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Short Communication

3-Acetyl-11-keto-β-boswellic acid loaded-polymeric nanomicelles for topical anti-inflammatory and anti-arthritic activity

Amit Goel[a], Farhan Jalees Ahmad[b], Raman Mohan Singh[a] and Gyanendra Nath Singh[a]

[a]Indian Pharmacopoeia Commission, CIPL campus, Ghaziabad, Uttar Pradesh and [b]Department of Pharmaceutics, Faculty of Pharmacy, Jamia Hamdard, Hamdard University, New Delhi-110062, India

Abstract

Objectives The aim of this study was to develop 3-acetyl-11-keto-β-boswellic acid (AKBA)-loaded polymeric nanomicelles for topical anti-inflammatory and anti-arthritic activity.

Methods Polymeric nanomicelles of AKBA were developed by a radical polymerization method using *N*-isopropylacrylamide, vinylpyrrolidone and acrylic acid. The polymeric nanomicelles obtained were characterized by Fourier transform infrared (FTIR), transmission electron microscopy (TEM) and dynamic light scattering (DLS). In-vitro and in-vivo evaluations of AKBA polymeric nanomicelles gel were carried out for enhanced skin permeability and anti-inflammatory and anti-arthritic activity.

Key findings TEM and DLS results demonstrated that polymeric nanomicelles were spherical with a mean diameter approximately 45 nm. FTIR data indicated a weak interaction between polymer and AKBA in the encapsulated system. The release of drug in aqueous buffer (pH 7.4) from the polymeric nanomicelles was 23 and 55% after 2 and 8 h, respectively, indicating sustained release. In-vitro skin permeation studies through excised abdominal skin indicated a threefold increase in skin permeability compared with AKBA gel containing the same amount of AKBA as the AKBA polymeric nanomicelles gel. The AKBA polymeric nanomicelle gel showed significantly enhanced anti-inflammatory and anti-arthritic activity compared with the AKBA gel.

Conclusions This study suggested that AKBA polymeric nanomicelle gel significantly enhanced skin permeability, and anti-inflammatory and anti-arthritic activity.

Keywords 3-acetyl-11-keto-β-boswellic acid; anti-arthritic; *N*-isopropylacrylamide; polymeric nanomicelles; transdermal delivery

Introduction

Besides the corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs), including cyclooxygenase inhibitors (COX-1 and COX-2), leukotriene antagonists and lipoxygenase inhibitors are often used in current treatments for inflammation and arthritis. The current therapeutic treatment of arthritis with modern anti-inflammatory drugs is associated with side effects. For that reason, novel compounds selectively inhibiting the 5-lipoxygenase pathway are gaining more and more importance.

Several plants used for the treatment of arthritis in different systems of traditional medicine have shown activity when tested in modern bioassays. The gum resins of *Boswellia serrata*, family Burseraceae, commonly known as salai guggal, have been used for a variety of therapeutic purposes such as cancer, analgesia, asthma inflammation, arthritis, colitis, Crohn's disease and hyperlipidaemia.[1–15] The main biologically active principles of *B. serrata* for anti-inflammatory and anti-arthritic activity are boswellic acids. 3-Acetyl-11-keto-β-boswellic acid (AKBA), with an IC50 value (concentration required for 50% inhibition) of 1.5 μM, proved to be the most potent 5-lipoxygenase inhibitor. It acted by a 5-lipoxygenase directed, nonredox, noncompetitive mechanism and therefore possessed little toxicity and limited side effects compared with other anti-inflammatory drugs.[16–18] However AKBA possesses poor oral bioavailability with an elimination half life of 4.5 ± 0.55 h.[19–21]

Correspondence: Amit Goel, Indian Pharmacopoeia Commission, CIPL campus, Sector 23, Rajnagar, Ghaziabad, Uttar Pradesh, India.
E-mail: radhamit2006@gmail.com

For the treatment of arthritis and a reduction in inflammation, a higher concentration is required at the site of inflammation. Furthermore, topical application proved to be beneficial to patients with rheumatism because it could be directly applied to the target tissues.[9] Thus topical delivery of AKBA seems to be a preferred alternative to an oral dosage form, which could provide a sustained and constant plasma level and reduce the frequency of administration. However, topical delivery of AKBA is problematic due to its high lipophilicity (Log P 8).[22] Permeation of the drug could be increased either by changing the properties of the drug or by using vehicles with a high permeation ability. In recent years vehicles with high permeation ability have attracted increasing attention. A novel carrier system to emerge is the polymeric nanomicelle, which offers controlled release, protection of active substances and is nano-sized. No studies have shown the effect of polymeric nanomicelles on transdermal and dermal delivery properties. Therefore, in this study AKBA-loaded polymeric nanomicelles of *N*-isopropylacrylamide, *N*-vinylpyrrolidone and acrylic acid were prepared. Their physiochemical properties, in-vitro release behaviour, in-vitro skin permeation characteristics and in-vivo transdermal anti-inflammatory and anti-arthritic effects have been evaluated.

Materials and Methods

Materials

N-Isopropylacrylamide (NIPAAM) was procured from Ranbaxy Acros (New Delhi, India). *N*,*N*′-Methylene *bis*-acrylamide was procured from Sigma (St Louis, MO, USA). Acrylic acid, *n*-hexane, sodium monohydrogen phosphate and dihydrogen phosphate, and ferrous ammonium sulphate were purchased from SRL (Mumbai, India). Vinylpyrrolidone was purchased from Fluka (Buchs, Switzerland). All other reagents were of analytical grade and purchased from Merck (Mumbai, India). AKBA was isolated from the methanolic extract of the resin of *B. serrata*.[23] Standard AKBA was procured from Natural Remedies (Bangalore, India).

Formulation of polymeric nanomicelles

Polymeric nanomicelles of NIPAAM, vinylpyrrolidone and acrylic acid were synthesized through a free radical polymerization mechanism. NIPAAM, vinylpyrrolidone and acrylic acid were used in a 65 : 30 : 5 molar ratio. Briefly, 680 mg NIPAAM, 305 μl freshly distilled vinylpyrrolidone and 33 μl acrylic acid was added to 100 ml water. Methylene *bis*-acrylamide 300 μl (0.049 g/ml) was added in the aqueous solution of monomers to cross-link the polymer chain. The dissolved oxygen was removed by passing nitrogen gas for 30 min. Ferrous ammonium sulphate (50 μl) and saturated ammonium persulphate (50 μl) solutions were added to initiate the polymerization reaction. The polymerization was performed at 30°C for 24 h in a nitrogen atmosphere. Total aqueous solution of polymer was then dialysed overnight using a spectrapore membrane dialysis bag (12-kDa cut-off). The dialysed aqueous solution of polymeric nanomicelles was lyophilized immediately to obtain a dry powder for subsequent use. The yield of polymeric nanomicelles was 90%.

Formulation of transdermal gel

Transdermal gel of AKBA and AKBA-loaded nanomicelles was prepared using Carbomer 940 (Carbopol 940). Carbomer 940 1% gel was prepared for the study by dispersing 1 g Carbomer 940 in 100 ml distilled water. After complete dispersion an equivalent quantity of AKBA (5 g) and AKBA (20 g)-loaded nanomicelles was added to the aqueous dispersion under overhead stirring at 800 rev/min. Carbomer dispersion was then neutralized using 0.05% (w/w) triethanolamine to form the gel.

Loading of AKBA to polymeric nanomicelles

AKBA loading in the polymeric nanomicelles was performed using a post-polymerization method. Briefly, 100 mg lyophilized powder of polymeric nanomicelles was dispersed in 10 ml water and was stirred well to reconstitute the nanomicelles. AKBA was then dissolved in absolute ethanol (50 mg/ml) and 1 ml alcoholic solution was added to the polymeric solution slowly with constant stirring at 1500 rev/min. AKBA was directly loaded into the hydrophobic core of the nanomicelles. The drug-loaded polymeric nanomicelles were then lyophilized to obtain a dry powder. Drug loading was found to be 45%.

Entrapment efficiency (*E*%)

For determination of *E*%, the AKBA-loaded polymeric nanomicelles were separated from unentrapped free AKBA by centrifugation at 3000 rev/min for 10 min. The unentrapped AKBA was redissolved in 1 ml methanol and the concentration was measured by HPTLC at 250 nm.[24] The *E*% was calculated as

$$E\% = ([\mathrm{AKBA}]_{\mathrm{total}} - [\mathrm{AKBA}]_{\mathrm{free}})/[\mathrm{AKBA}]_{\mathrm{total}} \times 100 \quad (1)$$

Characterization by FTIR spectrometry

The Fourier transform infrared (FTIR) spectrum of NIPAAM, vinylpyrrolidone, acrylic acid, drug-free polymeric nanomicelles and AKBA-loaded polymeric nanomicelles were obtained using a FTIR instrument (Perkin Elmer, spectrum 2000, MA, USA).

Measurement of size and size distribution

Dynamic light scattering (DLS)

The polymeric nanomicelles (100 mg) were dispersed in 50 ml water in a volumetric flask and thoroughly mixed with vigorous shaking. Droplet size was determined by photon correlation spectroscopy using a Zetasizer (1000 HS, Malvern Instruments, Worcestershire, UK).

Transmission electron microscopy (TEM)

Morphology and structure of the polymeric nanomicelles were studied using TEM, with TOPOCON 002B operating at 200 kV (Topcon, Paramus, NJ, USA).

In-vitro release behaviour of AKBA polymeric nanomicelles

Lyophilized polymeric nanomicelles (100 mg) loaded with AKBA were dispersed in 10 ml phosphate buffer, pH 7.4. The solution was divided in 20 microfuge tubes (500 μl each). The tubes were kept in a thermostable water bath set at room temperature. Free AKBA was completely insoluble in water; therefore, at predetermined time intervals the solution was centrifuged at 3000 rev/min for 10 min to separate the released AKBA from the loaded nanomicelles. The released AKBA was redissolved in 1 ml methanol and the concentration was measured by an HPTLC method at 250 nm.[24] The percentage of AKBA released was determined from the equation:

$$\text{Release}\,(\%) = [\text{AKBA}]_{\text{rel}}/[\text{AKBA}]_{\text{tot}} \times 100 \qquad (2)$$

where $[\text{AKBA}]_{\text{rel}}$ was the concentration of released AKBA collected at time t and $[\text{AKBA}]_{\text{tot}}$ was the total amount of AKBA entrapped in the nanomicelles.

In-vitro skin permeation studies

The permeation ability of AKBA through rat skin of the AKBA polymeric nanomicelles gel compared with the AKBA gel was determined in-vitro with a Franz diffusion cell system (FDC-6, LOGAN Instrument Corp., Somerset, NJ, USA). The effective diffusion area was 0.75 cm^2 and the receptor compartment volume was 5 ml.

Full thickness rat skin was excised from the abdominal region and hairs were removed with an electric clipper. The subcutaneous tissue was removed surgically and the dermis side was wiped with isopropyl alcohol to remove adhering fat. The cleaned skin was washed with distilled water and stored at −21°C until further use. The skin was brought to room temperature and mounted between the donor and receiver compartment with the dermal side facing the receiver compartment. Initially, the donor compartment was empty and the receiver chamber was filled with receptor media consisting of phosphate buffer saline (pH 7.4) and 40% polyethylene glycol 400. The receiver fluid was stirred with a magnetic rotor at a speed of 100 rev/min and the temperature was maintained at 37 ± 1°C (using an in-built thermostatic water bath). It has been reported that 40% polyethyleneglycol 400 has been used for poorly water soluble drugs in transdermal delivery to prevent any dissolution limiting permeation into the receiver medium and to maintain sink conditions.[25] It was also reported that the skin barrier function was not influenced by 40% polyethyleneglycol 400.[26] After stabilization of skin, 1 g formulation was applied on the skin surface in the donor compartment and sealed with paraffin film to provide occlusive conditions. Samples were withdrawn at regular intervals (1, 2, 3, 4, 5, 6, 7, 8, 10, 12 and 24 h), filtered through 0.45-μm membrane filter and analysed for drug content by HPTLC at 250 nm.[24]

Data analysis of skin permeation

Cumulative amount of drug permeated through per unit of skin surface area (mg cm^{-2}) was plotted as a function of time for each formulation. Drug flux (permeation rate) at steady state (J_{ss}) was calculated by the slope of the linear portion of the graph (mg/cm^2 per h).[27,28] Permeability coefficient (K_p) was calculated by dividing J_{ss} by the initial concentration of the drug in the donor cell (C_o).[27,28]

$$K_p = J_{ss}/C_o \qquad (3)$$

Enhancement ratio (E_r) was calculated by dividing J_{ss} of the AKBA polymeric nanomicelles gel by J_{ss} of the AKBA gel:

$$E_r = J_{ss} \text{ of AKBA polymeric nanomicelles gel}/J_{ss} \text{ of AKBA gel} \qquad (4)$$

Skin irritancy test

Skin irritation testing was carried out on male Swiss albino mice (25–30 g). The animals were kept under standard laboratory conditions (temperature: 25 ± 1ºC; relative humidity: 55 ± 5%). The animals were housed in polypropylene cages, six per cage, with free access to standard laboratory diet (Lipton Feed, India) and water was freely available. A single 10 mg dose of the polymeric nanomicelles gel was applied to the left ear of the mice, with the right ear as a control. The development of erythema was monitored for six days using a reported method.[29]

In-vivo anti-inflammatory activity

Approval to carry out in-vivo studies was obtained from the Jamia Hamdard Animal Ethical Committee (approval no: 173/CPCSEA, 28th Jan 2000) and their guidelines were followed throughout the studies. The anti-inflammatory action of the optimized formulations was evaluated by the carrageenan-induced hind paw oedema method developed by Winter *et al.*[30] in Wistar rats. Young male Wistar rats (180–220 g) were used for the study. The animals were housed in polypropylene cages, five per cage, with free access to standard laboratory diet (Lipton Feed) and water was freely available under standard laboratory conditions (temperature: 25 ± 2ºC; relative humidity: 55 ± 5%). Gel was applied to the left hind paw of rats. The area of application was occluded with bandages and it was left in place for 2 h. The dressing was then removed and the gel remaining on the surface was wiped off with cotton. Paw oedema was induced in the left hind paw by injecting 0.1 ml 1% w/w homogenous suspension of carrageenan in saline. A total of three groups were used: group 1 injected with carrageenan only and to serve as control; groups 2 and 3 received carrageenan plus topically-applied AKBA gel and carrageenan plus topically-applied AKBA polymeric nanomicelles gel, respectively. Paw volume was measured with a digital plethysmometer (UGO Basile 7140 Plethysmometer, Comerio VA, Italy).

The rate of oedema and percentage inhibition of each group was calculated as follows:

$$\text{Oedema rate}\,(E) = V_t\text{–}V_o/V_o \qquad (5)$$

$$\text{Inhibition}\,(\%) = E_c\text{–}E_t/E_c \times 100 \qquad (6)$$

where V_o was the mean paw volume before carrageenan injection, V_t was the mean paw volume after the carrageenan

injection at time t, E_c was the oedema rate of the control group and E_t was the oedema rate of the treated group at time t.

In-vivo anti-arthritic activity

The evaluation was in accordance with the method of Newbould.[31] Animals were immunized with an injection of 50 μl of 5 mg/ml (w/v) suspension of heat-killed *Mycobacterium tuberculosis* (Difco) in liquid paraffin into the left hind foot in the subplantar region. AKBA gel and AKBA polymeric nanomicelles gel were administered a day before the immunization to treatment groups 2 and 3, respectively, and continued until day 13. Group 1 received vehicle only. Paw volume was measured on day 0 and every alternate day until day 13 with a digital plethysmometer (UGO Basile 7140 Plethysmometer).

Results of anti-inflammatory and anti-arthritic activity were compared using Dunnett's test of one-way analysis of variance.

Results

Polymeric nanomicelles

The size and size distribution of the polymeric nanomicelles, by DLS and TEM, demonstrated that the particles had spherical morphology and low polydispersity with an approximate size of 45 nm diameter. The entrapment efficiency of AKBA within the polymeric nanomicelles was found to be 90% based on calculations described in the method.

FTIR study of NIPAAM–vinylpyrrolidone–acrylic acid polymeric nanomicelles

Figure 1 shows the FTIR spectra of the polymeric nanomicelles along with those of NIPAAM and vinylpyrrolidone monomers. Strong peaks in the spectra of the monomers in the range of 800–1000 cm^{-1} disappeared in the spectrum of the polymer. A broad and intense peak at 3435 cm^{-1} and a strong peak at 2928 cm^{-1} appeared in the spectrum of polymeric nanomicelles.

In-vitro release kinetic studies

The in-vitro release profiles of the loaded AKBA from poly (NIPAAM–vinylpyrrolidone–acrylic acid) nanomicelles at pH 7.4 was determined at room temperature. The results obtained showed that 23 and 55% of total drug was released from the nanoparticles at 2 and 8 h, respectively.

Skin irritancy test

The skin irritancy score for the nanomicelles formulation was found to be 2.14 ± 0.41.

Transdermal skin permeation studies

Transdermal skin permeation studies through excised rat abdominal skin indicated an approximate 3-fold increase (Figure 2) in drug permeation from AKBA polymeric nanomicelles gel compared with that of AKBA gel of same concentration (Table 1).

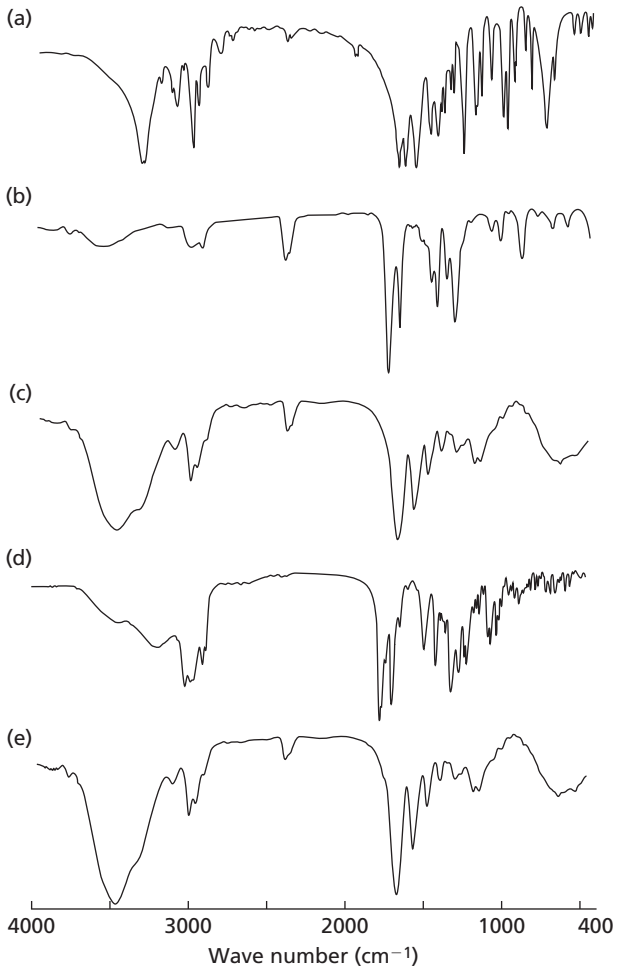

Figure 1 FTIR spectra of the polymeric nanomicelles and those of *N*-isopropylacrylamide and vinylpyrrolidone monomers. (a) *N*-Isopropylacrylamide (NIPAAM), (b) vinylpyrrolidone, (c) NIPAAM–vinylpyrrolidone–acrylic acid polymeric nanomicelles, (d) 3-acetyl-11-keto-β-boswellic acid (AKBA), (e) AKBA-loaded polymeric nanomicelles.

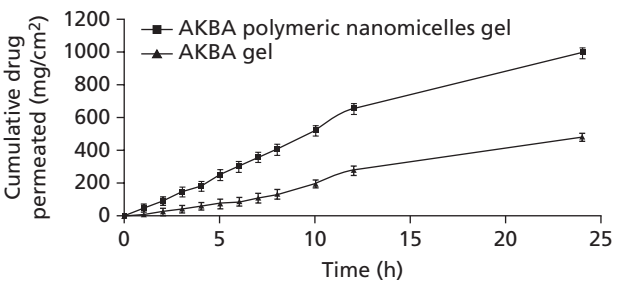

Figure 2 In-vitro skin permeation. 3-Acetyl-11-keto-β-boswellic acid (AKBA) gel vs AKBA polymeric nanomicelle gel.

In-vivo studies

The percent inhibition values of anti-inflammatory activity 24 h after administration was found to be high for AKBA polymeric nanomicelles gel (81.1% as compared with 44.1% for AKBA gel; Figure 3). The percent inhibition value of anti-arthritic activity was also found to be high for the AKBA

Table 1 Skin permeation parameters

Formulation	J_{ss} (mg/cm^2 per h)	$K_p \times 10^{-2}$ (cm/h)	E_r
AKBA gel	0.161 ± 0.035	0.341 ± 0.098	–
AKBA polymeric nanomicelle gel	0.488 ± 0.036	1.036 ± 0.151	3.031

AKBA, 3-acetyl-11-keto-β-boswellic acid; J_{ss}, drug flux (permeation rate) at steady state; K_p, permeability coefficient; E_r, enhancement ratio.

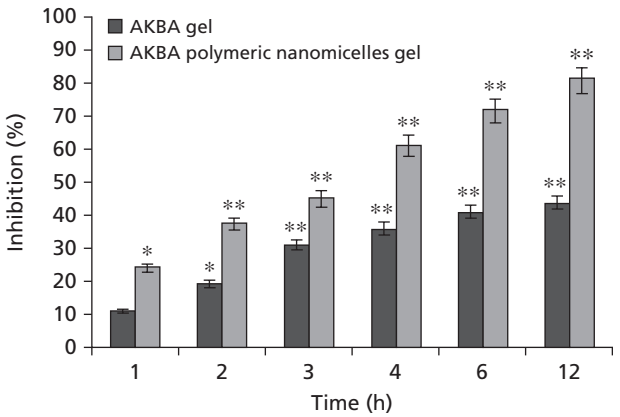

Figure 3 Effect of formulations on carrageenan-induced paw oedema. Values represent the mean ± SD of five animals for each group. $^{*}P < 0.01$ and $^{**}P < 0.05$ statistically significant from control.

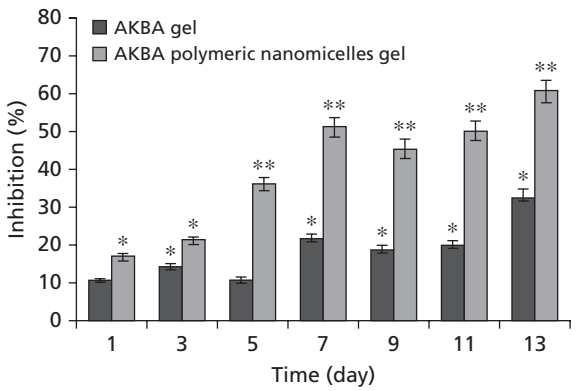

Figure 4 Effect of formulations on adjuvant-induced arthritis in rats. Values represent the mean ± SD of five animals for each group. $^{*}P < 0.01$ and $^{**}P < 0.05$ statistically significant from control.

polymeric nanomicelles gel after 13 days of activity (60.6% as compared with 33.3% for the AKBA gel; Figure 4).

Discussion

Random copolymerization of NIPAAM with vinylpyrrolidone and acrylic acid was performed using a radical polymerization process. Polymer formed in this way has amphiphilic characteristics with a hydrophobic core inside the nanomicelles and hydrophilic outer shell composed of hydrated amides, pyrrolidone and carboxylic groups projected from the monomeric units. The water insoluble drug, in this case AKBA, was encapsulated into the hydrophobic core of the polymeric nanomicelles.

As shown in the FTIR spectra (Figure 1), strong peaks in the range of 800–1000 cm^{-1} corresponding to the stretching mode of vinyl double bonds disappeared in the polymer spectrum indicating that polymerization had taken place. The water attached in the process of hydration of the polymer and proton exchange with the solvent gave rise to a broad and intense peak at 3435 cm^{-1}. The –CH– stretching vibration of the polymer backbone was manifested through a strong peak at 2928 cm^{-1}.

The in-vitro release profiles of the loaded AKBA from poly (NIPAAM–vinylpyrrolidone–acrylic acid) nanomicelles at pH 7.4 with 23 and 55% of total drug release from the nanoparticles at 2 and 8 h, respectively, showed that release occurred in a sustained manner.

The skin irritancy test was performed to confirm the safety of the optimized nanomicelles formulation. Van Abbe *et al.*[29] mentioned that a skin irritancy score of between 0 and 9 indicated that the applied formulation was nonirritant to human skin. From the skin irritancy score of 2.14 ± 0.41 it was concluded that the formulation was safe for transdermal drug delivery.

The interesting behaviour of increased transdermal skin permeation was thought to be due to slow dissolution of AKBA from the suspended crystalline material in AKBA gel and subsequent permeation of dissolved drug through the skin membrane. In the case of nanomicelles, the entrapped AKBA was hardly in the crystalline form. Therefore, the release of AKBA from the nanomicelles and subsequent dissolution in aqueous buffer was much faster. Moreover, the membrane uptake of the ultra-low size AKBA-entrapped nanomicelles could not be ruled out.

The enhanced in-vivo anti-inflammatory and anti-arthritic effect of AKBA polymeric nanomicelles gel could have been due to the enhanced permeation of AKBA through the skin as well as sustained release of the drug from nanomicelles.

Conclusions

Polymeric nanomicelles composed of NIPAAM, vinylpyrrolidone and acrylic acid were biocompatible and did not cause any skin irritation. Topical skin penetration of AKBA from nanomicelles gel was much higher compared with AKBA gel of equivalent concentration. The AKBA polymeric nanomicelles gel showed a much higher anti-inflammatory and anti-arthritic activity for a longer duration compared with that of AKBA gel. This could be attributed to the ultra-small size (45 nm diameter) of the polymeric micelles as well as their mucoadhesiveness. From in-vitro and in-vivo data it could be

concluded that the developed nanomicelles gel have great potential for transdermal drug delivery.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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