:1 Smix that when co-surfactant was added along with surfactant, the interfacial film became more fluid and no liquid crystalline area was found in the phase diagram. A large o/w Nanoemulsion area was observed. The maximum amount of oil that could be solubilized was 23% (*m*/*m*) with around 35% (*m*/*m*) of Smix. As the surfactant concentration was increased in Smix (ratio 2:1), a higher Nanoemulsion region was observed. It may be due to further reduction of the interfacial tension, increasing the fluidity of the interface, thereby increasing the entropy of the system. There may be greater penetration of the oil phase in the hydrophobic region of the surfactant monomers. As we further increased surfactant concentration in Smix to 3:1 the Nanoemulsion region decreased as compared to 1:1, the maximum concentration of oil that could be solubilized by this ratio was 24% (*m*/*m*) utilizing 36% (*m*/*m*) of Smix. When co-sufactant was increased as compared to surfactant in the Smix ratio of 1:2, the small area of Nanoemulsion further decreased and the liquid crystalline area started to appear in the phase diagram, which may be due to increased co-surfactant concentration. The maximum concentration of oil that could be solubilized with 53% of Smix was 23%. When co-surfactant concentration was increased from 1:1 to 1:2

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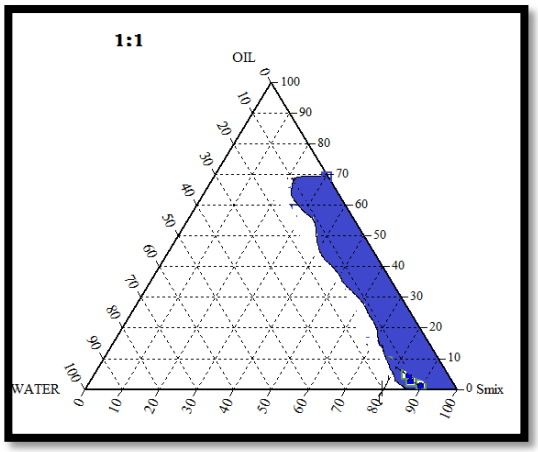
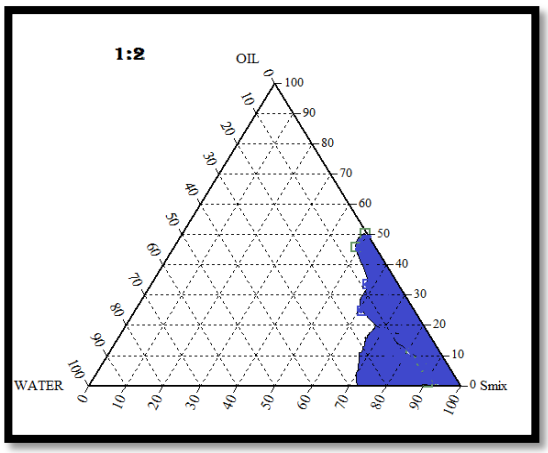
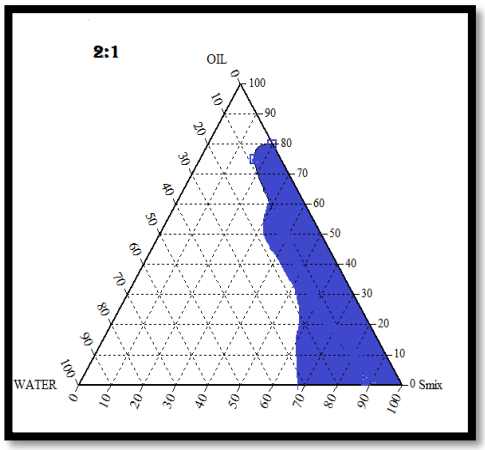
compared to surfactant, the Nanoemulsion area decreased. It is well known that large amounts of surfactants cause skin irritation, it is therefore important to determine the surfactant concentration properly and use the optimum concentration of surfactant in the formulation. From pseudoternary phase diagrams, the formulations in which the amount of oil phase completely solubilized the drug and which could accommodate the optimum quantity of Smix and distilled water were selected for the study. Formulation was selected from the nanoemulsion region of the constructed phase diagram to incorporate drug into oily phase. The best ratio was selected according to the Phase diagram which shows maximum emulsification area.[29]







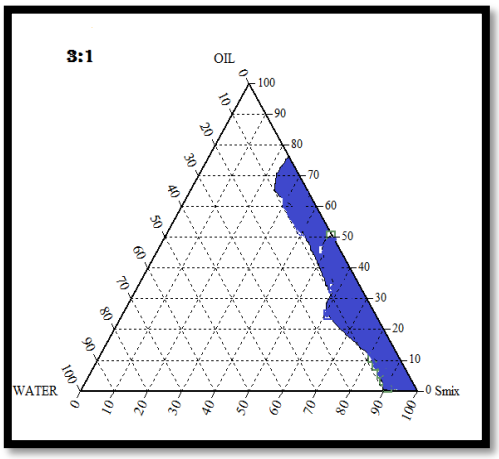


Figure 4: Pseudo-ternary phase diagram showing the o/w Nanoemulsion (shaded area) regions of CAPTEX 200 (oil), tween80 (surfactant), PEG 400 (cosurfactant) at S_{mix} ratio 1:1, 1:2, 2:1 & 3:1.

Table 4: Formulation selected from pseudo ternary phase diagram

CODE	Smix Ratio	Oil/Smix Ratio	Oil	Smix	Water
E-1	1:1	1:4	10	40	50
E-2	1:1	1:5	10	50	40
E-3	1:1	1:2	15	35	50
G-1	1:2	1:4	10	40	50
G-2	1:2	1:5	10	50	40
G-3	1:2	1:2	15	35	50
H-1	2:1	1:4	10	40	50
H-2	2:1	1:5	10	50	40
H-3	2:1	1:2	15	35	50
I-1	3:1	1:4	10	40	50
I-2	3:1	1:5	10	50	40
I-3	3:1	1:2	15	35	50

A total of 12 formulations were selected based on their ability to form oil in water (O/W) nanoemulsions which are selected from Pseudoternary phase diagram of each Smix as shown in the above table.

Dilution test

The prepared nanoemulsion formulation was diluted in 1:10, 1:50, 1:100 ratio with distilled water, the system does not show any sign of separation and found to be clear. So, its conform that prepared nanoemulsion is O/W type. [30]

Thermodynamic stability studies of nanoemulsion

Nanoemulsions are thermodynamically and physically stable systems and are formed at a particular concentration of oil, surfactant and water, making them stable to phase separation, creaming or cracking. It is the thermo stability that differentiates Nanoemulsion from emulsions with kinetic stability and eventually phase

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separation. Thus, the formulations were tested for their physical (dispersion) stability by using centrifugation, heating-cooling cycle and freeze-thaw cycle. Only those formulations which survived dispersion stability tests were selected for further study. The compositions of selected formulations are given in table 05. Except E3 and G_3 remaining all the formulation were passed the stability tests.

Table 5: Stability studies of Nanoemulsion

Code	Heating and cooling	Centrifugation	Freeze thaw cycle	Inference
E-1	Pass	Pass	Pass	Pass
E-2	Pass	Pass	Pass	Pass
E-3	Pass	Pass	Fail	Fail
G-1	Pass	Pass	Pass	Pass
G-2	Pass	Pass	Pass	Pass
G-3	Fail	Fail	Fail	Fail
H-1	Pass	Pass	Pass	Pass
H-2	Pass	Pass	Pass	Pass
H-3	Pass	Pass	Pass	Pass
I-1	Pass	Pass	Pass	Pass
I-2	Pass	Pass	Pass	Pass
I-3	Pass	Pass	Pass	Pass

Polydispersity index

Polydispersity index (PI) is the measure of particle homogenicity and it varies from 0.0 to 1.0. The closer to zero the polydispersity value the more homogenous are the particles. Optimum formulation (H-2) shows their PI 0.314 that indicates acceptable homogenicity. Nanoemulsion formulation consists of non-ionic components which show relatively neutral charge, it means it will not affected by body membrane charge during absorption [31].

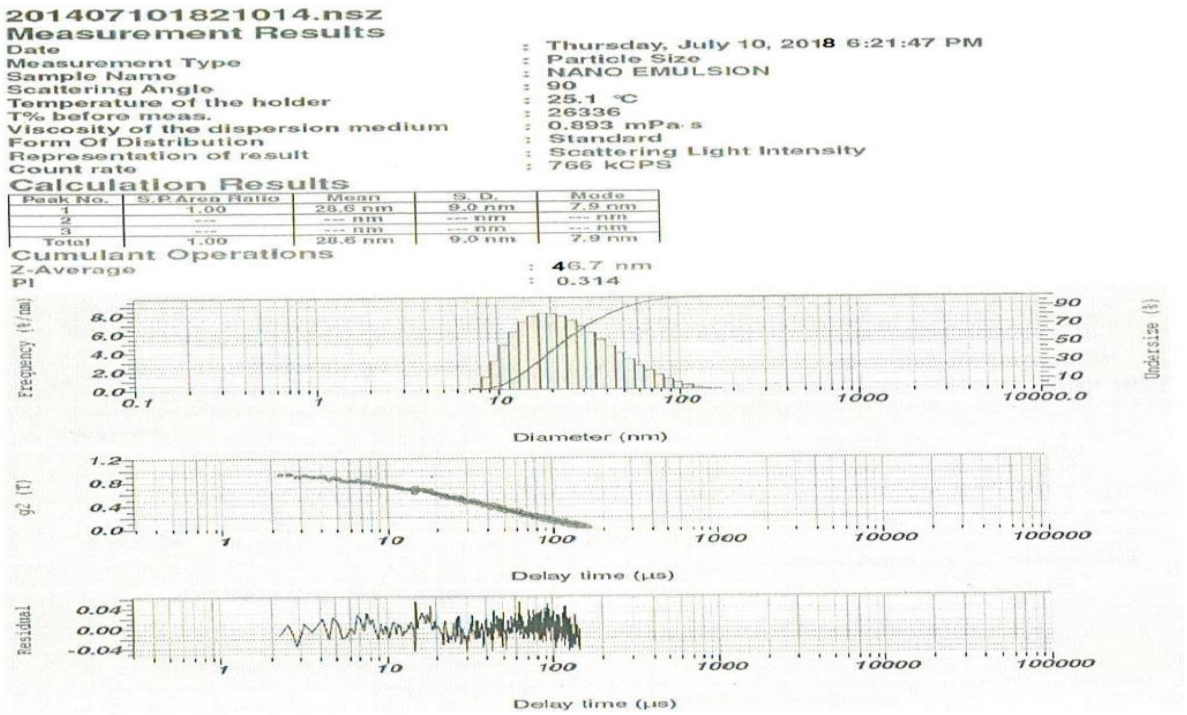

Figure 5: Polydispersity index of optimized formulation (H-2)

Zeta potential

Zeta potential of all Nanoemulsion formulation was found within range when diluted 100 times. Nanoemulsion formulation consists of non-ionic components which show relatively neutral charge, it means it will not affected by body membrane charge during absorption.[32]

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Table 6: Table showing different zeta potential of all formulations

Code	Zeta potential(mV)
E-1	-4.7
E-2	-5.3
G-1	-7.5
G-2	-9.1
H-1	-2.2
H-2	-16.3
H-3	-6.8
I-1	-2.4
I-2	-8.2
I-3	-10.5

Mean ± SD, n=3

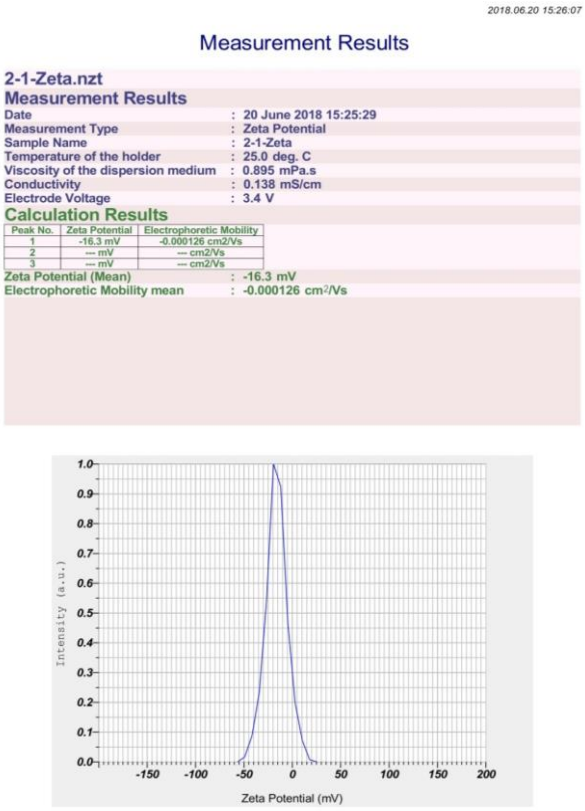

Figure 6: Zeta potential of optimized formulation

Viscosity and pH of Nanoemulsion

The table below shows the viscosity well as pH of all nanoemulsions and optimized nanoemulsion gel. The optimum pH value of nanoemulsion ranges between 6-7.5 as it is applied on skin and viscosity ranges between 20 - 90 for nanoemulsion and gels have high viscosity owing to their high viscous nature. The formulations pH ranges between 6.7-7.2 which shows they can be applied on skin and viscosity ranges between 28–80 which shows they have optimum viscosity whereas gel have viscosity of 2050 for its high viscous property.

Table 7: Viscosity and pH

Code	Viscosity(cP)	pH
E-1	45±0.15	7.16 ± 0.11
E-2	35±0.30	6.93 ± 0.05
G-1	47±0.23	7.08 ± 0.05
G-2	60±0.08	6.7 ± 0.10
H-1	35±0.33	7.06 ± 0.11
H-2	28±0.41	7.1 ± 0.10
H-3	54±0.11	6.83 ± 0.05
I-1	38±0.31	6.73 ± 0.05
I-2	68±0.09	7.1 ± 0.1
I-2	80±0.35	6.82 ± 0.11
Nanogel (NH-2)	2050±0.12	7.2±0.02

Mean ± SD, n=3

Refractive index

The refractive index of placebo formulations and drug loaded formulations was determined using an Abbes refractometer. The values of the refractive index of drug loaded formulations and placebo formulations are given Table 10. When the refractive index values for formulations were compared with those of the placebo, it was found that there were no significant differences between the values. Therefore it can be concluded that the nanoemulsion formulations were not only thermodynamically stable but also chemically stable and remained isotropic. Thus there were no interactions between nanoemulsion excipients and drug.[33]

Table 8: Comparison between refractive index of placebo and formulation

Code	Placebo	Formulation
E-1	1.324 ± 0.021	1.326 ± 0.001
E-2	1.313 ± 0.001	1.314 ± 0.001
G-1	1.328 ± 0.02	1.329 ± 0.01
G-2	1.333 ± 0.002	1.335 ± 0.001
H-1	1.371 ± 0.001	1.372 ± 0.002
H-2	1.374 ± 0.02	1.372 ± 0.04
H-3	1.379 ± 0.001	1.375 ± 0.001
I-1	1.382 ± 0.02	1.381 ± 0.002
I-2	1.413 ± 0.04	1.425 ± 0.02
I-3	1.401 ± 0.02	1.403 ± 0.01

Mean ± SD, n=3

In-Vitro diffusion studies of Nanoemulsions

In vitro skin permeation studies were performed to compare the drug release from ten different Nanoemulsion formulations. The study was carried out using pH 7.4 buffer in receiver compartment. *In vitro* diffusion studies were found highest in formulation H-2.The maximum release in H-2 could be due to the

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smallest particle size and lowest viscosity compared to other Nanoemulsions.[34] The presence of co-surfactant i.e PEG 400 which is also a permeation enhancer on its own affects the permeability of the skin and enhanced the transdermal drug delivery.

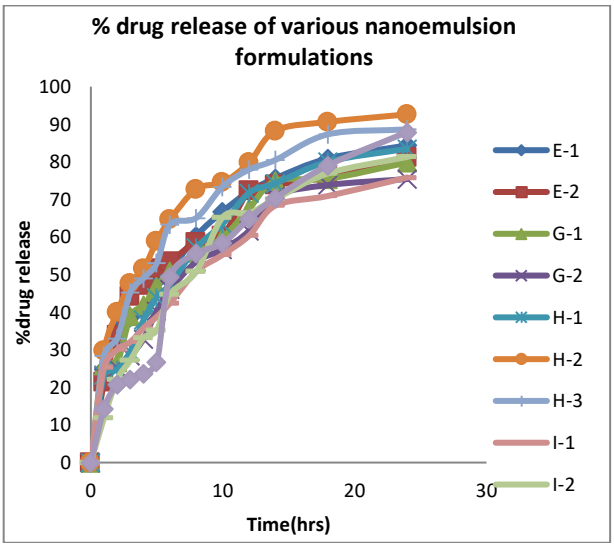

Figure 7: Percentage drug release of Nanoemulsion formulations

Scanning electron microscopy

The formulation was analyzed for scanning electron microscopy for particle size. From the results of particle size analysis H-2 was selected and analyzed for SEM the results of the analysis also confirms that the formulation shows least particle size that is less than 100nm which was within the required limit (5nm-200nm) as shown in figure below

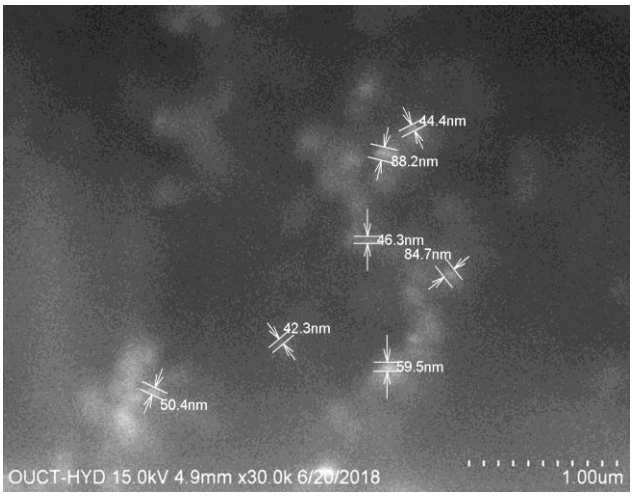

Figure 8: An image showing SEM report

Comparative drug release study between optimized nanoemulsion (h-2) and nanogel (nh-2)

In-vitro skin permeation studies were performed to compare the drug release of Nanoemulsion formulation (H-2) & Nanoemulsion gel (NH-2). The study was carried out using pH 7.4 saline buffer in receiver compartment. *In vitro* skin permeation was the highest in formulations H-2 and the lowest for gel. Optimized formulated nanoemulsion gel (NH-2) showed an intermediate skin permeation profile. The significant difference in ketoprofen permeation between Nanoemulsion formulation & Nanogel (NG-2) was probably due to the mean size of internal phase droplets, which were significantly smaller in case of Nanoemulsions and increased permeation characteristics in nanoemulsion and nanogel. The maximum release in H-2 could be due to the smallest particle size and lowest viscosity compared to other Nanoemulsions. To explain the probable mechanism by which Nanoemulsions enhance the skin permeation of drugs, the histological and histochemical structure of stratum corneum must be taken into consideration. Drugs permeate stratum corneum through two micro pathways, *i.e.*, intercellular and transcellular pathways. Of these, the intercellular pathway plays a major role in percutaneous uptake of drugs. It is well known that a complex mixture of essentially neutral lipids, which are arranged as a bilayer with their hydrophobic chains facing each others, forms a lipophilic bimolecular leaflet. Most of the lipophilic drugs pass through this region, and it is called a lipid pathway. The polar head group of lipids faces an aqueous region, forming a polar route that hydrophilic drugs generally prefer. A dermally applied Nanoemulsion is expected to penetrate the stratum corneum and to exist intact in the whole horney layer, alter both lipid and polar pathways. The drug dissolved in the lipid domain of the Nanoemulsions can directly penetrate the lipid of the stratum corneum, thereby destabilizing its bilayer structure. These interactions will increase the lipid pathway permeability to drugs. On the other hand, the hydrophilic domain of Nanoemulsions can hydrate the stratum corneum to a greater extent and play an important role in percutaneous uptake of drugs[35]. Nanoemulsion shows more drug release when compared to the Nanogel. Nanogel drug release was 84.31%, and Nanoemulsion shown 92.63% at the end 24 hrs. The drug release was found in the following order like Nanoemulsion > Nanogel.

Comparative drug release study between optimized nanogel (nh-2) & marketed gel

In vitro skin permeation studies were performed to compare the drug release from Nanoemulsion gel (NH-2) and marketed ketogel (fastum gel). The study was carried out using pH 7.4 saline buffer in receiver compartment. *In vitro* skin permeation was the highest in nanogel (NH-2) and the lowest for normal marketed gel. Nanogel drug release was 84.31% where as normal marketed gel of ketoprofen shows 72% at the end 24 hrs. This indicates that drug release was increased greatly when compared to the normal marketed gel. The drug release was found in the following order like Nanogel > Normal gel.

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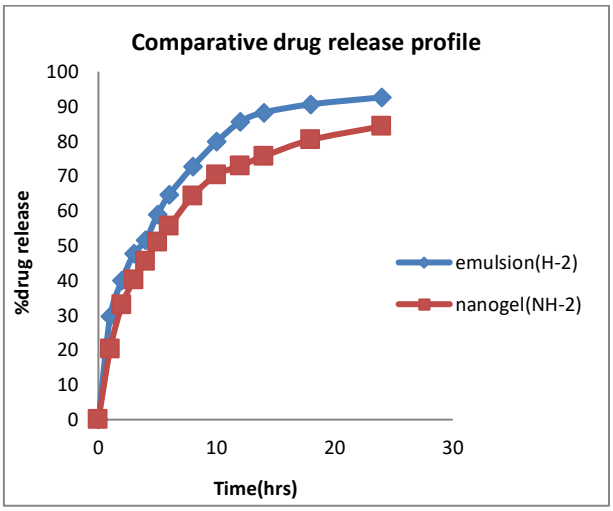

Figure 9: Comparative drug release of optimized nanoemulsion (H-2) and optimized nanogel (NH-2)

Using ANOVA test we found out that at there were significant differences (i.e $p< 0.05$) between the values of comparative drug release profile of nanogel and marketed gel. Therefore, it can be concluded that the nanoemulsion formulations have enhanced drug permeation characteristics as compared to normal marketed gel.

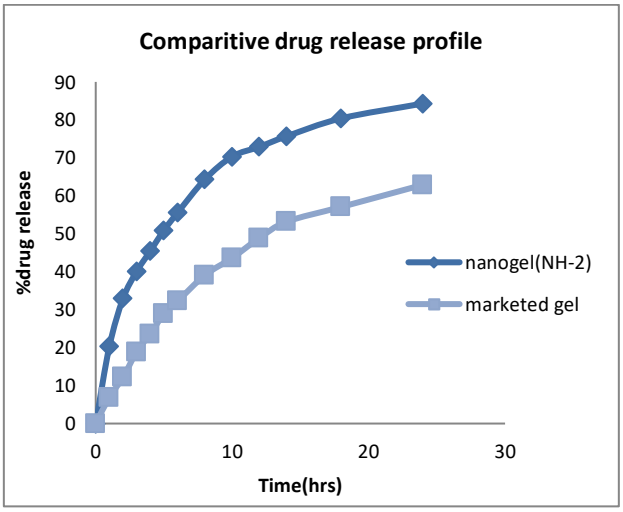

Figure 10: Comparative drug release of optimized nanogel (NH-2) and marketed gel

Permeation data analysis

Permeability parameters like steady-state flux (J_{ss}), permeability coefficient (K_p), and enhancement ratio (E_r) were significantly increased in Nanoemulsions and the Nanogel formulation as compared with marketed gel. This is because Nanoemulsions and Nanogel decreased drug loaded globule size. The permeability parameters of different formulations are given in the following table. Nano gel shows the steady state flux (511.13(µg/cm2/hr), permeability coefficient (0.255 cm/hr) and enhancement ratio was 3.30 when compared marketed gel.[36]

Table 9: Permeation data analysis of marketed gel and all formulation

Sample code	Jss±SD (µg/cm2/hr)	Kp± SD *10^{-2} (cm/hr)	Enhancement Ratio (Er)
Market ketoGel	154.45	0.077	--------
E-1	553.78	0.276	3.58
E-2	554.74	0.277	3.59
G-1	542.32	0.271	3.51
G-2	535.64	0.267	3.46
H-1	554.74	0.277	3.59
H-2	618.39	0.309	4.04
H-3	570.01	0.285	3.69
I-1	553.78	0.276	3.58
I-2	551.55	0.275	3.57
I-3	573.1	0.286	3.71
NanoGel (NH-2)	511.13	0.255	3.30

Skin irritation test

Based on *in-vitro* diffusion study nanoemulsion formulation H-2 containing drug was optimized. Further, Skin irritation test was performed with optimized formulation NH-2 by applying it to left ear of mice to study irritancy by comparing with its right ear to which formulation was not applied (control). The study was carried out for six days and it was found that the Nanogel of NH-2 causes no irritation or erythema [37]

Table 10: Skin irritation test of control and nanogel

S.No.	Treatment	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
1	Control	A	A	A	A	A	A
2	Nanogel	A	A	A	A	A	A

Where reaction if any was graded as follows;

A	No reaction
B	Slight, patchy erythema
C	Slight but confluent or moderate but patchy erythema
D	Moderate erythema
E	Severe erythema with or without edema

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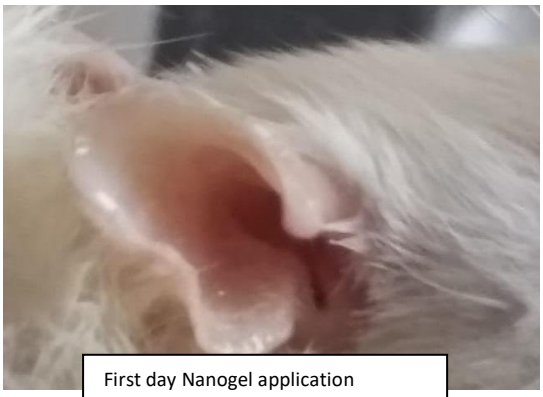

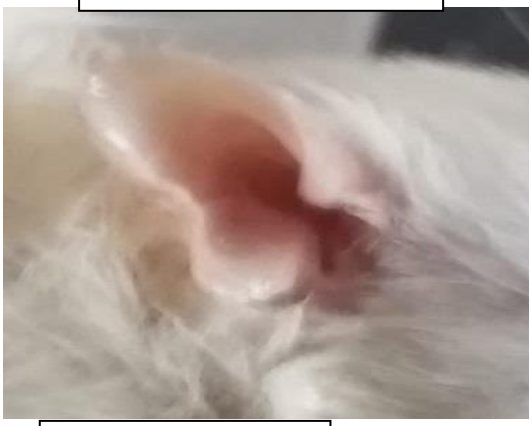

ritancy studies

***in-vivo* studies / anti-inflammatory activity**

Based on higher drug release, optimum droplet size, optimum viscosity, formulation H-2 was selected for the *in vivo* anti-inflammatory effects. The percent inhibition value after 24 h administration was found to be high for optimized nanogel NH-2, i.e., 81% as compared to marketed gel (62.91%). The enhanced anti-inflammatory effects of formulation NH-2 could be due to the enhanced permeation of ketoprofen through the skin. Based on higher drug permeation, smallest droplet size, optimum viscosity, optimum surfactant and co-surfactant concentration and higher solubility, formulation NH-2 was optimized as the Nanoemulsion formulation of gel for ketoprofen. After ANOVA test, it was found that there were significant differences (i.e $p< 0.05$) between the values. Therefore it can be concluded that the nanoemulsion formulations have enhanced anti-inflammatory activity. [38]

Table 11: Anti-inflammatory study of Group-1(control, carrageenean only):

Time (hr)	Edema (mean±SD, %)
1	32±1.2
2	46±1.3
3	81±1.2
6	51±1.5
12	43±1.5
24	15±1.3

Mean ± SD, n=3

Table 12: Anti-inflammatory study of Group-2(optimized NanoGel i.e NH-2)

Time (hr)	Edema (mean±SD, %)	Inihibition (%)
1	21±1.0	34
2	29.1±1.3	37
3	46.9±1.4	42
6	20.1±1.5	61
12	12.6±1.7	71
24	2.8±1.8	81

Mean ± SD, n=3

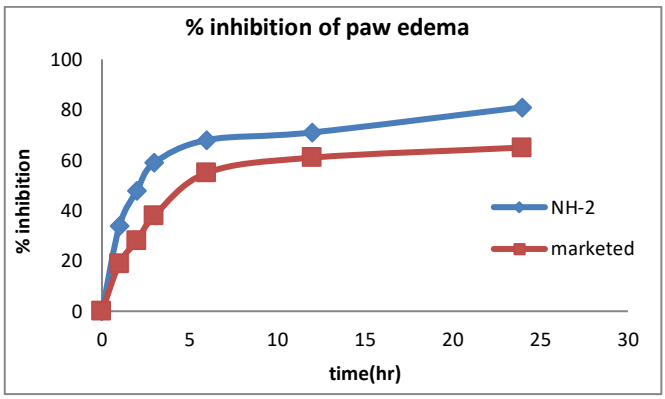

Figure 12: Percentage edema inhibition effect

CONCLUSION

In this work, a ketoprofen nanoemulsion gel for topical administration has been developed to deliver the anti-inflammatory agent i.e ketoprofen. The optimized formulation contained 10 % of oil phase (CAPTEX 200), 50 % of surfactant mixture (Tween 80 as surfactant and PEG 400 as co-surfactant) and 40 % of distilled water. From the studies, it is observed that the formulated Nanoemulsions and Nanoemulsion gel released up to 92.63 % and 84.31 % of the drug, respectively. The formulation was non sensitizing and safe for use prepared with non-irritating, pharmaceutically acceptable ingredients. No additional permeation enhancers were needed to be added since the excipients themselves acted as permeation enhancers. A high percent inhibition of edema was observed with the nanogel (81 %) as compared with the marketed normal gel (62.91 %). Thus, it can be concluded that the developed Nanoemulsion-based gel have a greater potential for topical drug delivery as compared to conventional formulations.

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