Transdermal delivery of solid lipid nanoparticles of ketoprofen for treatment of arthritis

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ABSTRACT
Ketoprofen (KP) is a 2-(3-benzolphenyl) propionic acid with anti-inflammatory, analgesic and antipyretic properties. It belongs to BCS Class II drug. It also has a short half-life of 120 minutes. Drugs acidic nature causes gastric irritation which is a major limitation. The present work aims to develop and evaluate solid lipid nanoparticles (SLN) to provide transdermal drug delivery. SLN loaded gel will enhance the solubility of Ketoprofen thereby increasing bioavailability giving controlled drug release. Solid lipid nanoparticles were prepared by solvent injection followed by probe sonication method. Cetyl palmitate was used as lipid and Tween80 as a surfactant. Batches were prepared by varying the concentration of the lipid phase and the surfactant phase. The solid lipid nanoparticles were evaluated for particle size analysis, drug entrapment efficiency, zeta potential and in vitro drug release study. Differential scanning calorimetry (DSC) and Powder X-ray diffraction (PXRD) study were done to study crystallinity behavior.SLN was studied for its anti-inflammatory activity. F4 batch of SLN was incorporated into gel and evaluated for drug content, pH, viscosity, in-vitro diffusion and ex-vivo diffusion study. SLN were successfully prepared. Among the batches F1-F9, F4 batch was selected based upon the size, entrapment efficiency, stability and drug release. The resultant solid lipid nanoparticles showed entrapment efficiency of 78.24%. The solubility was improved by 50 fold. The particle size was 250 nm, PDI was 0.398 and zeta potential -21.98mV. In-vitro drug release of gel from F4 SLN batch showed controlled drug release in 8 hours. Transdermal delivery of SLN retaining its anti-inflammatory activity was successfully developed.

Keywords: Nanoparticles, Solid lipid, Ketoprofen, Transdermal, Delivery

1. INTRODUCTION
Solid lipid nanoparticles (SLN) are colloidal carrier systems ranging from 50 to 1000 nm made up of a high melting point lipid as a solid core coated by surfactant. The increased attention for SLN is due to its unique properties like biocompatibility, feasibility of large scale production, stability, tolerability, lower cytotoxicity, site specific targeting and enhancement of bioavailability of drug. SLN combines the advantages and simultaneously avoid the limitations of polymeric nanoparticles, fat emulsion and liposomes [1, 2]. Compared with other vehicles such as cream, tincture and emulsion, SLN loaded gel combines advantages such as controlled release, negligible skin irritation and protection of active compounds. Especially, SLN can favor drug penetration into the skin, maintain sustained release to avoid systemic absorption, act as an ultraviolet (UV) sunscreen system and reduce skin irritation. SLN additionally found to own a skin targeting effect [3, 4].

As the science of SLN technology has progressed, several approaches for the preparation of SLN dispersions have been reported since these carriers were first described in the early 1990s. The preparation technique has a significant role in the performance of the colloidal formulation. The choice of preparation technique for SLN dispersions may be influenced by physiochemical property of drug, lipid, stability and production equipment [5].

The production techniques can be categorized into two groups; techniques which require high energy for dispersion of the lipid phase (such as high pressure homogenization, high shear homogenization and ultrasonication) and techniques which require precipitation of nanoparticles from homogenous systems (such as microemulsion, solvent-based techniques, membrane contactors and coacervation techniques). Selection of the technique depends upon several factors stated above and desired SLN properties. In current research work, solvent injection method approach is used for the successful formation of SLN [6-8].

Ketoprofen (KP) is a 2-(3-benzolphenyl) propionic acid with anti-inflammatory, analgesic and antipyretic properties. Ketoprofen belongs to NSAID category. It is used in the treatment of acute and chronic rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, primary dysmenorrhea and muscolotendinous trauma (sprains and strains), postoperative (including dental surgery) or postpartum pain. They are frequently used various application as prescribed prescription and over-the-counter medication. Despite of wide application, poor solubility, short half-life and high first-pass metabolism limits its therapeutic use [9].

Developing solid lipid nanoparticles of the drug will enhance solubility and thereby its efficacy. It will also be helpful in improving the dissolution profile of the drug and thereby the extent of action. This nanoparticle will protect the drug from gastric irritation if given orally and bypasses the same on transdermal delivery. Moreover, developing this SLN and incorporation of same in the gel will give controlled release. SLN avoids first pass metabolism reducing the frequency of dosing. It also improves the therapeutic efficiency of the drug. Ketoprofen penetrates the skin in amount to be effective for topical treatment
of localized inflammation and musculoskeletal diseases. Hence, it will lead to improvement in patient compliance [9]. The present work aims to develop and evaluate solid lipid nanoparticles to provide targeted drug delivery. SLN loaded gel will enhance the solubility of drug thereby increasing bioavailability with controlled drug release.

2. EXPERIMENTAL SECTION

Material.
Ketoprofen was gift sample from BEC Chemicals Pvt. Ltd. Mumbai. Precirol® ATO 5 was a gift sample from Gattefosse Pvt Ltd. Mumbai. Cetyl Palmitate, tween 80 and span 60 were purchase from Pure Chem laboratories. HPMC K4 was gift sample from Colorcon Asia Pvt. Ltd. Mumbai. All other chemicals were of analytical grade.

Methods.
Screening of lipids and surfactant for Solid lipid nanoparticles. The saturation solubility of Ketoprofen in different lipids as cetyl palmitate, Percirol ATO 5, compritol ATO 888, glyceryl monosterate and stearic acid was studied. Similarly solubility was studied in surfactant span 60, span 20, lecithin and tween 80. For saturation solubility study a fixed amount of lipid was taken in a test tube. Then ketoprofen was added in increments of 10 mg and test tube was heated in a water bath with shaking at a temperature above the melting point of solid lipid. The amount of ketoprofen in molten state solubilized in the lipid was noted. The complete dissolution was confirmed by the formation of a clear, transparent solution. The same method was used for the saturation solubility study of ketoprofen in lipophilic surfactant [10].

Preparation of solid lipid nanoparticles. Ketoprofen loaded SLN dispersions were prepared using a solvent injection method. The lipid phase was prepared by melting lipid and lipophilic surfactant together, i.e., cetyl palmitate and span 60 respectively. Cetyl palmitate and span 60 were varied in the ratio 1:10, 1:20 and 1:30 respectively. Ethanol was then added in a previously melted lipid phase. This phase was heated to 60°C to melt the lipid. Then the weighed amount of ketoprofen (10 mg) was added in above molten solution [10-11]. This solution was taken in a glass syringe and was injected rapidly into the aqueous phase containing 1% tween 80 (having the same temperature as that of lipid phase) and sonicated for 3 minutes using Probe Sonicator (Sonics Model VCX 750). The formed dispersion was stirred on magnetic stirrer (REMI) for 30 minutes at 600 rpm, to get nanoparticles dispersion. Supernatant was collected as it contains SLN and was then dried in Petri dish to obtain SLN. The composition of different batches is as shown in table 1.

Characterization of SLN.
Entrapment efficiency and drug loading efficiency. Free ketoprofen (unentrapped) in the SLN dispersion was sediment by controlled centrifugation at 4500 rpm for 60 minutes using Remi centrifuge. SLN dispersion was decanted without disturbing the ketoprofen pellet. SLN dispersion and Ketoprofen sediment were used for estimation of entrapped and unentrapped drug content, respectively. Fixed volume of SLN dispersion and ketoprofen sediment were dissolved separately in methanol and analyzed for entrapped and unentrapped drug content using UV spectrophotometer at 255 nm. Entrapment efficiency and drug loading efficiency was calculated using equation 1 & 2 respectively. This procedure was performed in triplicate [9].

Table 1. Composition of batches of SLN.

<table>
<thead>
<tr>
<th>Batch</th>
<th>Ketoprofen (mg)</th>
<th>Cetyl palmitate (mg)</th>
<th>Span 60 (mg)</th>
<th>Ethanol (ml)</th>
<th>1% Tween 80 (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>10</td>
<td>100</td>
<td>25</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>F2</td>
<td>10</td>
<td>200</td>
<td>25</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>F3</td>
<td>10</td>
<td>100</td>
<td>30</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>F4</td>
<td>10</td>
<td>100</td>
<td>50</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>F5</td>
<td>10</td>
<td>200</td>
<td>50</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>F6</td>
<td>10</td>
<td>300</td>
<td>50</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>F7</td>
<td>10</td>
<td>100</td>
<td>75</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>F8</td>
<td>10</td>
<td>200</td>
<td>75</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>F9</td>
<td>10</td>
<td>100</td>
<td>50</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>F10</td>
<td>10</td>
<td>75</td>
<td>25</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>F11</td>
<td>10</td>
<td>75</td>
<td>25</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Blank</td>
<td>-</td>
<td>100</td>
<td>25</td>
<td>5</td>
<td>10</td>
</tr>
</tbody>
</table>

Formula:

i. Entrapment efficiency

Entrapment efficiency = (W_initial ketoprofen – W_free ketoprofen) ÷ (W_initial ketoprofen) ×100 Equation 1

Where, W is quantity of Ketoprofen content in mg

ii. Drug loading

Drug loading (g%) = Ketoprofen entrapped in SLN × 100
Amount of ketoprofen entrapped+Amount of lipid added

Equation 2

Particle size analysis- Preliminary study. The size of the formulation was analyzed by an inverted microscope Carl Zeiss microscope (Primovert 124 DC). The dispersion of SLN was put on the clean slide and observed under microscope. The under live microscope window on ZEN 2 software by setting a parameter to get the desired image having particles distributed under microscope. This slide was focused on images and size distribution was analyzed by measuring the size of the particles. Measurement of particle size, polydispersity index (PDI) and zeta potential. The average size, polydispersity and zeta potential of F4 batch was determined by photon correlation spectroscopy (Malvern Zeta sizer, Nano ZS; Malvern Instruments). Measurements were carried with an angle of 90 degrees at 25°C[12].

Scanning electron microscopy. Globule size of F4 batch was measured by using FE-SEM (SEM, JOEL, JSM-700 1F). The sample was placed in sample holder of FE-SEM and images were captured at various magnification. Size was measured and morphology was studied [12].

Fourier transform infrared (FTIR) spectroscopy. To elaborate on drug excipient compatibility FTIR spectrophotometer was employed. Analysis of respective IR spectra for the peaks was obtained for ketoprofen, lipid Cetyl palmitate, SLNs and physical mixture of ketoprofen/lipid (1:10). The KBr pellets were prepared on KBr disc and then the spectra of pellets were taken on IR.
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spectrophotometer (Varian 4640). The IR spectra were compared and analyzed [12].

Differential scanning calorimetry (DSC). DSC thermogram was recorded using differential scanning calorimeter (Hitachi 7020). Approximately 2-5 mg of sample was heated in a pierced aluminum pan up to 200°C at a heating rate 10°C/min under a stream of nitrogen at a flow rate of 30 ml/min. Thermal data analysis was then done by DSC thermogram. The crystallinity indexes (CI %) of SLNs was calculated using the equation 3 [7,12].

\[
\text{Melting enthalpy of SLN (J/g)} = \frac{\text{Melting enthalpy of bulk material without KP (J/g)} \times \text{Concentration of lipid phase (%)}}{100}
\]

Equation 3

Powder X-Ray diffraction (PXRD). The crystalline nature and characteristics of the Ketoprofen and SLN were analyzed by powder x-ray diffraction. Ketoprofen and Ketoprofen loaded SLN were studied for X-ray diffraction spectra using a diffractometer. The scan parameters were set at scan speed. Samples were placed on glass sample holder and scanned from 5° to 60° with an angular scan speed of 5 second [10,12].

Refractive index. Refractive index of a substance is ordinarily determined by measuring the change in direction of collimated radiation as it passes from one medium to another. Refractive index of SLN dispersion was measured by using Abbe’s Refractometer [7].

Solubility measurement of Ketoprofen and ketogenic-SLN. The equilibrium solubility of the pure ketoprofen in water was determined by the traditional shake flask method. According to this method the ketoprofen was added in surplus to 2 ml of water and shaken for 24 hours at 37 °C until equilibrium reached. The saturation was confirmed by observation of the presence of undissolved material. After separation of the solid by filtration through Whatmann filter paper, the concentration of the ketoprofen in the filtrate was determined by UV Vis Spectrophotometer at 260 nm against known standard concentration preparation. For the estimation of the soluble fraction of ketoprofen in Ketoprofen loaded SLN, 5 ml of the suspension was taken and filtered through Whatmann filter paper. Thereafter, a filtered solution containing Ketoprofen SLNs, solubility of ketoprofen was determined from the above solution against the known standard at 260 nm spectrometrically. This procedure was performed in triplicate [10-11].

In-vitro drug release (Dialysis study). Drug release study through dialysis membrane. Dialysis bag method was used to study the drug release using a Phosphate buffer 7.4 as the dissolution medium. The dialysis bag (Dialysis membrane 50) was soaked in phosphate buffer 7.4 for 12 hours before use. Two milliliter of SLN dispersion (equivalent to 1-2 mg/ml as per entrapment efficiency) was poured into the dialysis bag. The bag was placed in a beaker containing 50 ml dissolution medium and stirred at a rate of 50 rpm. Aliquots of the dissolution medium were withdrawn at different time intervals of 15, 30, 45, 60, 120, 240, 360 and 480 minutes and were replaced with the same volume of fresh medium to maintain the sink conditions. The samples were analyzed for spectrophotometrically at 259 nm [13].

Release kinetics from SLN. Drug release from all batches was evaluated for the best fit model as zero order, first order, Higuchi, Hixson Crowell and Korsmeyer Peppas. The correlation coefficient ($R^2$) for all these models was found out. PCP disso v3 software was used for this release kinetic study.

Ex-vivo drug release. Ex-vivo drug release is a parameter to determine the difference in ketoprofen release from a plain ketoprofen solution and from SLN dispersion. The ex-vivo drug release from plain ketoprofen solution and that from SLN dispersion were determined. For this purpose, fresh chick intestine was brought in tyrode solution from the slaughter house and the ileum portion of the chick intestine was separated carefully. This ileum part was rinsed with tyrode solution and then placed in fresh tyrode solution. One end of the ileum was tightly sealed with a thread. Through the other open end of the ileum, plain ketoprofen solution was filled in the ileum such that the filled amount was equivalent to 10 mg of Ketoprofen. Similarly, another ileum was filled with SLN dispersion and both the ileum portions were sealed. These portions were tied to a loop and were separately placed in two jars of USP dissolution apparatus (type 2). The jars were filled with 900 ml of distilled water and the dissolution apparatus was run for a period of 2 hours with continuous aeration. Dissolution media used was 900 ml of distilled water. The temperature of the bath was maintained at 37±0.5°C with aeration speed 1-2 bubbles/sec. Samples were withdrawn at predetermined times interval. The amount of ketoprofen released from the SLN dispersion and plain ketoprofen solution was estimated spectrophotometrically at 260 nm by taking distilled water as blank. This procedure was performed in triplicate [14].

Dissolution study. Solubility test was performed with 1 mg of Ketoprofen drug and 1.5 ml (equivalent to 1 mg) SLN dispersion F4 batch was introduced in the vessels with 900 ml distilled water. The above solutions were then set in the dissolution system with 50 rpm for 2 hours in a water bath kept at 37.0±0.05°C. The sampling was done at an interval of 15, 30, 45, 60 and 120 minutes. Concentrations were determined by measuring absorbance at 260 nm and interpolated from respective calibration curves. This procedure was performed in triplicate [10-11].

In-vitro anti-inflammatory activity by protein denaturation inhibition.

The reaction mixture consists of 0.45ml of bovine serum albumin (5% aqueous solution) and 0.05 ml of ketoprofen solution of different concentration namely 3, 6,9,12 and 15 µg/ml. pH of the above solution was using IN HCl. These samples were incubated at 37°C for 20 minutes. Further they were heated for 5 minutes at 57°C. All the samples were cooled at room temperature and 2.5 ml of phosphate buffer solution was added in all test tubes. Turbidity was measured spectrophotometrically at 273 nm. Control test was prepared by taking 0.05 ml of distilled water instead of ketoprofen solution whereas product control test lacked bovine serum albumin. A similar procedure was followed for Marketed gel ketoprofen (Fastum gel® manufactured by Menarini India
Pvt.Ltd.) and SLN to evaluate its anti-inflammatory activity [15]. This procedure was performed in triplicate. The percent inhibition of protein denaturation was calculated using equation 4.

\[
\text{% inhibition} = 100 \times \frac{\text{Absorbance of sample} - \text{Absorbance of blank}}{\text{Absorbance control}}. 
\]

Equation 4

The control represents 100% of protein denaturation. The result was compared with Diclofenac sodium [15].

**Stability study.** Short term accelerated stability of SLN was studied after 1 month whereas long term stability studies were carried out after 3 months at (30 °C ± 2 °C, 65% RH ± 5% RH) and accelerated (40 °C ± 2 °C, 75% + 5% RH). After storage, samples were tested for their physical appearance, consistency, entrapment efficiency and ketoprofen loading.

**Preparation of gel batches.**

Gel was prepared by adding HPMC in warm distilled water. HPMC was soaked and kept overnight for complete hydration of polymer. Then the equivalent of SLN dispersion (0.1% w/w) and dried SLN were added to the weighed amount of gel. It was added with the aid if stirring to ensure uniform dispersion. Triethanolamine was slowly added drop by drop to form a fine gel after complete dispersion to balance the pH of the gel. Similarly, ketoprofen loaded gel was formed. SLN batch F4 and F5 were coded as G4 and G5 for the SLN loaded gel batches respectively. The batches were prepared as shown in table 2. This procedure was performed in triplicate [11].

### Table 2. Composition of gel.

<table>
<thead>
<tr>
<th>Batch</th>
<th>Method and SLN code</th>
<th>HPMC %</th>
<th>Plain ketoprofen (w/w) %</th>
<th>SLN (Qty of ketoprofen equivalent to w/w) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>Gel</td>
<td>6</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>G2</td>
<td>Gel</td>
<td>6</td>
<td>0.1</td>
<td>1</td>
</tr>
<tr>
<td>G3</td>
<td>Gel</td>
<td>10</td>
<td>0.1</td>
<td>0.1 (Dispersion)</td>
</tr>
<tr>
<td>G4</td>
<td>SLN (F4)</td>
<td>8</td>
<td>-</td>
<td>0.1 (Dispersion)</td>
</tr>
<tr>
<td>G5</td>
<td>SLN (F5)</td>
<td>8</td>
<td>-</td>
<td>0.1 (Dispersion)</td>
</tr>
</tbody>
</table>

**Evaluation of gel.**

**Appearance.** Prepared SLN based gels were inspected for their color, homogeneity, consistency.

**Viscosity.** The viscosity of SLN loaded gel was determined by using Brookfield’s viscometer.

\( pH \) : pH of gel was checked by using a digital pH meter at room temperature. pH meter was calibrated and then electrode of pH meter was dipped in 1% solution of gel and the pH was noted.

**Spreadability.** Gel (0.5 g) was placed beneath the glass slide. A weight of 2 g was allowed to rest on the upper glass slide for 1 minute. The increase in the diameter due to spreading of the gel was noted. Spreadability was then calculated by using the equation 5.

\[
S = \frac{M.L}{T} \quad \text{Equation 5}
\]

Where, \( S \) = Spreadability, \( M \) = weight attached to upper slide, \( L \) = length of spread, \( T \) = time taken

**Drug content.** Ketoprofen content in SLN loaded gel was determined by dissolving a known quantity of the formulation in methanol by sonication. Solution was stirred for 5 hours on magnetic stirrer to ensure complete dissolution of formulation in methanol. Later, the solution was filtered through Whatmann filter paper and their absorbance is measured at 255 nm using UV-Vis spectrophotometer. Ketoprofen content was expressed as a percent of ketoprofen concentration obtained. Ketoprofen concentration was calculated using the following equation 6. This procedure was performed in triplicate.

\[
y = mx + c 
\]

Equation 6

Where, \( x = \) concentration in μg/ml. \( y = \) absorbance of solution at 255 nm \( c = \) intercept of calibration curve. \( m = \) slope of calibration curve

**In-vitro diffusion (release study).**

i. **In-vitro diffusion study: Cellophane membrane:**

Franz diffusion cell was used for permeation study. Cellophane membrane number 12, having an average pore size of 2.4 nm, mol. wt. approx. 12,000 Dalton and capacity of approx. of 1.61 ml/cm was utilized in permeation. Cellophane membrane (cut to suitable size) was stored in phosphate buffer pH 7.4 for 24 hours before use. Cellophane membrane was placed in between donor and receptor compartment. Equivalent amount of gel containing 1 mg of ketoprofen loaded SLN was accurately weighed and spread on cellophane membrane (donor compartment). Entire surface of membrane containing formulation was in contact with receptor compartment containing 25 ml of phosphate buffer pH 7.4. Cell was agitated by a magnetic stirrer at 50 rpm and maintained at 37±1°C. Aliquots of 2 ml were withdrawn at intervals of 15, 30, 45, 60, 120, 180, 240, 300, 360, 420 and 480 minutes and each time was replaced with an equal volume of fresh phosphate buffer pH 6.8, previously heated to 37±1°C. The absorbance of samples was measured at 260 nm. This procedure was performed in triplicate.

ii. **In-vitro diffusion study: Egg membrane.** Similar to in-vitro diffusion study, another study was carried out using egg membrane. Raw egg was taken and a small hole was made at bottom to remove all its contents. Then egg shell was dipped into 0.1 N HCl for 2 hours. Egg shell was dissolved and membrane was collected. Concentrated HCl was added. Fresh egg membrane was then washed with distilled water. Freshly separated egg membrane was used each time for the study. All experimental conditions were similar to cellophane membrane studies. This procedure was performed in triplicate [11].

iii. **In-vitro diffusion study: Goat skin.** Fresh dorsal skin of goat was obtained from slaughter house. Skin hair was removed and hydrated by keeping it in a physiological salt solution. Subcutaneous fat was carefully removed with scissors. This skin of goat was mounted on donor compartment with epithelium facing donor compartment. Receptor compartment was filled with phosphate buffer solution pH 7.4 and maintained at 37±0.5°C and cell was agitated by using magnetic stirrer. 1 gm of SLN loaded gel was placed over it and spread evenly and ketoprofen permeation study was carried out in the similar to cellophane membrane. This procedure was performed in triplicate [11].
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Permeation data analysis (Flux). Permeation rate of drug i.e. flux of ketoprofen was also found by using PCP disso v3 software. Flux is the amount of drug permeated through skin per unit time at a steady state.

Similarity factor. Similarity factor was calculated using the cumulative % drug release through cellophane membrane for batch F4 and the marketed formulation (Fastum Gel® manufactured by Menarini India Pvt.Ltd.). Ketoprofen release rate was calculated and compared by finding out the similarity factor (f2) which was calculated by using BIT SOFT software.

Stability studies of SLN loaded Gel. Short term accelerated stability of SLN loaded gel was carried for 1 month whereas long term stability study was carried out after 3 months at (30 °C ± 2 °C, 65% RH ± 5% RH) and accelerated (40 °C ± 2 °C, 75% + 5% RH). After storage, samples were tested for their physical appearance, consistency and ketoprofen content.

3. RESULTS SECTION

Screening of lipids and surfactant for Solid lipid nanoparticles.
The data of solubility study in different lipid and surfactant is depicted in table 3 below.

Table 3. Saturation solubility data in lipids and surfactant.

<table>
<thead>
<tr>
<th>Sr.no.</th>
<th>Lipid</th>
<th>Solubility (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10 mg</td>
</tr>
<tr>
<td>1</td>
<td>Cetyl palmitate</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Percitol ATO 5</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Compritol ATO 888</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Glycerol Monosterate (GMS)</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Stearic acid</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>Solubility (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 mg</td>
</tr>
<tr>
<td>6. Span 60</td>
<td>+</td>
</tr>
<tr>
<td>7. Span 20</td>
<td>+</td>
</tr>
<tr>
<td>8. Tween 80</td>
<td>+</td>
</tr>
</tbody>
</table>

+ denotes solubility of Ketoprofen and – denotes insolubility of Ketoprofen

The solubility study was performed to assess the solubility of Ketoprofen in the lipid as a qualitative test. Table no 3 gave a clear idea that cetyl palmitate and stearic acid had high solubility as compared to other lipids. It was performed on observation whether the solution remains clear or become turbid on the addition of Ketoprofen. Thus higher solubility of the Ketoprofen was observed for Cetyl palmitate and was further used for evaluation. It was observed that Ketoprofen and Span 60 had good solubility respectively [16-17].

Preparation of solid lipid nanoparticles.
Drug-free and drug loaded SLN were successfully prepared from solvent injection method. Cetyl palmitate was used as solid lipid core material. Two simultaneous effects contribute to the effective formation of SLN. Firstly, gradual solvent diffusion out of lipid-solvent droplets into water causes reduction of droplet size and simultaneously increases lipid concentration. Secondly, diffusion of pure solvent from the lipid-solvent droplet causes local variations in the interfacial tension at droplet surface, inducing a reduction of size of droplets. In this process particle size of SLNs can be influenced and controlled by variation of process parameters such as injected solvent, lipid concentration, injected volume of solvent, lipid concentration in the solvent phase and viscosity of the aqueous phase [10,13].

This involved use of cetyl palmitate as lipid in batches based on solubility analysis study and Span 60 as lipophilic surfactant in the lipid phase. Tween 80 was used as a surfactant in aqueous phase.

Batches were initially prepared at 600 rpm which gave SLN size in microns. Further increase of speed to 1000 rpm gave results in nanometer. Use of 1:3 and 1:5 ratio had turbidity with low entrapment efficiency. Ratio of 1:10 for a drug: lipid proved to be efficient batch. This method was further explored for various concentrations of lipid and lipophilic surfactant. Lipid concentration did not affect the entrapment efficiency to a greater extent. It was observed that as the concentration of lipid increased, the size of the nanoparticles increased which was the main reason behind the difference in the entrapment efficiency. Thus the lowest concentration i.e 1:10 ratio seems to be effective. The prepared batches were evaluated for appearance, entrapment efficiency, loading efficiency, size and dialysis studies. F4 had the highest entrapment efficiency and the nanoparticles were stable on storage. Batch F1, F2, F4 and F5 were found to be good and similar to desirable characteristics namely entrapment efficiency, size and drug release (table 4). F1 and F2 batch had large particle size and shown slight turbidity. F1 showed sign of phase separation on storage after 30 days. Further drying of this dispersion F4, F5, F8 and F9 batches shown good characteristics and were stable. The criterion of pattern of release and duration was also taken into consideration. Batch F7, F8 and F9 showed the lowest entrapment (table 4). F4 and F5 batch was further selected as it had a smaller size distribution and shown good reproducibility. It was stable for long duration and had the necessary characteristics for stable SLN. These batches of SLN were incorporated in gel and were further evaluated for various parameters.

Characterization of SLN.

Entrapment efficiency. The entrapment efficiency and drug loading of all the batches are reported (table 4). Ketoprofen is a hydrophobic drug that showed maximum solubility in cetyl palmitate and was selected to achieve higher entrapment and minimum drug leakage [9]. In addition it was found that the addition of span 80 increased the entrapment efficiency due to reduction on particle crystallinity. Thus, F4 batch highest entrapment efficiency and drug loading efficiency along with the lowest particle size.

Particle size analysis: Preliminary study. The morphology and size of SLN were analyzed using an inverted HDcam microscope for preliminary determination of particles size. The photos were taken and Average size was calculated by taking measurement of few particles. F4 batch had the least particles size (table 4).

Particle Size, poly dispersity index and Zeta potential. F4 batch showed a particle size of 368 nm and PDI value of 0.398 which
are in acceptable limit. This indicated narrow distribution of particle size (Figure 1A). These values are in range which impacts the release and stability of the SLN system. The zeta potential was found to be -21.8 mV which depict the physical stability of the SLN (Figure 1B).

In principle, smaller particles are more likely to remain stable and have higher encapsulation ability. It is based on the basic principle that smaller particle has a higher surface area that imparts physical stability by allowing more of surfactant molecules to cover the particles.

PDI is measure of particle homogeneity and it varies from 0.0 to 1.0. If PDI value closer to 0.0, it indicates narrow size distribution. Zeta potential indicated electrostatic stability [9, 12].

The ideal value of zeta potential ranges from -30 mV to +30 mV. It is believed that when the zeta potential is value more positive than +30 mV or more negative than -30 mV are normally considered as stable. The general dividing line between stable and unstable colloidal dispersion is taken at either +30 or -30 mV. In this case, the values suggest that formulation possess good physical stability and particle aggregation may not occur due to electrostatic or steric repulsion between the particles [18].

**Table 4.** Entrapment efficiency of SLN batches.

<table>
<thead>
<tr>
<th>Batch</th>
<th>Mean particle size (µm)</th>
<th>Entrapment efficiency (%)</th>
<th>Drug loading (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>6.62</td>
<td>69.24±1.44</td>
<td>6.47</td>
</tr>
<tr>
<td>F2</td>
<td>3.135</td>
<td>72.61±2.45</td>
<td>3.503</td>
</tr>
<tr>
<td>F3</td>
<td>2.963</td>
<td>99.31±2.44</td>
<td>3.35</td>
</tr>
<tr>
<td>F4</td>
<td>1.75</td>
<td>82.24±0.74</td>
<td>6.50</td>
</tr>
<tr>
<td>F5</td>
<td>1.94</td>
<td>37.76±1.33</td>
<td>3.48</td>
</tr>
<tr>
<td>F6</td>
<td>1.8</td>
<td>72.33±1.11</td>
<td>3.03</td>
</tr>
<tr>
<td>F7</td>
<td>2.0</td>
<td>31.33±1.89</td>
<td>3.04</td>
</tr>
<tr>
<td>F8</td>
<td>1.95</td>
<td>38.90±2.75</td>
<td>1.90</td>
</tr>
<tr>
<td>F9</td>
<td>1.7</td>
<td>40.27±3.45</td>
<td>1.32</td>
</tr>
<tr>
<td>F10</td>
<td>7.65</td>
<td>25.52±2.44</td>
<td>6.50</td>
</tr>
<tr>
<td>F11</td>
<td>6.89</td>
<td>30.62±1.14</td>
<td>6.30</td>
</tr>
<tr>
<td>Blank</td>
<td>1.9</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Figure 1.** Particle size and zeta potential measurement A. Particle size B. Zeta potential.

Scanning electron microscopy. FE-SEM image of dried SLN batch F4 showed particle size 250 nm and had a uniform size distribution (Figure 2). Many researchers have used SEM to study the morphology of the SLNs. The SLNs may not maintain their shape and integrity in FESEM analysis due to the high energy electrons used in the analysis and the vacuum applied in the FESEM chamber. The results obtained here, therefore, should be considered qualitative and not an absolute estimation of the actual shape and size of the particles [9].

**Figure 2.** SEM image for SLN.

Infrared Spectrophotometry. The IR spectra of Ketoprofen, lipid and SLN prepared were studied to characterize the band of the functional group of all the three respectively.

**Figure 3.** IR spectra of Drug, lipid and SLN.

Scan A (Figure 3) of pure drug Ketoprofen had characteristics band at1437, 1595, 1695, 2930,730 cm⁻¹ as described above.

Scan B (Figure 3) of cetyl palmitate had characteristics band at 2950, 1640, 1000 cm⁻¹.

Scan C (Figure 3) of SLN had band at 3000, 1640, 1560 and 900 cm⁻¹.

In IR spectra of SLN Figure 3C, it was observed that some of the bands of drug are of reduced intensity and some bands had disappeared which indicates that Ketoprofen is incorporated in the lipid thus forming SLN. The doublet band at 1695 cm⁻¹ of Ketoprofen was converted to singlet. Also, the multiple bands in 900-1000 cm⁻¹ were diminished in SLN spectra. A decrease in intensity of band at 1695 cm⁻¹ was observed for the Ketoprofen. Also, the peak at 3000 cm⁻¹ and 1640 cm⁻¹ of cetyl palmitate was observed in SLN spectra.

The diminished band of a drug in SLN spectra signifies the masking of a drug molecule with lipids matrix thereby proving physical interaction between SLN and drug. In SLN preparation it was observed by FTIR that when Ketoprofen was encapsulated, these characteristic peaks disappeared. This is mostly due to interactions between the reactive groups of Ketoprofen and the lipid matrix. Hence these typical bands probably were masked by the matrix and proving definitively physical and not chemical interaction between the excipients and Ketoprofen. Similar finding has been previously reported by Pindato for the rosmarinic acid formulation of SLN and Witepsol was used as lipid. Also, it was studied for Ketoprofen with polymeric drug particles [7, 12,16].

Differential scanning calorimetry. Differential scanning calorimetry (DSC) was used to study and obtain the information
on the polymorphism and the degree of crystallinity of SLN. DSC profile of Ketoprofen, lipid and SLN are depicted in figure 4 and data obtained from these profiles are summarized in table 5.

Table 5. DSC results of SLN.

<table>
<thead>
<tr>
<th>Material</th>
<th>T\textsubscript{onset} (°C)</th>
<th>T\textsubscript{max} (°C)</th>
<th>H (mJ/mg)</th>
<th>RI %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ketoprofen</td>
<td>92.5</td>
<td>94.5</td>
<td>93.45</td>
<td>-</td>
</tr>
<tr>
<td>Cetyl palmitate</td>
<td>51.2</td>
<td>57.3</td>
<td>197.0</td>
<td>100</td>
</tr>
<tr>
<td>Drug loaded SLN</td>
<td>50.9</td>
<td>53.6</td>
<td>29.2</td>
<td>49.40</td>
</tr>
</tbody>
</table>

The melting transitions of the DSC profiles indicate that they are solid in nature irrespective of drug loading. This is due to their melting at a temperature above or near 40° C. It confirms its presence in solid nature at normal body temperature. F4 batch has the melting transition 50.9 °C and RI value of 49.40.

Moreover, the reduction in melting transition observed for cetyl palmitate as compared to Ketoprofen-SLN can be attributed to reduce particle size resulting in the increased surface area i.e. small size or Gibbs-Thomson effect. It relates the particle size with thermal properties. A similar effect has been studied previously and reported [7]. The shift in melting transition of the endotherms could be due to the entrapped/adsorbed drug and surfactant molecules in the SLN. The DSC profiles of SLN showed transition at 50.9°C and did not show any melting transition at the melting point of the Ketoprofen at 94°C. As SLN did not show any melting transition at ketoprofen’s temperature it is evident that Ketoprofen was successfully incorporated into the lipid phase in a solubilized or dispersed form during the preparation of the SLN. DSC data shown in the table 6 showed a reduction in melting enthalpies of SLNs [7, 12].

The Reduced crystallinity (RI%) allows the understanding of the thermal behavior of materials, and is directly correlated with compound incorporation and release rate, where thermal behavior is different for lipid cetyl palmitate and the SLNs. Reduced crystallinity was calculated in terms of Recrystallization index (RI). Reduction of melting enthalpies (i.e. energy required for melting) attributes to an increased number of imperfection in the crystal structure of lipid RI (Reduced crystallinity). The reduced crystallinity is often correlated to increase in entrapment efficiency and drug loading capacity as observed.

In case of Ketoprofen it has a sharp endothermic peak at 94.01°. SLN of Ketoprofen showed endothermic peak at 50.9°. These all results indicate that Ketoprofen no longer present in the crystalline form may have got converted into the amorphous form. It also suggests that solubility of amorphous form better than crystalline form which is further confirmed by XRD study.

Powder X-Ray diffraction (PXRD). PXRD was performed to confirm the presence of degree of crystallinity. Pure drug (Ketoprofen) XRD showed high crystalline nature with multiple peaks and the principal peak of high intensity at 2θ=14.11°, 18.12° and 22.53° and medium intensity peak at 26.74° and 27.64° (Figure 5). These clear and sharp peaks indicated the presence of Ketoprofen in crystalline state. The XRD pattern of cetyl palmitate revealed one sharp peak at 21.2°, medium-intensity peaks at 6.7°and 24.3°. In XRD of SLN it was observed that peaks of lipid were reduced and shifted whereas the peak of Ketoprofen was disappeared indicating its presence in an amorphous or dispersed state. Absence of several peaks in SLN as compared to Ketoprofen indicated the formation of SLN. Various researchers studied this effect of analytical technique to confirm crystallinity by DSC and PXRD method had similar finding for the oryzanol drug. Clusters of small crystallites do not give strong peaks [19]. Also, these changes may be attributed to formulation parameter and heating/cooling processes. The peaks of SLN were present at 2θ value of 21.83° and 24.03°.

Where A is drug Ketoprofen, B is Cetyl palmitate and C is SLN

Thus, the above results indicate that Ketoprofen has sharp peaks at diffraction angles (2θ) between 0-60 showing a typical crystalline pattern. However, all major characteristic crystalline peaks appearing the diffractogram of SLN but of low intensity. Also, some of the peak gets disappeared. This indicates that some amount of Ketoprofen converts to its amorphous form [7, 19].

Refactrometry. RI value is ratio of the velocity of light in vacuum to its velocity in a specified medium. Refractive index of dispersing media for SLN i.e. water was 1.33 while that of SLN F4 batch was 1.412. From literature the refractive index value of lipid used Cetyl palmitate was found to be 1.455. The slight shift of refractive index value may be due to the presence of other components of the SLN formulation.
Solubility measurement of Ketoprofen and KP-SLNs. The solubility results showed that the aqueous solubility of Ketoprofen is 0.014761 mg/ml which indicates the practically insoluble nature of the drug. After the formation of SLN’s the solubility of KP-SLNs of batch F4 and F5 increased to 0.7156 and 0.4936 mg/ml, respectively which indicate an increase in solubility of 48.48 and 33.57 fold respectively [15].

In-vitro drug release (Dialysis study).

The dialysis procedure is routinely used to study the release of nanoparticles as there is no technique approved by the regulatory authority for the assessment of drug release of nanoparticles. Release data is depicted in Figure 6 for all the batches at a specified time interval. The release of all the batches of SLN and plain drug dispersion was studied by dialysis method. The release pattern of initial burst effect for 1 hour followed by a sustained release for next 7 hours was observed. Batch F1 and F2 showed complete release. Batch F3 had a much sustained release and only 50% of the drug was released within 8 hours. Batch F4- F6 had a complete release for 8 hours in a pattern of burst release followed by the sustained release [20-21].

Release kinetic [7]. Correlation coefficient value (R^2) of each formulation for zero order, first order, Matrix, Hixson Crowell and value of release exponent from Korsmeyer- Peppas model is as shown in table 6. The selection of a suitable model that fits release data is useful for evaluation of release characteristics of the system. Batch F1, F2 and F4 showed matrix type of release pattern. While all other batch, F3, F5, F6, F8 and F9 have Korsmeyer Peppas model. When the value of n is highest amongst all value, formulation follows Korsmeyer-Peppas model. ‘n’ is release exponent. Value of ‘n’ is used to characterize drug release mechanism. If n=0.5 transport mechanism is Fickian diffusion, 0.5-1 it is Non Fickian diffusion and 1 depicts Class II transport mechanism as per value of n and type of release as per Korsmeyer-Peppas model [22].

Ex-vivo release (chick ileum study). Ex-vivo drug release is a parameter which was used to study the difference in drug release from plain drug solution and from SLN. It was observed that the drug release from plain drug dispersion at the end of 2 hours was 11.67%. The drug release from SLN dispersion by F4 batch at the end of 2 hours was 86.39%. Releases of the drug from both the sample in studies are depicted in figure 7A below at regular interval. This indicates that drug release increases significantly in the SLN dispersion as compared to the drug release from plain drug dispersion. The increase in the release was due to reduced particle size which increases surface area and dissolution. Also the presence of surfactant improves the release of the drug from SLN as compared to the plain drug [23].

Dissolution study. The release of plain drug and SLN dispersion was 32.34% and 92.85% respectively within 2 hours (figure 7B). The dried nanoparticles of dispersion batch F4 had 71.53% release within 30 minutes. The increased release by 60% depicts that SLN prepared had improved solubility. The increase in release was due to reduced particle size which increases surface area and dissolution rate of the drug from SLN. Also, analytical studies like DSC and PXRD confirms the presence of SLN into amorphous form resulting in improvement of solubility.

Anti-inflammatory activity. Anti-denaturation study was performed using Bovine serum albumin (BSA). The basic principle of the study is that when BSA is heated, it undergoes denaturation of protein which results in expression of antigen related with hypersensitivity type III reaction which is related to disease like rheumatoid arthritis, serum sickness etc. Rheumatoid arthritis has protein denaturation as a cause of disease documented in literature. The autoantigen produced in this disease is due to protein denaturation. Mechanism of protein denaturation involves alteration of electrostatic hydrogen, hydrophobic and disulphide bonding. Ketoprofen samples at different concentrations provided significant protection against denaturation of proteins. The result of Ketoprofen under study was compared to standard drug diclofenac sodium. There was 99.8% inhibition at 15µg/ml of Diclofenac sodium used as a standard drug. Inhibition percentage at various concentrations of 3, 6, 9, 12 and 15µg/ml for pure drug Diclofenac Sodium and Ketoprofen were studied. SLN F4 Batch and Marketed Ketoprofen gel (Fastum Gel) were evaluated for the inhibition and had 94.55 and 92.52 % respectively (Figure 8). The results depicted that SLN prepared had better anti-inflammatory activity as compared to marketed preparation and pure ketoprofen drug. From the result of the present study, it can be stated that SLN maintains the activity as compared to plain drug and marketed preparation. Therefore it was concluded that SLN maintains the activity by controlling the production of auto antigen and thereby inhibiting protein denaturation [15].

![Figure 6: Drug release by dialysis method.](image1)

![Figure 7: A) Ex-vivo release study; B) Dissolution study.](image2)

![Figure 8: Comparison of protein denaturation (% inhibition) Ketoprofen solution, SLN and marketed preparation.](image3)
Stability study. From stability studies for F4 it was observed that there was no significant change in evaluation parameters before and after study. It showed entrapment efficiency of 82.15±2.00% and drug loading 6.43 after 3 months which was periodically evaluated with the interval of 30 days at various temperatures. Also, it is indicated that the appearance of the formulation was clear colloidal dispersion and homogenous consistency.

Evaluation of SLN loaded gel. Results of physical evaluation and other parameter described further. Batches G4 and G5 showed formation of a firm gel for topical application with pH near to skin pH. All the batches of Gel showed clear and viscous appearance with no grittiness. All the batches had pH in range of 6.2-6.4 near to skin pH. Spreadability of final batch G3 and G4 was 18.66 and 20.71 gm.cm/s respectively. The viscosity of final batch G3 and G4 are 7000 and 8890 cps respectively [20,22].

In-vitro drug release study. The release study for the SLN loaded gel was performed using Cellophane, egg and goat skin membrane. The release data for this study is shown in figure 9. % cumulative drug release was calculated for all batches. The drug release for all the final batches of SLN loaded gel was between 85-100 % within 8 hours for drug 95.21 % in 8 % HPMC. For G4 (SLN-F4) and G5 (SLN-F5) had drug release of 110.30 % and 64.60 % respectively. The results were compared with marketed gel (fastum gel) which had drug release of 39.03% in 8 hours. From in-vitro study performed with cellophane membrane, G4 batch was used for other membranes. Formulations G4 showed slight decrease in drug release through egg membrane than cellophane membrane after 8 hours. Also, in goat skin ex-vivo showed similar release. Drug release from batches of SLN matches to ideal drug release range given hour wise for extended-release capsules in USP-NF [21].

Permeation data analysis (Flux). The flux of drug through cellophane membrane with area 7.00 cm² was studied. Diffusion flux measures the amount of substance that will flow through a small area during a small interval. Flux was obtained from the slope values plotted for amount diffused per unit area against time. Flux of final batches of gel through the cellophane membrane was determined. Batch G4 had a permeation flux of 0.06 µg/cm²/min.[22]

Similarity factor. The similarity factor f2 is a measure of the similarity in the percent of drug release between two release profiles. For the profiles to be considered “similar” f1 should be less than 15 and f2 should be greater than 50 according to current FDA guidelines for comparison of “changed” and “approved” products [24]. Cumulative % drug release through cellophane membrane for the final batch of SLN namely F4 and M4 and the marketed formulation (FASTUM® GEL) was calculated and compared by finding out the similarity factor (f2) which was calculated by using BIT SOFT software. The similarity factor for F4 SLN batch was 28. Hence it was found that SLN loaded gel was not similar to the marketed formulation.

Stability studies of SLN loaded Gel. In Stability studies for G4 it was observed that there was no significant change in evaluation parameters before and after study. The drug content was 98.25±1.80 evaluated at 3 months at various stability conditions. In the stability study it is indicated that the appearance of formulation was smooth and clear and viscous consistency.

### Table 6. Mathematical modeling of % drug release data.

<table>
<thead>
<tr>
<th>Batch code</th>
<th>Zero order (R²)</th>
<th>First order (R²)</th>
<th>Matrix (R²)</th>
<th>Korsmeyer- Peppas (R²)</th>
<th>Korsmeyer- Peppas</th>
<th>Hixson- Crowell (R²)</th>
<th>Best fit model</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>0.7805</td>
<td>0.7820</td>
<td>0.9772</td>
<td>0.9572</td>
<td>0.5838</td>
<td>0.0277</td>
<td>0.7815</td>
</tr>
<tr>
<td>F2</td>
<td>0.8917</td>
<td>0.8928</td>
<td>0.9985</td>
<td>0.9958</td>
<td>0.5114</td>
<td>0.0372</td>
<td>0.8924</td>
</tr>
<tr>
<td>F3</td>
<td>0.9542</td>
<td>0.9548</td>
<td>0.9802</td>
<td>0.9855</td>
<td>0.7090</td>
<td>0.0128</td>
<td>0.9546</td>
</tr>
<tr>
<td>F4</td>
<td>0.6814</td>
<td>0.6829</td>
<td>0.9557</td>
<td>0.9348</td>
<td>0.5570</td>
<td>0.0256</td>
<td>0.6824</td>
</tr>
<tr>
<td>F5</td>
<td>0.3714</td>
<td>0.3775</td>
<td>0.9157</td>
<td>0.9428</td>
<td>0.3724</td>
<td>0.0939</td>
<td>0.3755</td>
</tr>
<tr>
<td>F6</td>
<td>0.6553</td>
<td>0.6578</td>
<td>0.9725</td>
<td>0.9830</td>
<td>0.4413</td>
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<td>0.9797</td>
<td>0.5132</td>
<td>0.0666</td>
<td>0.7703</td>
</tr>
<tr>
<td>F8</td>
<td>0.7363</td>
<td>0.7392</td>
<td>0.9877</td>
<td>0.9928</td>
<td>0.4513</td>
<td>0.0762</td>
<td>0.7382</td>
</tr>
<tr>
<td>F9</td>
<td>0.6768</td>
<td>0.6812</td>
<td>0.9687</td>
<td>0.9884</td>
<td>0.3682</td>
<td>0.1246</td>
<td>0.6797</td>
</tr>
</tbody>
</table>

R² is Correlation coefficient; n is Diffusion exponent; k is Rate constant.

### 4. CONCLUSIONS

The present work could be concluded as a successful formulation of SLN using cetyl palmitate as lipid, span 60 as a lipophilic surfactant and tween 80 as a hydrophilic surfactant. SLN loaded gel for transdermal delivery for Ketoprofen has been developed. SLN loaded gel exhibited good in-vitro drug release and viscosity. SLN loaded gel will act as a depot of drug which will release the drug in a controlled manner at the targeted site. Hence the optimized formulation F4 may be used to treat the anti-arthritis diseases and commercially viable alternative to marketed product.
5. REFERENCES

6. ACKNOWLEDGEMENTS
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