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Oral pH-triggered colon-specific ketoprofen loaded microspheres for the better management of early morning symptoms associated with rheumatoid arthritis. Part II: Pharmacokinetic and pharmacodynamic assessment in rats

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ABSTRACT

Objective: To evaluate the effectiveness of oral pH-triggered colon-specific ketoprofen-loaded microspheres (C-SKLMs) in managing early morning symptoms of rheumatoid arthritis (RA) through pharmacokinetic and pharmacodynamic assessments.

Methods: Pharmacokinetic parameters, including T_{max}, C_{max}, and mean residence time (MRT), were analyzed in animals treated with C-SKLMs and compared with pure ketoprofen. Pharmacodynamic evaluations assessed the ability of C-SKLMs to address early morning RA symptoms effectively by aligning drug release with the body's circadian rhythm.

Results: The T_{max} for the C-SKLMs group (9.33 ± 1.63 h) was significantly prolonged compared to pure ketoprofen (2 h), indicating delayed drug release tailored to circadian needs. The C_{max} for C-SKLMs (5.94 ± 1.20 μg/mL) was lower than that of pure ketoprofen (12.4 ± 3.00 μg/mL), demonstrating a controlled-release profile. Additionally, the MRT for C-SKLMs (12.96 ± 1.42 h) was approximately 1.4 times longer than for pure ketoprofen (9.44 ± 0.69 h), emphasizing extended drug release. Pharmacodynamic evaluations supported the superior effectiveness of C-SKLMs in managing early morning RA symptoms compared to pure ketoprofen.

Conclusion: C-SKLMs demonstrated significant potential to improve the management of early morning symptoms associated with RA through controlled, extended, and circadian-tailored drug release, making them a promising therapeutic approach.

1. Introduction

Ketoprofen is classified as a nonsteroidal anti-inflammatory drug (NSAID). It is a well-established therapeutic agent often recommended to treat a wide range of inflammatory illnesses, including rheumatoid arthritis (RA), and is a commonly prescribed drug.[1–5] Gastric intolerance is a typical adverse reaction associated with all NSAIDs.[6–9] However, there exists room to improve the risk-benefit assessment for such medicines by improving management efficiency.

RA is a chronic inflammatory condition that impairs joint health. This condition creates joint pain and functional impairment, particularly during the early morning hours. The above signs are induced by diurnal changes in proinflammatory cytokine levels in the blood, such as interleukin-6 and tumor necrosis factor-α.[10–14] The notion of chronotherapy can be applied to improve RA treatment by keeping the most medicine in the systemic circulation throughout the early morning hours. In this scenario, colon-specific or purposefully delayed absorption may be preferred to provide a consistent pharmacological activity because medication release occurs after a lag period, so the treatment can be presented in an appropriate dose for the most prevalent need. This approach helps prevent the most intense pain and stiffness, leading to better patient compliance.[15–19] The chronotherapy-based drug delivery

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approach has demonstrated promise in treating nocturnal asthma,[20–23] nocturnal angina,[24–26] nocturnal hypertension,[27–29] and RA.[22,30–32]

The research team aims to develop a novel chronotherapeutic formulation strategy targeting rheumatoid arthritis symptoms, specifically addressing early morning manifestations commonly experienced by patients. The novel chronotherapeutic formulation is proposed to provide therapeutic effects during late-night hours, aligning with the typical timing of patients' medication intake before bedtime. The formulation design integrates the chronotherapeutic concept using pH-triggered colon-specific ketoprofen-loaded microspheres (C-SKLMs). The research involves screening key variables influencing microsphere properties using a Plackett-Burman screening design[33] and optimizing the formulation with the Box-Behnken design.[34] The screening phase highlighted key factors, including the amounts of ketoprofen and Eudragit® S-100, along with paddle speed, that significantly influenced drug entrapment efficiency and release profiles. Optimization efforts led to the development of pH-triggered C-SKLMs with desirable characteristics, including particle size and % cumulative drug release at the 5th and 10th h. Findings on the solid-state characterization and stability of the drug in its final formulation, as well as the surface morphology of the microspheres, were reported.[33–35]

The potential of the developed formulation was confirmed through ex vivo permeation tests using isolated segments of the rat gastrointestinal tract.[34] Current research includes *in vivo* characterization, aimed at demonstrating the delayed response through pharmacokinetic evaluation in healthy rats and pharmacodynamic assessment using the carrageenan-induced rat paw edema model. The effectiveness of the new formulation was further verified by testing its anti-arthritic potential in a Complete Freund's adjuvant (CFA)-induced arthritic rat model.

2. Materials

Ketoprofen was provided as a free sample from Ciron Drugs & Pharm. (Mumbai, India). Polyvinyl alcohol and carrageenan were purchased from Himedia Laboratories (Mumbai, India). Complete Freud's adjuvant and dichloromethane were bought from Sigma Aldrich Chemicals Pvt. Ltd. (Bangalore, India); ethanol was purchased from Byahut Scientico (Jaipur, India), and the AET Laboratories, Hyderabad, India, kindly provided the Eudragit® S-100 as a generous sample.

3. Methods

3.1. Preparation of oral pH-triggered colon-specific ketoprofen-loaded microspheres

Ketoprofen (189.09 mg) was dissolved with Eudragit® S-100 (600 mg) in a solvent mixture of ethanol (7.5 mL) and dichloromethane (7.5 mL). The resultant mixture was added to a 250 mL glass beaker consisting of 100 mL of 1% w/v polyvinyl alcohol in water while being stirred with a three-blade lab stirrer at 400 rpm. The agitation was continued until the invisible liquid emulsion globules converted into visible solid microspheres. The solid product was filtrated and rinsed three times using distilled water. The final product was desiccated at room temperature for 24 h.[33–37]

3.2. In vivo evaluation of oral pH-triggered colon-specific ketoprofen-loaded microspheres

The selected male albino Wistar rats for *in vivo* experimentation had a body weight range of 200–220 g. Rats were randomly divided into groups with six animals in each and housed individually in well-spaced, ventilated cages. They were kept in temperature-controlled circumstances, and the animals were unrestricted from taking the water and eating a nutritious fixed diet. The research was carried out with previous authorization from the IAEC, School of Pharmacy, Anurag University, Hyderabad, India (formerly Anurag Group of Institutions), bearing CPCSEA registration number 1412/A/11/CPCSEA.

3.2.1. In vivo pharmacokinetic studies

3.2.1.1. Fabrication of stock solutions and quality control samples. Stock solutions of ketoprofen (1 mg/mL) and paracetamol (1 mg/mL) were prepared by dissolving 30 mg of each drug in 30 mL of the mobile phase comprised of 90:10 (% v/v) acetonitrile and mixed phosphate buffer (pH 3) separately. Suitable dilutions from the standard preparations fabricated paracetamol (60 µg/mL) internal standard solution. Ketoprofen (40, 35, 30, 25, 20, 17.5, 15, 12.5, 10, 7.5, 5, 2.5, 1.25, and 0.625 µg/mL) functioning preparations were fabricated by serial dilution of a standard solution of ketoprofen. The resulting individual working solutions contained 10 µL of corresponding stock, and the volume was made up to 200 µL with Wistar rat plasma. For the method validation, quality control (QC) samples such as lower quality control (LQC), medium quality control (MQC), and high quality control (HQC) of 2.3, 21, and 32 µg/mL were fabricated by adequate dilutions from the ketoprofen standard solution. Among all the stocks, the lowest concentration, 0.625 µg/mL, was selected as LLOQ.

3.2.1.2. Instruments and conditions for sample detection. All plasma samples from the pharmacokinetic study were analyzed for ketoprofen using high-performance liquid chromatography (HPLC) (Shimadzu, Nakagyo-Ku, Kyoto, Japan). The HPLC system used for the study was equipped with a Rheodyne injector with a 20 µL sample injection capacity, LC-20AD pumps for accurate solvent supply, a DGU-20A3 degasser to assure bubble-free operation, and an SPD-M20A photodiode array detector for multi-wavelength monitoring. The chromatographic separation was successfully carried out using a C_{18} column with specifications of 250 mm in length, 4.6 mm internal diameter, and a particle size of 5 µm, ensuring high efficiency in the process. To maintain consistency and reproducibility, the column temperature was precisely controlled at 25 °C throughout the analysis. The detection of analytes was performed at a wavelength of 260 nm, which was selected to optimize sensitivity and provide reliable results. The mobile phase consisted of a 90:10 (% v/v) mixture of acetonitrile and phosphate buffer (pH 3), and a flow rate of 0.4 mL/min was maintained for sample elution. Processed plasma samples (20 µL) were introduced into the column. All the chromatographic data were acquired and analyzed using LC Solutions 1.25 software.

3.2.1.3. Plasma sample preparation or extraction procedure. All the prepared working solutions (mentioned under Section 3.2.1.1) of each 200 µL and 25 µL of 60 µg/mL paracetamol (internal standard) were shifted into new tips. The resultant was mined with 400 µL of acetonitrile after vortex mixing on a vortex mixer for 60 s and centrifuging for 10 min on a laboratory microcentrifuge (RM-12C, REMI Instruments, Vasai, India) at 4000 rpm. Aliquots of 20 µL of supernatant organic phase from each sample were injected into the HPLC column to measure ketoprofen and paracetamol peak areas and calculate concentrations of ketoprofen from the standard plot. The same procedure was repeated for the rat plasma samples of the pharmacokinetic study.

3.2.1.4. Method validation. To validate the suggested HPLC technique, all necessary and recommended validation parameters were comprehensively determined, including specificity, sensitivity, accuracy, precision, matrix effect, and recovery. These characteristics were thoroughly tested to guarantee that the technique was reliable, robust, and appropriate for its intended analytical applications. The blood samples were collected from six different albino Wistar rats, then processed for plasma and utilized as blank plasma samples for the screening to establish the specificity of the proposed Reverse Phase (RP)-HPLC method. To measure the sensitivity of the developed RP-HPLC method, the HPLC column was alternatively injected with the lower limit of quantification (LLOQ) and six separate plasma blank samples, and the area of ketoprofen and

paracetamol peaks was measured in the chromatograms at the corresponding retention times. Six LLOQ ketoprofen samples were tested to confirm the sensitivity of the newly proposed method. The accuracy of the method was evaluated by calculating the percentage standard deviation (% RSD) of the HQC, MQC, and LQC samples. This approach ensured a comprehensive assessment of the reliability of the method across different concentration levels. In addition, the intra-day precision was thoroughly examined by analyzing six replicates of HQC, MQC, and LQC samples within the same day under identical experimental conditions. This evaluation provided valuable insights into the consistency and reproducibility of the method over a short time frame, further strengthening its validation. Inter-day accuracy was obtained by measuring these samples ($n = 18$) on three consecutive days to ensure the dependability and consistency of the method. The HQC ($n = 3$) and LQC ($n = 3$) samples derived from six distinct Wistar rat plasmas, which were previously screened chromatographically, were injected to study the effect of the matrix for the developed ketoprofen RP-HPLC method. Recovery was established by injecting all three QC samples of ketoprofen. The comparison of plasma-extracted ketoprofen/corresponding aqueous-extracted ketoprofen peak area ratios calculated the extraction recovery.

3.2.1.5. Animal treatment and sample collection. The pharmacokinetic characteristics were determined using two groups of rats, six animals in each. The 1st group received 10 mg/kg of pure ketoprofen, whereas the 2nd group received an equivalent amount of pH-triggered C-SKLMs. Both dosages were supplied orally after being dispersed in 0.5% w/v sodium carboxymethyl cellulose (SCMC). Blood samples were taken at predefined intervals into heparinized tubes by terminal retro-orbital hemorrhage. The collected blood samples were immediately subjected to centrifugation at 4000 rpm for 10 min, and the separated plasma was placed in a deep freezer for further examination.

3.2.2. Pharmacodynamic evaluation

3.2.2.1. Carrageenan-induced acute inflammatory rat model. The anti-inflammatory activity of ketoprofen from the pH-triggered C-SKLMs was evaluated using a carrageenan-induced acute inflammatory rat model, as reported in earlier research.[38–40] This study used four different groups of rats to estimate the acute anti-inflammatory activity of the prepared C-SKLMs formulation. In groups 1, 2, and 3, inflammation was caused by inserting 100 µL of a 1% w/v carrageenan solution into the plantar region of the right hind paw of every rat. This experimental approach guaranteed that edema was consistently induced, offering a trustworthy method for measuring the anti-inflammatory effects of the prepared formulation.

Group 4 received no carrageenan and served as the normal (non-induced, vehicle-treated) control. Group 2 was given the dose (10 mg/kg) of pure ketoprofen; Group 3 received the dose equivalent amount of prepared pH-triggered C-SKLMs, suspended in 0.5% w/v SCMC and was ingested by oral administration 1 h before the carrageenan administration. Group 1 was vehicle-treated and served as the negative (induced, but vehicle-treated) control. The edema of the paw was assessed at specific time points to evaluate the anti-inflammatory effects across the treatment groups. The change in paw volume of the right hind paw was considered at pre-specified time intervals using a digital plethysmometer (VJ Instruments, Maharashtra, India), and % change was calculated using equation (1).

3.2.2.2. CFA-induced arthritis in albino Wistar rats. The CFA-induced arthritis model was utilized to assess the anti-inflammatory effects of ketoprofen from the developed C-SKLMs in a chronic inflammation setting.[21,38–41] A total of four groups of animals were taken in this investigation to understand the chronic anti-inflammatory performance of the fabricated C-SKLMs. All the animals from groups 1, 2, and 3 had arthritis induced by administering a single dose of 100 µL of CFA into the sub-plantar region of the right hind paw. Group 4 received no CFA and served as the normal (non-induced, but vehicle-treated) control. Group 2 received the dose (10 mg/kg) of pure ketoprofen; Group 3 received the dose equivalent amount of prepared pH-triggered C-SKLMs, dispersed in 0.5% w/v SCMC, administered orally 1 h before the CFA injection. Group 1 was vehicle-treated and served as the negative (induced, but vehicle-treated) control. The animals were treated orally with pure ketoprofen and the corresponding fabricated C-SKLMs once daily until the 21st day. Simultaneously, parameters including the percentage alteration in paw volume and body weight, arthritic score, and fall-off time for each animal across all groups were monitored and recorded throughout the treatment period.

3.2.2.2.1. Percentage change in paw volume

$$\%\ Change\ in\ Paw\ Volume = \frac{V_t - V_0}{V_0} \times 100 \quad (1)$$

Here, V_t and V_0 represent the paw volume at specified time points after CFA administration and the initial paw volume immediately after CFA administration, respectively.

3.2.2.2.2. Percentage change in body weight

$$\%\ Change\ in\ Body\ Weight = \frac{A - B}{B} \times 100 \quad (2)$$

Where A, body weight at '0' time before injection; B, body weight at predetermined time points after CFA delivery.

3.2.2.2.3. Arthritic score. The severity of the arthritis was assessed by scoring systems reported previously[21,38–41]: normal paw = 0; mild swelling and erythema of digits = 1; swelling and erythema of digits = 2; severe swelling and erythema = 3; gross deformity and inability to use limb = 4 on respective days.

3.2.2.2.4. Fall off time. The time in seconds taken for each animal to fall from the rotating rod was recorded, with a maximum observation limit of 60 s. Each animal from the respective groups was individually placed on the rotating rod of the rota-rod apparatus (Techno Rota-Rod, Lucknow, India) to measure their fall latency.

3.2.2.2.5. Radiological analysis. The radiological examination and record of radiographs of the hind paw of each anesthetized albino Wistar rats from each group were carried out using an X-ray machine (Model Number: 10063923, Seimen Ltd., Goa, India) on the 21st day of the study. The operation conditions of the machine during the examination were 45 kV peak, 6 mA, the exposure time of 0.1 s, and the focus length was adjusted to 40 ″.[35]

3.2.2.2.6. Histological analysis. On the 21st day of the study, one animal from each group was sacrificed. The CFA-injected hind paw was decalcified in 5% formic acid after being placed in a 10% buffered formalin solution separately for 24 h, followed by detachment from the ankle joints. The specimens collected were fixed in paraffin, and thin slices of 4–5 µm thickness were prepared. The processed thin-sliced samples were analyzed using a light microscope to evaluate synovial hyperplasia, pannus formation, and alterations in joint space after staining with hematoxylin and eosin.[35]

3.3. Statistical analysis

The results of the carrageenan-induced paw edema model for acute inflammation and the CFA-induced arthritis model for chronic inflammation were given as mean ± SD. A two-way ANOVA followed by a Bonferroni post hoc test was used to assess the anti-inflammatory properties of different groups, since these tests are appropriate for examining interactions between numerous components and controlling type I error during multiple comparisons. In albino Wistar rats, pharmacokinetic characteristics such as C_{max}, $AUC_{0\text{-}24}$, $AUC_{0\text{-}\alpha}$, $T_{1/2}$, MRT, and T_{max} were studied following oral administration of ketoprofen in its pure form and developed C-SKLMs. A paired *t*-test was employed for normally distributed data to assess significant differences between paired samples, and the Wilcoxon nonparametric test was utilized for data that did not fit

normality, guaranteeing robust and reliable statistical analysis of the pharmacokinetic results.

4. Results and discussion

The earlier findings of this study showed successful development and optimization of pH-triggered C-SKLMs using Eudragit® S-100 as a pH-sensitive polymer to target early morning symptoms associated with RA.[34] The formulation was optimized with a Box-Behnken design, achieving high drug encapsulation efficiency, desirable particle size, and a controlled *in vitro* drug release profile with significant release in the colon. Stability studies showed no significant degradation over the storage period, indicating good formulation stability. Additionally, solid-state characterization using DSC, XRD, and FTIR established the stable dispersion of ketoprofen in the polymer matrix without chemical interactions. *Ex vivo* permeation studies further confirmed the colon-targeting potential of the formulation, demonstrating limited presentation of the drug in the upper segment of the gastrointestinal tract. These findings laid the groundwork for the present study, which focuses on *in vivo* pharmacokinetic and pharmacodynamic evaluations to further characterize the efficacy of the developed formulation in rat models.

4.1. In vivo pharmacokinetic study

4.1.1. RP-HPLC method validation for estimation of ketoprofen in rat plasma

The specificity experiments' data revealed no considerable interference at the Retention Time (RT) of ketoprofen and paracetamol for all the analyzed Wistar rat plasma samples. The peak areas of the ketoprofen and paracetamol at their respective RTs in blank plasma samples were less than 20.00% and 5.00%, respectively, when compared with the peak areas of the ketoprofen and paracetamol from the extracted corresponding LLOQ sample. The corresponding results were tabulated in Table S1. The observed precision and accuracy for the sensitivity study were 3.17% and 107.23%, respectively. The corresponding results were tabulated in Table S2. For the matrix effect parameter, the calculated percentage coefficient of variance (%CV) of HQC and LQC samples were 1.98% and 7.14%, respectively. The results were as per acceptance criteria (less than 15.00%). The corresponding results are represented in Table S3. The LLOQ was defined as "the lowest concentration at which both precision and accuracy were ≤20%" which was 0.625 μg/mL for ketoprofen. The standard plot was constructed by taking concentration (μg/mL) and peak as ratios on the X-axis and Y-axis, respectively. The obtained equation and correlation factor were $Y = 0.065x - 0.013$ and 0.997, respectively. The standard plot constructed while validation was linear for the standard concentration ranging from 0.625 to 40 μg/mL, obtained from the 2nd precision and accuracy batch. The standard linearity plot is represented in Fig. S1. Validation of the suggested approach yielded acceptable chromatographic results, as shown in Fig. S2 for blank plasma, HQC, MQC, LQC, and LLOQ. The %CV evaluated the accuracy of the RP-HPLC technique for several QC samples (LQC, MQC, and HQC) during validation. Intra-day (within batch) precision was represented by computing %CV for concentrations of HQC, MQC, and LQC ranging from 1.30 to 8.33 for batch I, 2.41 to 5.50 for batch IIA, 0.77 to 3.94 for batch IIB, 2.78 to 5.99 for batch IIC, and 3.01 to 6.02 for batch III, which is ≤ 15.00% (within the acceptable limit). And inter-day (between batches) precision was represented by calculating %CV for concentrations of HQC, MQC, and LQC ranging from 2.05 to 5.36, which is ≤ 15.00% (within the acceptable limit). The data is represented in Table S4.

The accuracy of the established method was tested by comparing the measured mean values of QC samples to their nominal values, represented as percentages. The percentage mean accuracy of the measured concentrations for HQC, MQC, LQC, and samples varied from 96.23% to 107.69% for batch I, 96.61%–110.57% for batch IIA, 92.83%–113.39% for batch IIB, 92.18%–101.30% for batch IIC, and 98.12%–10.58% for batch III. These readings are within the allowed range of 85.00%–115.00%, demonstrating the method's accuracy within individual batches. The between-batch accuracy of measured concentrations for HQC, MQC, and LQC samples varied from 96.68% to 102.84%, reaching the acceptable ranges of 85.00%–115.00%. The comprehensive data supporting these conclusions is included in Table S4, demonstrating the method's resilience over numerous batches.

The recovery of ketoprofen was measured by calculating the percentage mean recoveries of extracted plasma QC samples compared to the matching aqueous un-extracted QC samples. The % mean recoveries for LQC, MQC, and HQC were found to be 83.32%, 83.38%, and 92.62%, respectively, for an overall % mean recovery of 84.44%. This recovery evaluation indicates the method's capacity to properly extract ketoprofen from plasma samples, further validating its suitability for the pharmacokinetic study. The data are represented in Table S5.

4.1.2. Pharmacokinetic data analysis

The blood samples collected from the healthy male albino Wistar rats showed different pharmacokinetic parameters for the pure ketoprofen and C-SKLMs. All the possible parameters are represented in Table 1, and the mean plasma concentration-time profile curves of pure ketoprofen and C-SKLMs are represented in Fig. 1.

The obtained T_{max} from the C-SKLMs treated animals (9.33 ± 1.63 h) was longer than that of the pure ketoprofen treated animals (2 h). In contrast, the obtained C_{max} from C-SKLMs treated animals (5.94 ± 1.20 mg/mL) was smaller than that of the pure ketoprofen treated animals (12.4 ± 3.00 mg/mL). This may be attributed to more rapid absorption from the initial region of the digestive tract than the colon. The MRT value of ketoprofen from the C-SKLMs group (12.96 ± 1.42 h) was about 1.4 times that of the pure ketoprofen group (9.44 ± 0.69 h). This indicates that the average residence time of ketoprofen for microsphere formulation is longer than that of pure ketoprofen. There was an insignificant difference ($P > 0.05$) in $AUC_{0\text{-}24}$ and $AUC_{0\text{-}\alpha}$ values between microsphere formulation (61.352 ± 6.134 and 66.283 ± 8.775) and pure ketoprofen (66.574 ± 6.630 and 72.634 ± 6.903). The absorption pattern for colon target microspheres is different from pure ketoprofen. Delayed ketoprofen absorption was observed with C-SKLMs since it takes longer to arrive in the colon after oral administration. Importantly, there was no significant ($P > 0.05$) change in the half-life ($t_{1/2}$), which aligns with the pharmacokinetic behavior expected from a delayed-release formulation. These results support that while the formulation effectively modulates the rate and onset of drug absorption, it does not alter the intrinsic elimination properties of the drug.[42–45]

4.2. In vivo pharmacodynamic study

Carrageenan-induced paw edema and CFA-induced arthritis are prominent clinical rat models utilized for evaluating various substances' anti-inflammatory and anti-arthritic activities for many years. Injecting carrageenan and CFA into albino Wistar rats led to several changes, including joint swelling, infiltration of inflammatory cells, bone degradation, erosion of joint cartilage, and remodeling, ultimately compromising joint integrity and function. In this study, the C-SKLMs (10 mg/

Table 1
The summary of the pharmacokinetics of pure ketoprofen and C-SKLMs from 12 healthy male Wistar rats given a single oral dose (10 mg/kg).

PK parameter	Pure ketoprofen	Optimized formulation for C-SKLMs
C_{max} (mg/mL)	12.4 ± 3.00	5.94 ± 1.20**
T_{max} (h)	2.00 ± 0.00	9.33 ± 1.63***
$AUC_{0\text{-}24}$ (μg·mL^{-1}·h)	66.574 ± 6.630	61.352 ± 6.134$^{\#}$
$AUC_{0\text{-}\alpha}$ (μg·mL^{-1}·h)	72.634 ± 6.903	66.283 ± 8.775$^{\#}$
$t_{1/2}$ (h)	6.68 ± 0.50	5.69 ± 1.63$^{\#}$
MRT (h)	9.44 ± 0.69	12.96 ± 1.42**

$^{\#}P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, compared with the pure ketoprofen group.

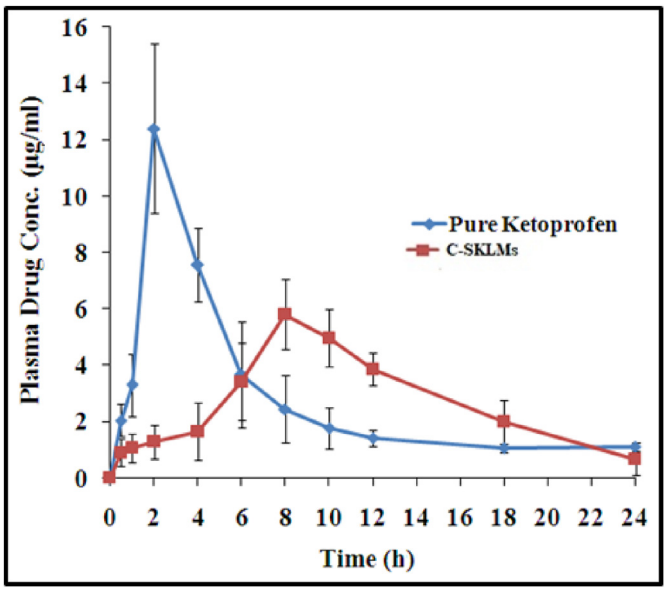


Fig. 1. Mean plasma concentration-time curve of ketoprofen in Wistar rats after oral administration of pH-triggered C-SKLMs and pure ketoprofen at a dose equivalent to 10 mg/kg ($n = 6$).

mL) demonstrated anti-inflammatory and anti-arthritic effects similar to those of pure ketoprofen (10 mg/kg) by targeting various inflammatory mediators.

4.2.1. Effects on carrageenan-induced hind paw edema in albino Wistar rats

The anti-inflammatory effects of fabricated C-SKLMs were tested in albino Wistar rats utilizing a carrageenan-induced paw edema model. It is a two-phase process, with the initial acute phase (first 2 h) marked by the elevation of mediators of inflammation, including histamine, bradykinin, and serotonin (5-HT). The secondary chronic phase (2–5 h) is characterized by neutrophil infiltration and continuous generation of prostaglandins or nitric oxide, which contribute to continued inflammation.[35,46] The % alteration in paw volume for the pure ketoprofen (control), C-SKLMs (test), and vehicle (vehicle control) treatment

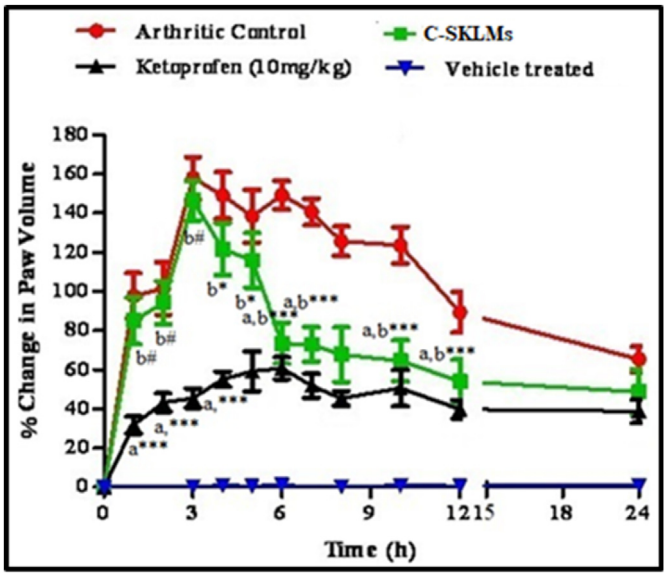


Fig. 2. Percentage change in paw volume of carrageenan-induced rat paw edema. $^{\#}P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; a: comparison between pure ketoprofen treated and arthritis induced group; b: comparison between C-SKLMs and arthritis induced group.

groups was computed and shown in Fig. 2. This represents the average paw edema volume measured at various time intervals. The pure ketoprofen (control) exhibited a faster anti-inflammatory effect, as evidenced by its superior paw edema inhibition compared to the C-SKLMs. The developed C-SKLMs demonstrated a significant ($P < 0.001$) anti-inflammatory effect after 7 h post-administration, similar to that observed in pure ketoprofen treated albino Wistar rats. This suggests that the C-SKLMs had a delayed onset of action, likely due to their controlled release properties. This is related to the pH-triggered feature of produced C-SKLMs, which may control preterm delivery of ketoprofen in the upper part of the gut region (pH < 5) and can promote ketoprofen release in the colon region (pH < 5). This postponed (~7 h) onset of anti-inflammatory action may be useful for the chronotherapy of RA early morning symptoms.

4.2.2. Effects on CFA-induced arthritis in albino Wistar rats

CFA is an inactivated and dried mycobacteria primarily responsible for stimulating immunity mediated by cells and, as a result, increasing the synthesis of specific immune globulins. CFA-induced arthritis is a primary and secondary chronic arthritis.[41,47–51] Primary chronic arthritis is divided into two phases: the inflammatory phase, which generates prostaglandins, and the secondary immunological phase, which produces autoantibodies. The onset of arthritis is characterized by a considerable increase in paw volume following CFA injection, indicating an inflammatory response. In CFA-induced arthritis, treatment with pure ketoprofen (10 mg/kg) and C-SKLMs (10 mg/kg) decreased paw volume substantially, suggesting an anti-inflammatory effect.

4.2.2.1. Percentage change in paw volume. Over a period of 12 days, rats treated with pure ketoprofen (10 mg/kg) and C-SKLMs (10 mg/kg) showed a significant reduction in paw volume ($P < 0.001$) compared to the arthritic control group. In contrast, the arthritic control group exhibited a significant increase in paw volume ($P < 0.001$) when compared to the vehicle-treated group. Furthermore, paw volume in the arthritic control rats remained significantly elevated ($P < 0.001$) throughout the study and persisted until the conclusion of the research (Fig. 3A).

4.2.2.2. Percentage change in body weight. Recovery from a sickness or medical condition is implicitly limited by body weight. Leptin, a cytokine-like hormone, regulates inflammation and immunity, and impacts body weight, food intake, and metabolism. Leptin has been shown to enhance T cell proliferation and amplify TH1 responses, which show a vital role in the progress of autoimmune diseases.[47] In CFA-induced arthritis, systemic intensities of leptin were rapidly elevated, leading to loss of body weight due to anorexia within 24 h following the injection of CFA.[52] The findings of our present study are consistent with those reported in prior research.[53] In CFA-induced arthritic rats, treatment with pure ketoprofen (10 mg/kg) and C-SKLMs (dosage corresponding to 10 mg/kg) dramatically reduced body weight loss. CFA's immune response leads to a considerable drop in body weight ($P < 0.001$) in the arthritic control group, compared with the body weight evaluated in the vehicle-treated group. The weight loss in the arthritic control group was steady, with body weight continuously decreasing until the 21st day of the trial. Whereas, rats ingested with pure ketoprofen (10 mg/kg) or C-SKLMs (dosage corresponding to 10 mg/kg) showed a substantial improvement in body weight ($P < 0.001$). These data demonstrate the effectiveness of both ketoprofen therapies in reducing weight loss associated with CFA-induced arthritis. There was no significant ($P > 0.05$) difference in body weight (up to 9th d) in rats from pure ketoprofen (10 mg/kg) as compared with C-SKLMs (dose equivalent to 10 mg/kg) treated group; significant ($P < 0.001$) surge in body weight in rats from C-SKLMs as compared to pure ketoprofen treated group (Fig. 3B).

4.2.2.3. Arthritic score. The arthritis score indicates joint inflammation

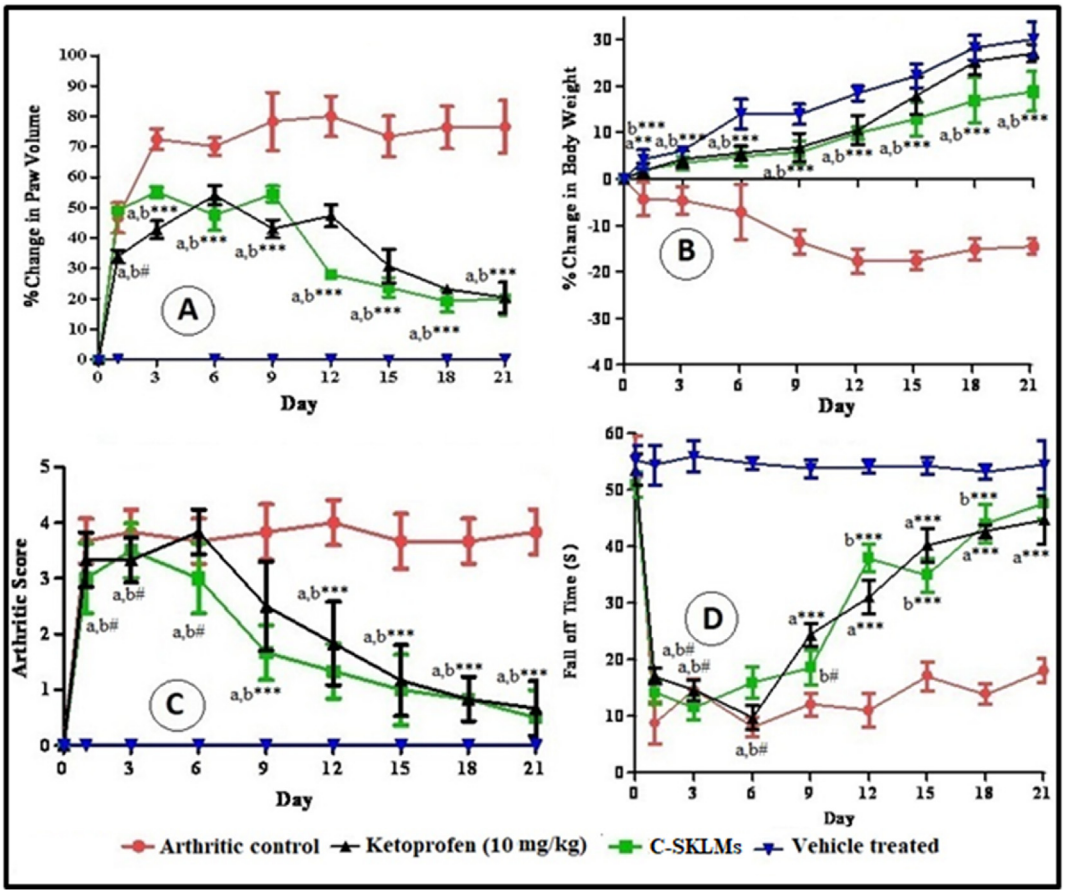


Fig. 3. A. Percentage change in paw volume; B. Percentage change in body weight; C. Arthritic score; D. The motor incoordination in Freund's adjuvant-induced arthritis rats. $^{\#}P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; a: comparison between pure ketoprofen treated and arthritis induced group; b: comparison between C-SKLMs and arthritis induced group.

following vaccination in CFA-caused arthritis. Subplantar administration of CFA resulted in a considerable ($P < 0.001$) increase in arthritic score in all animals from the CFA-treated group, which persisted expressively elevated until the completion of the trial as evaluated against the animals from the vehicle control group. Animals from the pure ketoprofen (10 mg/mL) and C-SKLMs (dose corresponding to 10 mg/kg) treated groups exhibited a substantial reduction in arthritic score ($P < 0.001$) from day nine onwards until the completion of the trial (Fig. 3C).

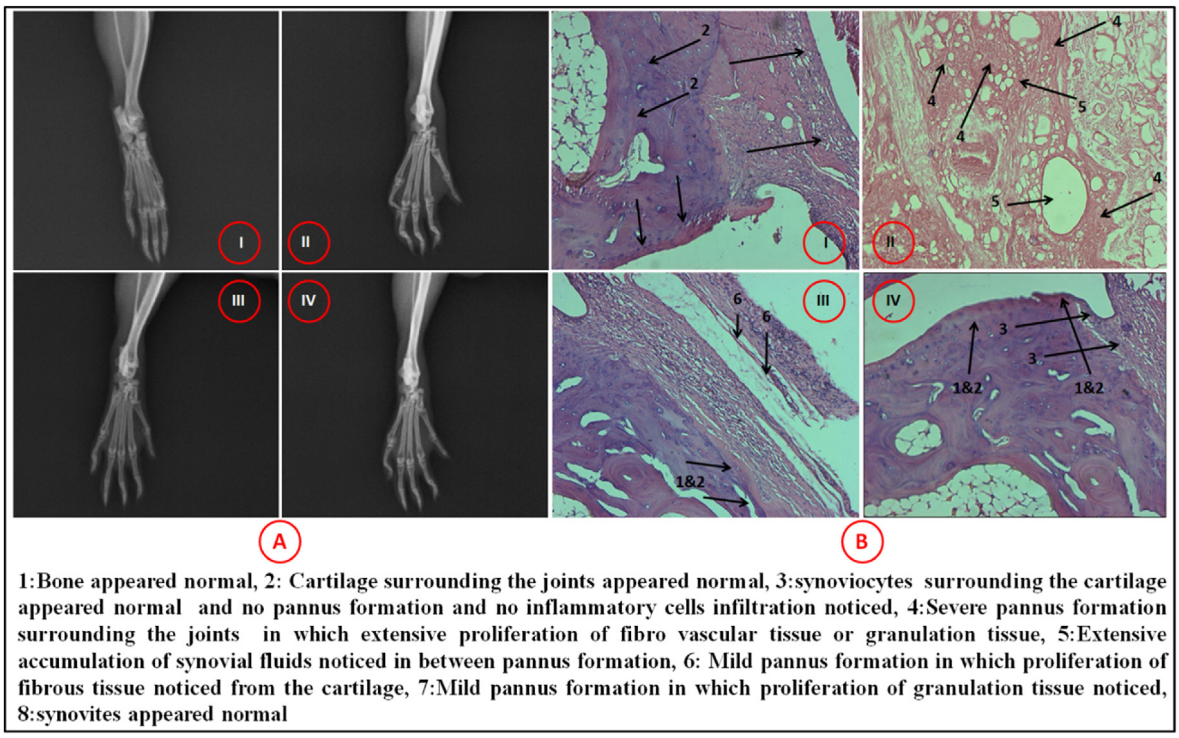
4.2.2.4. Fall off time. The mean fall-off time, an indicator of motor incoordination, was measured using a rota-rod test instrument. Rats treated with CFA had considerably shorter fall-off times ($P < 0.001$) compared with the vehicle-treated rats, indicating severe motor impairment. Compared to the arthritic control group, rats ingested with pure ketoprofen (10 mg/kg) showed considerable improvement ($P < 0.001$) in fall-off time beginning on day nine. Similarly, rats treated with the ketoprofen formulation (10 mg/kg) displayed a substantial increase ($P < 0.001$) in fall-off time when compared to arthritic control animals, with considerable improvement evident by day 12. Over the course of the trial, there was no significant difference ($P > 0.05$) in fall-off time between rats given pH-triggered C-SKLMs and those given pure ketoprofen (10 mg/kg). These findings show that the pH-triggered C-SKLMs formulation and pure ketoprofen are both effective in treating arthritis-related motor incoordination (see Fig. 3D).

4.2.2.5. Radiological analysis. The radiological analysis of rat joints from the normal control group showed intact morphology of joints (Fig. 4A.I). The rats in the CFA-administered group had apparent intertarsal joint gap reduction, widespread soft tissue enlargement, and more substantial bone deterioration (Fig. 4A.II). These arthritis-associated joint alterations were not observed or less pronounced in both ketoprofen (10 mg/kg) (Fig. 4A.III) and C-SKLMs (dose equivalent 10 mg/kg) treated rats (Fig. 4A.IV). According to radiological findings, oral therapy with both pure ketoprofen and C-SKLMs reduced arthritis-related joint changes in rats.

4.2.2.6. Histological analysis. The histology of normal control rats' tibiotarsal joints revealed undamaged bone and cartilage around the joints and synoviocytes enclosing the cartilage. Pannus formation was not observed around the joints or any fibrovascular or granulation tissue growth (Fig. 4B.I). CFA-treated rats exhibited severe pannus around the joints, with substantial proliferation of fibrovascular or granular tissue and massive synovial fluid accumulation evident in the intervals in the middle of pannus formation (Fig. 4B.II). Bone and cartilage seemed normal in the rat given pure ketoprofen (10 mg/kg), with little pannus formation and fibrous tissue proliferation (Fig. 4B.III). Bone and cartilage seemed usual in rats given the C-SKLMs (10 mg/kg), with little pannus formation and granulation tissue proliferation (Fig. 4B.IV). There was no buildup of synovial fluids between pannus development in rats treated with pure ketoprofen or C-SKLMs.

5. Conclusion

In conclusion, this study demonstrates the superiority of pH-triggered C-SKLMs in addressing the early morning symptoms of RA through enhanced chronotherapeutic drug delivery. This system not only



1:Bone appeared normal, 2: Cartilage surrounding the joints appeared normal, 3:synoviocytes surrounding the cartilage appeared normal and no pannus formation and no inflammatory cells infiltration noticed, 4:Severe pannus formation surrounding the joints in which extensive proliferation of fibro vascular tissue or granulation tissue, 5:Extensive accumulation of synovial fluids noticed in between pannus formation, 6: Mild pannus formation in which proliferation of fibrous tissue noticed from the cartilage, 7:Mild pannus formation in which proliferation of granulation tissue noticed, 8:synovites appeared normal

Fig. 4. A. Radiographic analysis in CFA-induced arthritis in rats; **B**. Histopathological representation of tibiotarsal joints in CFA-induced arthritis in rats and stained with hematoxylin and eosin: I) Vehicle treated, II) Arthritis induced, III) Pure ketoprofen, IV) C-SKLMs; Dose equivalent to 10 mg/kg treated are typical and representative of each group.

synchronizes drug release with circadian rhythms but also achieves significantly prolonged pharmacokinetic parameters, with a T_{max} of 9.33 ± 1.63 h and an extended MRT of 12.96 ± 1.42 h, compared to pure ketoprofen. This prolonged T_{max} ensures that drug release aligns precisely with early morning symptom peaks, offering sustained therapeutic action while reducing systemic exposure, as evidenced by a lower C_{max} of 5.94 ± 1.20 μg/mL. Additionally, our pharmacodynamic studies confirm the efficacy of C-SKLMs in managing RA symptoms, providing a robust advantage over traditional systems.

Unlike compression-coated tablets (CCTs) developed by Sunil et al. (2013), which require a predefined lag phase for drug release, prepared microspheres provide a more flexible and responsive approach with superior pharmacokinetic parameters.[54] While Khan et al. (2013) achieved dual-pulse release with a multilayered tablet system,[55] prepared C-SKLMs formulation offers a simpler design with enhanced temporal control and consistency. Additionally, compared to Maqbool et al. (2020), who utilized celecoxib-β-cyclodextrin complexes for colon targeting,[19] prepared C-SKLMs demonstrate an innovative yet straightforward formulation technique without requiring complexation, while still achieving effective and prolonged drug delivery. Collectively, these advancements establish C-SKLMs as a promising and superior chronotherapeutic strategy for improved rheumatoid arthritis management.

CRediT authorship contribution statement

Krishna Sanka: Writing – original draft, Investigation. **Prabhakar Reddy Veerareddy:** Writing – review & editing, Methodology, Conceptualization. **Rajeswara Rao Pragada:** Writing – review & editing, Methodology, Conceptualization.

Data availability

All the relevant data are reported within the paper and supplementary files.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhip.2025.03.001.

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