ORIGINAL ARTICLE

Development and Evaluation of a Chitosan-based Topical Gel of Dinalbuphine sebacate for Targeted Management of Rheumatoid Arthritis

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Abstract

Aim: The aim of the study is to develop and evaluate sustained release formulations containing dinalbuphine sebacate (DNS) for the treatment of rheumatoid arthritis (RA). **Materials and Methods:** A DNS-loaded microemulsion was optimized using pseudoternary phase diagrams and Minitab software. This was incorporated into a 2% w/v chitosan gel for enhanced skin delivery. The formulation was characterized for particle size transmission electron microscopy, rheology, and stability. *In vitro* skin permeation was tested using Franz diffusion cells with human cadaver skin, and *in vivo* anti-arthritic efficacy was evaluated in complete Freund's adjuvant-induced arthritic rats. **Results and Discussion:** The DNS-loaded chitosan gel showed favorable rheology, sustained release, and enhanced skin retention. Treated arthritic rats had a significant reduction in paw swelling, with one gel reducing inflammation by 57.5%. Inflammatory and liver biomarkers improved, and histology confirmed preserved joint structure with minimal inflammation. The formulation remained stable over 6 months. **Conclusion:** DNS-loaded chitosan gel offers a promising localized treatment for RA, providing prolonged pain relief while reducing systemic exposure and opioid side effects. It presents a non-invasive alternative for managing chronic inflammatory pain.

Key words: Complete Freund's adjuvant, Dinalbuphine sebacate, Liquid chromatography-tandem mass spectrometry, Pseudoternary phase diagrams, Response surface methodology, Transmission electron microscopy

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune condition characterized by persistent inflammation of the synovial lining, leading to joint degradation and significant discomfort. Conventional treatments, including non-steroidal anti-inflammatory drugs, antirheumatic drugs, and opioid-based pain relievers, primarily offer symptom control but are frequently linked to systemic side effects and variable treatment outcomes.[1,2] In response to these challenges, localized drug delivery systems evolved as an important strategy to improve drug concentration at the inflamed joints while reducing systemic absorption and associated adverse effects.[3,4]

Dinalbuphine sebacate (DNS), a long-acting prodrug of nalbuphine, has shown significant promise in the treatment of inflammatory pain. Studies have highlighted its ability to provide prolonged pain relief while minimizing the side effects commonly linked to traditional opioid therapies.[5] Investigations involving DNS injections in RA models have reported extended analgesic duration; however, systemic delivery continues to pose challenges due to drug clearance and potential adverse effects. Although these results are encouraging, there is still a lack of widespread research on the use of DNS in topical formulations aimed at direct application to inflamed joints, which may offer a more precise, efficient, and user-friendly treatment approach.[6,7]

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Chitosan-based topical gels offer an excellent platform for DNS delivery due to their biocompatibility, skin penetration-enhancing ability, and controlled drug release capabilities. Chitosan, a natural polysaccharide, enhances skin permeability and forms a semi-occlusive film over the application site, which supports prolonged drug retention and localized delivery – making it a suitable carrier for RA therapy.[8,9] By incorporating DNS into a chitosan-based gel, the aim is to improve local drug bioavailability, extend analgesic duration, and reduce systemic opioid exposure.

This study aims to develop and assess a chitosan-based topical gel formulation of DNS for the RA management. The formulation will be assessed for physicochemical properties, drug release profile, permeation behavior, and therapeutic efficacy. By leveraging previous research on DNS's analgesic potential and the advantages of chitosan-based delivery, this work aims to introduce an innovative approach to RA pain management, offering a safer and more efficient alternative to traditional systemic therapies.

MATERIALS AND METHODS

Materials

DNS, nalbuphine and naltrexone materials, Castor oil®, Sesame oil®, Oleic acid®, Stearyl heptanoate®, Isopropyl myristate®, Tocopherol®, Phosphatidylcholine®, Dipalmitoylphosphatidylglycerol® (DPPG), Glyceryl Stearate®, Cetyl alcohol®, Tween 80®, and other chemicals and reagents were kindly supplied by Navin Saxena Research and Technology (NSRTC) Center. Indomethacin was used as standard drug (Merck Chemicals, India). Complete Freund's adjuvant (CFA) was procured from Sigma-Aldrich Corporation, Mumbai. All remaining materials were analytical grade and used as received.

Characterization of oil, cosolvent, and surfactant combinations for microemulsion (ME) region

Solvents with optimum solubility potential for DNS were identified by evaluating the solubility of DNS in a variety of oils till saturation, including castor oil, sesame oil, oleic acid, stearyl heptanoate, isopropyl myristate, tocopherol, and benzyl alcohol. In addition, the solubility was assessed in surfactants such as phosphatidylcholine (PC), DPPG, glyceryl stearate, cetyl alcohol, and Tween 80.

An additional amount of DNS was introduced into a combination of oil phase, surfactants, and co-surfactants within 10 mL volumetric flask. The flasks were vortexed for 72 h at room temperature, followed by centrifugation at 4,000 rpm for 20 min, and the DNS concentration in the superficial layer was analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) API 4000, AB SCIEX with Analyst Software 1.7.3. and HPLC with SIL-30ACMP Autosampler. Solubility of DNS in oil and surfactant mixtures is presented in Table 1.

Phase behavior analysis using pseudoternary diagrams

Pseudoternary phase diagrams were constructed through a titration-based approach to map the ME region and establish the compositional boundaries of oil phase, surfactant/cosolvent, and water required for ME formation.[10,11] The Smix mixture ratios were adjusted to 1:1, 2:1, 3:1, 1:2, and 1:3, and corresponding combinations were prepared. The Smix formulations were subsequently combined with the oil/oil-solvent mixtures in a range of weight ratios, including 9:1, 8:2, 7:3, 6:4, 1:1, 4:6, 3:7, 2:8, and 1:9, to explore their influence on ME formation.[12]

The aqueous titration method involved the slow, dropwise addition of distilled water to the oil-Smix mixture while continuously stirring at room temperature. After the addition of each proportion, the mixture was visually inspected to assess clarity and detect any signs of turbidity. The point of turbidity was considered the titration endpoint, and the corresponding volume of water required was noted.[13]

Table 1: Solubility of DNS in oil-cosolvents/surfactant mixtures

Solvents/mixture of solvents (w/w)	Percentage Cosolvents/surfactants ratio	DNS solubility (mg/mL)
70–80% Oleic acid	20–30% Stearyl heptanoate	32±0.5
50–60% Oleic acid	40–50% Diethylene glycol monoethyl ether	56±0.2–75±0.6
70–80% Oleic acid	20–30% Isopropyl myristate	42±0.4–46±0.1
70–80% Oleic acid	20–30% Tocopherol	14±0.2
60% Benzyl alcohol	40% Phosphate buffer saline 5.5	442±2.5
60% Benzyl alcohol	40% Phosphate buffer saline 6.8	458±1.6
60% Benzyl alcohol	40% Phosphate buffer saline 7.4	476±1.5
Oleic acid (90% w/w)	Phosphatidylcholine (PC) (10% w/w)	36±0.2
Oleic acid (90% w/w)	Glyceryl stearate (10% w/w)	36±0.6
Oleic acid (90% w/w)	Cetyl alcohol (10% w/w)	32±0.4
Oleic acid (98% w/w)	Tween 80 (10% w/w)	42±0.2

DNS: Dinalbuphine sebacate

Following this, the relative proportions of oil, Smix, and water were calculated. This process was repeated across various Smix ratios to map the compositional boundaries for ME formation. The data obtained were used to develop using TriPlot Todd Thompson software (version 4.1.2) for pseudoternary phase diagrams. ME regions, indicated by clear and isotropic formulations, were distinctly outlined within the phase diagrams, as illustrated in Figure 1.

Formulation development

An optimal experimental design was developed to explore the effects of multiple formulation and experimental course parameters on the emulsion properties and to identify optimal conditions for achieving the desired characteristics. A response surface design of experimental design was employed, considering a three-component system comprising an oil phase (C1), a surfactant-cosolvent mixture (Smix, C2), and an aqueous phase (C3, water), with a combined concentration of 100%.[14] The concentration ranges, determined based on prior phase diagram studies, were 10–30% for the oil phase, 30–60% for the surfactant-cosolvent mixture, and 10–30% for the aqueous phase. Five independent variables were selected: Oil phase concentration (oleic acid, isopropyl myristate, diethylene glycol monoethyl ether, stearyl heptanoate, and tocopherol), surfactant mixture concentration (PC, glyceryl stearate, cetyl alcohol, Tween 80), aqueous phase concentration (distilled water), emulsification speed (1,000–4,000 rpm), and emulsification time (10–40 min). The measured parameters comprised globule size, zeta potential, viscosity, pH, and formulation stability. The experimental design consisted of 32 runs, and the responses of all model formulations were analyzed using Minitab software version 22.2.0. Contour plots and 3D response surface plots were produced for illustration of the relation among factors and responses, with specific focus on optimizing particle size, zeta potential, pH, viscosity, and stability. The final formulation was selected based on optimal oil and Smix ratios, ensuring the smallest particle size, highest stability, and maximum encapsulation efficiency, thereby enabling a precise and systematic optimization of the emulsion formulation. Ternary phase diagrams are displayed in Figure 2.

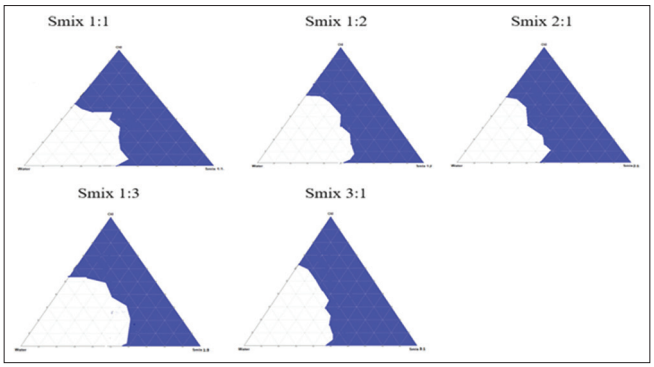

Figure 1: Ternary phase compilation of topical gels

Formulations of microemulsions

As per pseudoternary phase systems, the Smix ratio that displayed the leading ME region was preferred for the formulation. The oil phase and selected surfactant mixture were combined in predefined proportions and mixed using a magnetic stirrer at 1,000 rpm for 30 min to achieve a clear and homogenous oil-DNS-surfactant blend. Subsequently, the aqueous phase was introduced gradually while maintaining continuous high-speed homogenization using a Remi RQ-140/DE homogenizer at 4,000 rpm for 40 min. This process facilitated the formation of fine emulsion droplets, resulting in a stable ME system. To prepare the gel formulation, a 2% w/v chitosan aqueous solution was obtained by solubilizing chitosan polymer in distilled water using 0.1% v/v acetic acid under continuous magnetic stirring at 25 ± 2°C for 4 h to ensure homogeneity. The ME was then mixed with the chitosan solution at 500 rpm for 1 h, allowing the formation of a gel network around the oil droplets, stabilizing the microgel system. The final pH was adjusted to 5.5 using 0.1% v/v acetic acid. All formulations were stored at ambient temperature.

Characterization and assessment of formulated MEs

Evaluation of ME globule size

The droplet size, expressed in nanometers, was determined using dynamic light scattering, which assesses variations in light scattering resulting from the particles in suspension. Measurements were conducted at room temperature (25°C) using a Zetasizer Nano-ZS (Malvern Instruments Ltd., Version 6.20), with the detector positioned at a 90° angle to the incident laser beam. This technique enabled precise evaluation of the average globule size within the ME formulations.

Determination of zetapotential

The zeta potential was measured using electrophoretic light scattering to assess the surface charge and stability of the formulation. The analysis was conducted with a Zetasizer Nano-ZS (Malvern Instruments Ltd., version 6.20) at room temperature (25°C), with measurements recorded at a 90° angle to the incident beam.

Determination of % drug content

A precise aliquot of 0.1017 g of the sample was accurately weighed using a calibrated analytical balance and transferred into a clean glass vial container. To facilitate the extraction process, 1 mL of dichloromethane was added to the vial, and subsequently, the addition of 0.050 mL of a 100 ng/mL Naltrexone solution was added as an internal standard to ensure accurate quantification. The mixture was vortexed for

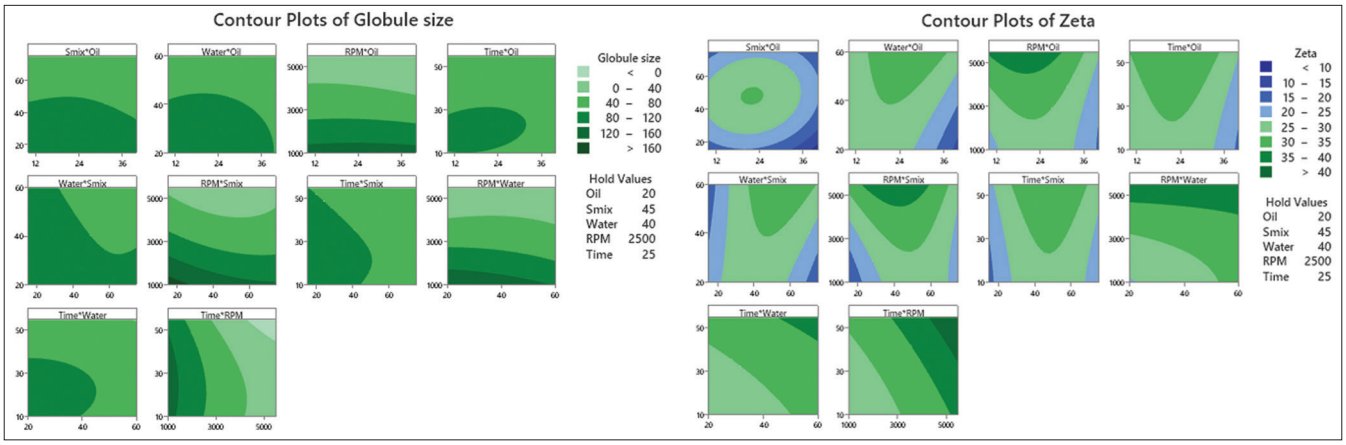

Figure 2: Contour plots of globule size and zeta potential for fresh samples

15 min to achieve complete extraction of the analyte from the sample matrix.

Following extraction, the solution underwent nitrogen evaporation until complete dryness. The dried residue was subsequently reconstituted in 0.500 mL of acetonitrile and vortexed for 5 min for complete dissolution, and the reconstituted solution was filtered through a 0.22 µm hydrophilic syringe filter and transferred into autosampler vials in the autosampler (5°C) to maintain sample integrity before analysis.

The quantification of the drug content was performed using an LC-MS/MS system under optimized chromatographic conditions. The analytical method was validated for specificity, linearity, accuracy, precision, and robustness to ensure reliable quantification of the drug content in the samples. Chromatographic separation was attained using a HILIC Plus Column (2.1 × 100 mm, 3.5 µm) maintained at 35°C, with a mobile phase composed of 2 mM ammonium formate buffer containing 0.1% formic acid in water (A) and acetonitrile (B) in a 40:60 v/v ratio. 10 µL of sample was injected into the system operated at a flow rate of 0.6 mL/min and a total run time of 4.5 min. Detection was carried out using electrospray ionization in positive polarity under multiple reaction monitoring mode, monitoring Q1 → Q3 transitions of 358.3 → 340.2 for nalbuphine, 881.6 → 863.7 for DNS, and 342.2 → 324.5 for naltrexone as the internal standard.

Transmission electron microscopic (TEM) analysis DNS-loaded microemulsions

A small aliquot of sample was carefully located onto a copper grid, and any surplus was gently blotted away using filter paper. A 2% (w/v) phosphotungstic acid solution was then added to the sample for negative staining, and it was left undisturbed for 30–60 s to achieve sufficient contrast.[15,16] Excess stain was similarly removed with filter paper. The prepared grids were then analyzed under a TEM (FEI Themis 60–300) equipped with an energy-dispersive X-ray spectroscopy detector.

Rheology study

The rheological study of chitosan-based topical gels was evaluated using a frequency sweep test to assess their stability and viscoelastic behavior at pH 5.5 and pH 7.5. The gels were prepared by dissolving chitosan and adjusting the pH using hydrochloric acid or sodium hydroxide. The formulations were equilibrated for 24 h before analysis to ensure uniform gel network formation.

Rheological measurements were performed using a rotational rheometer operating with a parallel plate geometry (25 mm diameter) at a controlled temperature of 25°C. Frequency sweep analysis was performed across an angular frequency range of 0.1–100 rad/s, maintaining a constant strain within the linear viscoelastic region to ensure reliable rheological measurements. The storage modulus (G') and loss modulus (G') were recorded to evaluate the structural strength and viscoelastic behavior of the gel formulations. Each test was conducted in triplicate to confirm reproducibility, and statistical comparisons were made across different pH conditions to assess formulation consistency and performance.

In vitro permeability test

The *in vitro* Franz diffusion cell model, employing human cadaver skin and a finite dose approach, serves as a robust method for evaluating percutaneous absorption and cutaneous pharmacokinetics of topically applied drugs. *Ex vivo* human cadaver skin was mounted on donor cells under controlled temperature and humidity conditions to mimic *in vivo* environments. To prevent bacterial degradation, 0.1% sodium azide was incorporated as an antimicrobial agent in the receptor solution.[17] The receptor compartment was having mixture of phosphate-buffered saline (pH 7.4) and benzyl alcohol in a 40:60% v/v ratio, pre-equilibrated to

physiological temperature and conditions. A precisely weighed 1.017 g of the developed formulations (DNSSF-RA-01 to DNSSF-RA-09) was applied to the donor chamber (7 mL volume), with the system maintained at 32 ± 1°C. Aliquots were collected at predetermined intervals from 0.5 to 24 h, with fresh medium replenished to maintain sink conditions. The collected samples were filtered through 0.22 µm syringe filters and analyzed using a validated analytical method to ensure accuracy and reproducibility. From another set, the sample was removed from the donor chamber after 4 h, and aliquots were collected at predetermined intervals and processed as described previously.

Stability evaluation

The chitosan-based DNS-loaded ME was subjected to stability testing by storing it at 40°C and 75% relative humidity for a duration of 6 months, in accordance with ICH guidelines.[18] During and after the storage period, the formulations were evaluated for any changes in visible clarity and drug content, using the same analytical methods applied to freshly prepared samples. In addition, the microstructural integrity of the stored formulation was investigated using TEM to detect potential morphological alterations over time, as depicted in Figure 3.[19]

In vivo skin irritation study

To evaluate potential skin irritation, three male albino Wistar rats weighing 120–130 g were kept in a controlled environment with unrestricted access to food and water. A 100 µL single dose of the chitosan-formulated DNS gel was gently massaged onto the outer surface of the left ear of each rat. The untreated right ear acted as an internal control for comparative evaluation. The treated areas were observed over a 24-h period for any signs of erythema, following which the gel was gently removed. The degree of skin irritation was assessed visually using a standardized scoring scale, categorizing erythema as absent, mild, moderate, marked, or severe.[20-22]

In vivo study of the anti-arthritic activity

Adjuvant-induced arthritis was initiated by a sub-plantar injection of 100 µL of CFA into the right hind paw of rats, excluding those in the normal control group, as described in Table 2. Each group consisted of six rats, which were pre-treated with either the test formulations or vehicle twice daily for 1 week before CFA administration, designated as day 0. Paw edema was evaluated on days 0, 4, 8, 12, 16, and 20 post-induction using a plethysmometer to monitor disease progression.

On day 21, blood samples were collected for biochemical evaluation of serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), albumin, calcium, phosphorus, and rheumatoid factor levels. On day 22, the rats were placed under anesthesia and humanely sacrificed using a CO_2 chamber. The ankle joints were carefully harvested, preserved in 10% neutral buffered formalin, and subsequently processed by decalcification. Tissues were then sectioned and stained with hematoxylin and eosin for microscopic examination of histopathological changes. Results were reported as mean ± standard error of the mean (SEM) (SEM, $n = 6$). Statistical comparisons were performed using one-way analysis of variance, followed by Dunnett's multiple comparison test, with a significance threshold set at $P < 0.05$.

RESULTS AND DISCUSSION

Evaluation of composition for DNS topical gels

The saturated solubility of DNS was assessed in various oil phases, cosolvents, surfactants, cosurfactants, and their combinations, as depicted in Figure 1. Among the tested oil components, oleic acid exhibited the highest solubility for DNS and was selected for further investigations. In the case of cosolvents and penetration enhancers, diethylene glycol monoethyl ether, isopropyl myristate, and stearyl heptanoate demonstrated superior solubility for DNS. In addition, PC and glyceryl stearate were chosen as non-ionic surfactants due to their favorable solubility profiles and low toxicity. A combination of cetyl alcohol and Tween 80, serving as a co-surfactant and stabilizer, was found to be highly effective in solubilizing DNS. The finalized components were chosen for formulating DNS-loaded MEs with the aim of improving the drug's encapsulation efficiency.

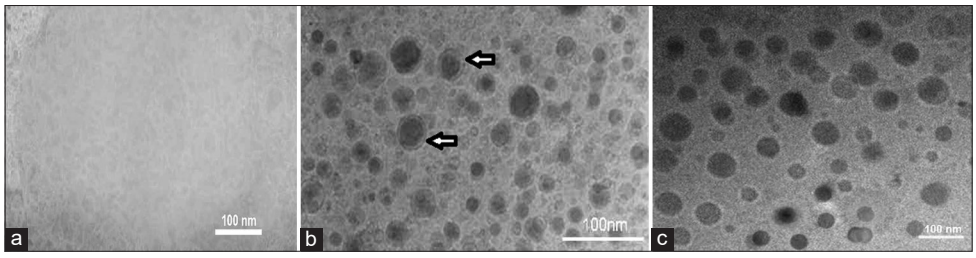

Figure 3: Zeta potential of dinalbuphine sebacate TG formulation (a) Blank sample, (b) Fresh sample, (c) Stability sample

Table 2: Treatment methodology of antiarthritic activity evaluation

Groups	Treatment
I	Normal control
II	Disease control (0.1 mL CFA administered on day 0)
III	Indomethacin (10 mg/kg) for 28 days+0.1 mL CFA administered on day 8
IV	DNSSF-RA-4 for 28 days+0.1 mL CFA administered on day 8
V	DNSSF-RA-8 for 28 days+0.1 mL CFA administered on day 8

CFA: Complete Freund's adjuvant

Evaluation of ternary systems

The combination of diethylene glycol methyl ether and oleic acid demonstrated a significantly higher emulsification area than pure oleic acid, indicating improved solubilization and emulsification efficiency. Among the tested surfactants, Tween 80 exhibited a broader emulsification area than Tween 20, likely due to its higher hydrophilic-lipophilic balance, which enhances ME formation and stability. A 3:1 or 2:1 surfactant-to-co-surfactant ratio with Tween 80 provided a broader ME region than Tween 20, though excessive surfactant increased irritation risks, limiting pharmaceutical or cosmetic applications. The system remained highly transparent, but a slight rise in viscosity affected handling. At a Smix ratio of 2:1, the phase diagram revealed an optimum emulsification area with minimal turbidity, ensuring improved ME formation and stability at lower Smix concentrations. Overall, the combinations of oleic acid, phosphatidylcholine, and glyceryl stearate system with Tween 80 demonstrated superior emulsification performance compared to combinations with Tween 20.

Formulation development of ME using response surface design

The formulation design of DNS topical gel and ME was systematically optimized using response surface methodology (RSM) to evaluate the impact of critical formulation parameters on key emulsion characteristics. The study investigated the influence of oil phase concentration (15–30%), surfactant mixture (40–55%), aqueous content (35–45%), emulsification speed (3,000–4,000 rpm), and mixing time (35–40 min) on globule size, zeta potential, viscosity, encapsulation efficiency, and stability. RSM analysis revealed that increasing the surfactant mixture concentration and emulsification speed significantly reduced globule size, enhancing stability. The optimized formulation exhibited a well-balanced globule size with minimal % difference over 3 months, indicating robust stability. Similarly, zeta potential values remained within the ideal range (−30–−50 mV), suggesting electrostatic stability. The non-turbidity zone, defined within oil (15–30%), Smix (40–50%), water (30–40%), revolutions per minute (3,000–4,500), and time (30–40 min), confirmed optimal phase behavior without phase separation. These findings demonstrate that precise control of emulsification conditions and component ratios is essential for achieving a stable, efficient, and pharmaceutically suitable ME system.

Evaluation of globule size and zeta potential analysis

The optimized DNS-loaded ME formulation displayed a mean globule size in the range of 60–70 nm, confirming its nanoscale characteristics, which are essential for enhanced drug solubility and dermal penetration. The small and uniform globule size facilitates better skin permeation and sustained drug release. The zeta potential of the formulation ranged from –26 mV to –18 mV, indicating moderate-to-good colloidal stability due to sufficient electrostatic repulsion between droplets. This negative surface charge plays a crucial role in preventing aggregation and maintaining the physical stability of the formulation over time.

Stability studies

The optimized formulations remained stable when stored at 40°C/75% RH for 3 months, with no noticeable changes in visual appearance. The %drug content of the fresh and stored formulations of the optimized drug-loaded ME was 98.64 ± 1.48%, 96.80 ± 3.16%, 96.54 ± 1.82%, respectively. The pH values of the fresh and stored optimized formulations remained within the range of 5.5–6.5, indicating no phase separation means stability. Morphological analysis through TEM confirmed that the optimized drug-loaded ME retained its structure after storage. TEM images [Figure 3] demonstrated that the globules in the developed MEs were spherical, discrete, and exhibited uniform droplet size distribution and zetapotential, closely matching with the values obtained.

Rheology study

The rheological analysis of the DNS-loaded chitosan-based topical gels using frequency sweep tests provided valuable insights into the influence of pH on gel stability and viscoelastic behavior as shown in Figure 4. At pH 5.5, the gel exhibited a pronounced solid-like character, as evidenced by a consistently higher storage modulus (G') compared to the loss modulus (G") across the tested angular frequency range (0.1–100 rad/s). This indicates strong gel integrity, enhanced elasticity, and network formation, which are essential for sustained drug release and prolonged residence at the application site. In contrast, at pH 7.5, the gel displayed predominantly liquid-like behavior, with G" values surpassing G', suggesting a weakened structural matrix and higher

viscous flow. This shift in viscoelastic profile is attributed to the reduced protonation of chitosan's amino groups at neutral to alkaline pH, leading to diminished electrostatic interactions and hydrogen bonding that are critical for gel formation. Consequently, the formulation at pH 5.5 offers superior gel strength, better adhesion to skin, and enhanced mechanical resistance, making it more suitable for topical delivery where sustained drug release and retention are desired. On the other hand, the pH 7.5 formulation, due to its increased fluidity, may be advantageous in scenarios requiring faster drug diffusion but is less favorable for long-term topical application. These findings underscore the importance of pH optimization in designing chitosan-based topical gels for targeted therapeutic efficacy.

In-vitro permeability test

Among all the tested formulations, the optimized drug-loaded topical gels exhibited the greatest cumulative permeation of DE over a 24-h period as 343.0 µg/cm^2 ± 3.61, 326 µg/cm^2 ± 2.81, and for sample removed after 4 h 205.0 µg/cm^2 ± 1.63, 195 µg/cm^2 ± 1.49. Oil phase mixture such as oleic acid, Isopropyl myristate, Diethylene Glycol Monoethyl Ether, and stearyl heptanoate combinations significantly enhanced the transport of drug through the skin. Furthermore, the nanoscale globule size of the microemulsion also affects the percutaneous absorption of the drug. Nano globule size can interact with a fixed area of stratum corneum in increasing the efficiency in percutaneous uptake. Thus, the high skin transport of DNS from microemulsion is mainly due to the amount of drug solubilized in small oil globules that easily transport through the lipid of stratum corneum of the skin.

Skin irritation evaluation

The rat study was conducted with prior approval from the Institutional Animal Ethics Committee of V.L. College of Pharmacy approval no. 557/02/c/CPCSEA. To ensure the topical safety of the developed DNS-loaded ME gel, a dermal irritation study was carried out using healthy Wistar rats. The optimized formulation was gently applied to the dorsal surface of the animals, and the skin was examined periodically for 24 h post-application for any signs of irritation such as redness, swelling, or rashes. No visible signs of erythema were observed, including those exposed to the reference marketed product. The absence of adverse skin reactions confirms that the formulation is non-irritant and dermatologically suitable for topical application, supporting its safe use in transdermal therapeutic approaches.

In-vivo study of the anti-arthritic activity

The study evaluated the effects of DNSSF-RA-4 and DNSSF-RA-8 in CFA-induced arthritis in rats by measuring paw volume, biochemical parameters, and histological changes.[19,23] The disease group exhibited a significant increase in paw volume over 20 days, indicating severe inflammation, whereas indomethacin treatment effectively reduced swelling, achieving a 67.2% reduction by day 20. Both DNSSF-RA-4 and DNSSF-RA-8 demonstrated dose-dependent anti-inflammatory effects, with DNSSF-RA-8 being more effective, reducing paw volume by 57.5% compared to 52.1% for DNSSF-RA-4. Biochemical analysis revealed that the disease group had elevated SGOT, SGPT, and rheumatoid factor levels, along with decreased albumin, calcium, and phosphorus, indicating liver dysfunction, systemic inflammation, and bone deterioration [Table 3 and Figure 5]. Indomethacin treatment improved most parameters, bringing them closer to normal values.[23]

Histological observations [Figure 6] supported these findings, where normal control rats exhibited standard tissue architecture, while CFA-induced arthritis led to severe cartilage degradation, detachment of the epidermal layer, mononuclear inflammatory infiltration, synovial hyperplasia, increased vascularization, pronounced edema, and necrotic changes. Indomethacin-treated rats showed preserved cartilage, minimal hyperplasia, well-defined metatarsal bones, and an almost normal paw structure. DNSSF-RA-4 treatment resulted in mild edema, reduced inflammation, and decreased vascularity, whereas DNSSF-RA-8 treatment preserved cellular architecture, maintained tarsal integrity, and significantly reduced vascularization, further confirming its superior anti-arthritic efficacy.

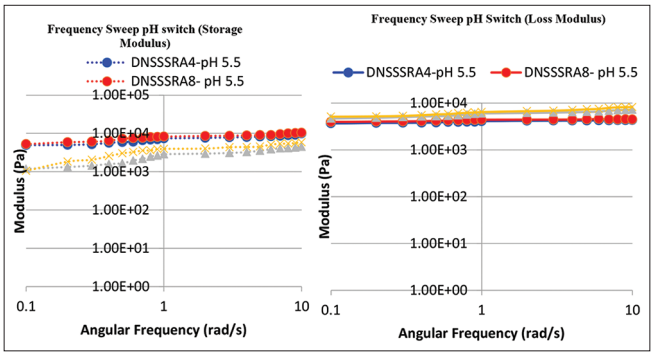

Figure 4: Rheology frequency sweep for pH switch of topical gels

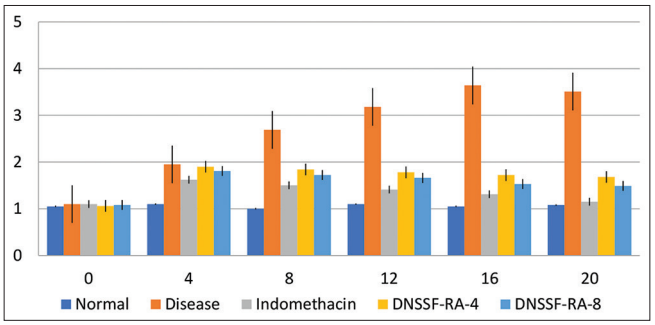

Figure 5: Effect of DNSSF-RA-4 and DNSSF-RA-8 in complete Freund's adjuvant-induced arthritis

Table 3: Influence of DNSSF-RA-4 and DNSSF-RA-8 on biochemical parameters in CFA-induced arthritis

Groups	SGOT (IU/L)	SGPT (IU/L)	Albumin (g/dL)	Calcium (mg/dL)	Phosphorus (mg/dL)	Rheumatoid factor (IU/L)
Normal	62.14±0.47	32.5±0.42	4.87±0.23	11.39±0.2	7.81±0.19	8.66±0.17
Disease	107.16±0.30*	88.16±0.3*	2.38±0.24*	4.89±0.37*	2.53±0.18*	18±0.50*
Indomethacin	67±0.44*	41.6±0.4*	4.52±0.26	9.61±0.15*	7.11±0.25	9.28±0.21
DNSSF-RA-4	76 80±0.55*	54±0.36*	3.82±0.18*	8.68±0.24*	6.02±0.19*	10.4±0.39*
DNSSF-RA-8	80±0.36*	61.16±0.47*	4.13±0.21	7.93±0.21*	5.38±0.24*	12.25±0.22*

Values were expressed as mean±SEM (n=6). Statistical analysis was performed by using ANOVA followed by Dunnett's t-test by comparing with normal control. Significant values were expressed as control group (*P<0.05). SGOT: Serum glutamic-oxaloacetic transaminase, SGPT: Serum glutamic pyruvic transaminase, ANOVA: Analysis of variance, SEM: Standard error of the mean

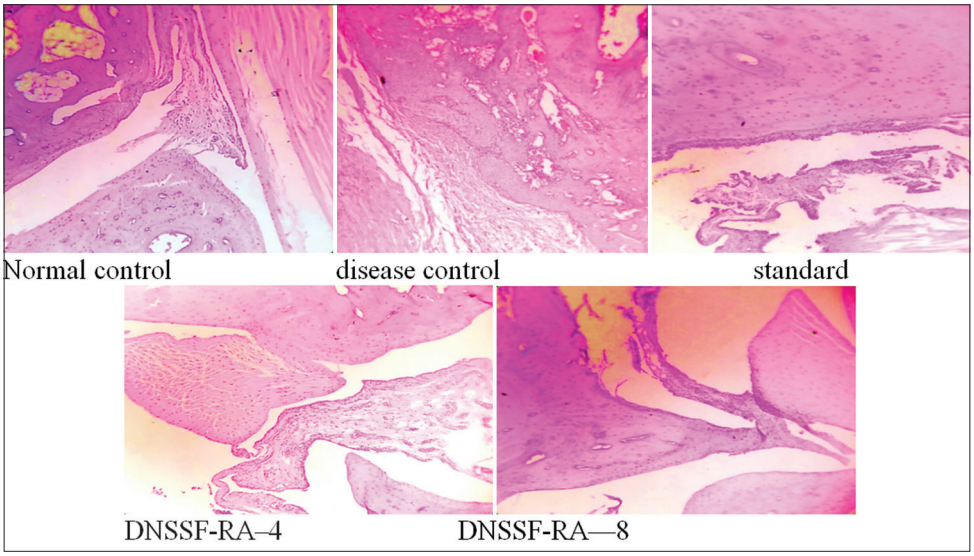

Figure 6: Histopathological studies of formulations

CONCLUSION

The optimized DNS topical gel and ME formulations demonstrated significant potential for enhanced drug delivery and therapeutic efficacy. RSM enabled the systematic optimization of key formulation parameters, ensuring stable emulsions with nanoscale globule sizes, optimal zeta potential, and sustained drug release. The incorporation of penetration enhancers, such as isopropyl myristate and diethylene glycol monoethyl ether, facilitated improved transdermal drug permeation, while PC and glyceryl stearate contributed to formulation stability and controlled drug diffusion. The *in vitro* permeation study confirmed prolonged drug release over 24 h, highlighting the formulation's potential for extended therapeutic action. In addition, the CFA-induced arthritis model validated the anti-inflammatory efficacy of DNSSF-RA-4 and DNSSF-RA-8, with DNSSF-RA-8 demonstrating superior anti-arthritic activity by reducing paw inflammation, improving biochemical markers, and preserving joint histology. Overall, the formulation of a chitosan-based topical gel containing DNS showed considerable potential for future research. Advancements may include refining the formulation to improve skin absorption and enhance localized delivery to affected joints. This approach could serve as a non-invasive substitute for injectable therapies in managing RA. Subsequent investigations might focus on incorporating nanocarriers, conducting animal studies, and evaluating clinical performance to support its effectiveness in treating chronic inflammatory disorders.

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