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Formulation, characterization and *In Vitro-In Vivo* evaluation of Ketorolac-loaded Solid lipid nanoparticles for transdermal delivery

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ABSTRACT

The present study was aimed at developing aqueous dispersion of Ketorolac-loaded solid lipid nanoparticles (SLN) by high speed stirring followed by ultrasonication technique and then incorporation into freshly prepared gel for transdermal delivery. A statistical central composite design was applied to study the effect of drug/lipid ratio and soya lecithin concentration on particle size and entrapment efficiency. The prepared formulations were characterized for in vitro release study. The anti-inflammatory effect of ketorolac-loaded solid lipid nanoparticles gel was assessed by carrageenan-induced paw edema model in rats and compared to ketorolac conventional gel. Both the ketorolac conventional gel and ketorolac-loaded SLN gel possessed a sustained drug release over period of 24 h but the sustained effect was more pronounced with the ketorolac-loaded solid lipid nanoparticles gel.

Keywords: Solid lipid nanoparticles (SLN), Ketorolac, anti-inflammatory activity, transdermal delivery.

INTRODUCTION

Solid lipid nanoparticles (SLN) are colloidal particles composed of lipid matrix that is solid at both room and body temperature and exhibit size range in between 50-1000nm [1]. They are composed of biodegradable lipids and lipidic stabilizers that are compatible with damaged and inflamed skin and are capable of incorporating both lipophilic and hydrophilic drugs [2,3]. SLNs like nanostructured lipid carriers (NLC) can be used to enhance the drug entraoment efficiency [4]. SLN's possess many significant advantages like high biocompatibility, high bioavailability, controlled release, suitability for large scale production, increased drug stability, feasibility for entrapment of lipophilic and hydrophilic drugs, avoidance of organic solvents and low or no toxicity [5].

Drug delivery from colloidal system such as solid lipid nanoparticles dispersed in a hydrogel appears to be unique and advantageous for transdermal application [6]. In the recent past, SLN have been in limelight due to several attractive features for transdermal drug delivery. SLNs have distinct occlusive properties due to formation of an intact film on the skin surface upon drying, which decreases transepidermal water loss. Prevention of transepidermal water loss increases skin hydration which further causes loosening of corneocytes cell junctions and thus favours drug penetration through the stratum corneum. Apart from a nonspecific occlusion effect on penetration, the small particle sizes of SLN ensure that the nanoparticles are in close contact with the stratum corneum, thus promoting the amount of drug which penetrates into the skin [7,8].

The aims of the present study were to 1) formulate SLN of ketorolac acid by applying central composite design, 2) develop ketorolac-loaded SLN gel for transdermal delivery, 3) evaluation of efficacy of ketorolac-loaded SLN gel

for anti-inflammatory activity in rats. The purpose was to provide the delivery of drug at a controlled rate across intact skin to improve bioavailability and inflammation control for longer period from ketorolac-loaded SLN gel.

MATERIALS AND METHODS

Materials

Ketorolac tromethamine was obtained as gift sample from Ranbaxy Research Laboratories Pvt. Ltd. (Gurgaon, India). Stearic acid and carrageenan were purchased from SD Fine-Chem. Ltd. (Mumbai, India). Soya lecithin was supplied by Hi-Media Laboratories Pvt Ltd. (Mumbai, India). All other chemicals used were of analytical grade.

Methods

Extraction of ketorolac from ketorolac tromethamine

An aqueous solution of ketorolac tromethamine (1%w/v) was treated with hydrochloric acid (0.1N), to precipitate ketorolac. The precipitated ketorolac was purified by first crystallization from ethyl acetate followed by recrystallization from mixture of ethyl acetate and ether (1:1) as reported earlier [9,10]. The proposed mechanism is detailed in figure 1.

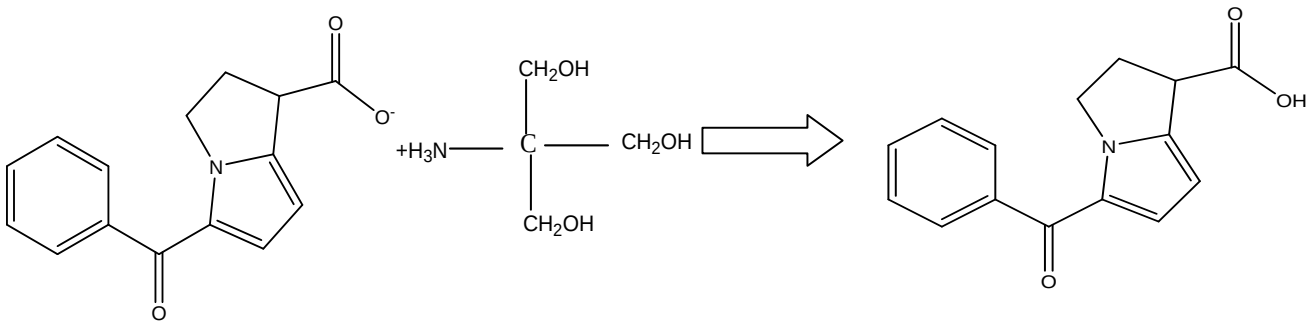

Fig. 1. Acidification of ketorolac tromethamine to ketorolac acid

Preparation of ketorolac-loaded solid lipid nanoparticles (SLN)

SLN containing ketorolac acid were prepared by high speed stirring- ultrasonication method using stearic acid as lipid carrier and soya lecithin as surfactant [11,12]. Briefly, an aqueous dispersion of soya lecithin was kept at 65±5ºC. To this oily phase comprising of stearic acid and ketorolac acid (40 mg) dispersed in 2ml of ethanol (95%) was added. The oil phase was added dropwise to hot aqueous phase with stirring at 1100 rpm while maintaining the temperature at 65-70°C for 30 minutes. The oil in water emulsion so formed was sonicated using bath sonicator (Power Sonic 405) for 25 minutes to obtain emulsion of SLN. Different batches of SLN were formulated by varying drug/lipid ratio and soya lecithin concentration as decided by central composite design for optimization of SLN.

Experimental Design

Design Expert (Version 7.1.6, Stat-Ease Inc, and Minneapolis, MN) was used for designing experimental plan for the preparation of ketorolac-loaded solid lipid nanoparticles. A two factor, three level central composite design (CCD) was employed for the optimization. The drug/lipid ratio i.e. ketorolac/stearic acid (X_1) and concentration of soya lecithin (X_2) were selected as independent variables. Particle size (Y_1) and entrapment efficiency (Y_2) were selected as response factors. All other processing variables were kept invariant throughout the study. The 13 experimental runs were carried out as described in table 1. The central point (0, 0) was studied in pentet. Further, the statistical assessment of the data was done using Design Expert.

Formulation of ketorolac gel

Preparation of gel of optimized batch of SLN

The optimized batch of ketorolac-loaded SLN was formulated as a gel employing Carbopol 934P as a gelling polymer [13]. Briefly, Carbopol 934P was dispersed in distilled water (80 ml) and allowed to hydrate overnight. An aqueous dispersion of optimized batch of ketorolac-loaded SLN (20 ml) was added to the hydrated Carbopol gel under constant stirring. The gelling of polymer was accomplished by adding triethanolamine (0.5%w/w).

Preparation of ketorolac conventional gel
Ketorolac conventional gel was formulated as a gel using Carbopol 934P as a gelling polymer. Carbopol 934P was dispersed in distilled water (80 ml) and allowed to hydrate overnight. An aqueous dispersion of ketorolac (20 ml) was added to the hydrated Carbopol gel under constant stirring. The gelling of polymer was accomplished by adding triethanolamine (0.5%w/w).

Characterization
Melting point determination
Melting point of ketorolac tromethamine and ketorolac acid was determined by melting point apparatus [8].

Fourier Transform Infra Red Analysis (FT-IR)
The IR spectra of ketorolac tromethamine and ketorolac (acid) was recorded in FTIR spectrophotometer (Perkin Elmer Spectrum BX, USA) by KBr pellet method at moderate scanning speed between the wavelength range of 4000-400 cm^{-1}.

Particle size analysis
The average particle size and polydispersity index of SLN was measured by dynamic light scattering technique using a particle size analyser (Nano ZS, Malvern Instruments, Malvern, UK).

Entrapment efficiency of SLN
The entrapment efficiency of ketorolac-loaded SLN was estimated by taking an aliquot of 10 ml of the SLN dispersion and centrifuging for 45 minutes at 13,500 rpm using REMI cooling centrifuge (C-24 BL) [14]. The supernatant was separated and the amount of free ketorolac was estimated spectrophotometrically by measuring the absorbance at 322 nm in UV-visible spectrophotometer (UV-visible double beam spectrophotometer 2203, Systronics).

$$\boldsymbol{Entrapment\ efficiency = \frac{KTt - KTf}{KTt} \times 100}$$

Where, KTt = total amount of ketorolac acid used in preparation of SLN, KTf = free ketorolac present in supernatant.

In vitro release
In vitro release of ketorolac from optimized batch of SLN-loaded Carbopol gel and ketorolac conventional gel formulation was studied using dialysis sac method [14]. The test formulations containing ketorolac equivalent to 4 mg were taken in dialysis bag and put it into dissolution flask containing 250 ml phosphate buffer (pH 7.4). An aliquot (3.0 ml) of samples were withdrawn at different time intervals and replaced with equal volume of release media. The absorbance of the samples was determined by UV-Visible Spectrophotometer (Shimadzu, Japan) at λmax 322 nm and percent drug release at different time intervals was plotted against time. The cumulative percent drug release at different time intervals was determined. The mechanism of drug release from the gel formulations was determined by fitting the release data into various kinetic models [15].

Evaluation of ketorolac-loaded SLN gel formulation for anti-inflammatory activity
Anti-inflammatory activity of transdermally applied SLN-loaded Carbopol gel and ketorolac conventional gel was determined in albino wistar rats (180-220 g) using carrageenan-induced hindpaw edema method [16]. The experimental protocol was implemented according to the recommendation of the university committee for research and ethical issues and the guidelines of IAEC/ CPCSEA. The overnight fasted animals were divided into three groups containing four in each. Group I was given normal saline (control), group II received ketorolac conventional gel and group III received SLN-loaded Carbopol gel. After 30 minutes of transdermal application of preparations, rats of all three groups were challenged by a subcutaneous injection of 0.1 ml of 1% (w/v) carrageenan solution, into subplantar region of the right hind paw of rats. The paw thickness was measured using digital vernier caliper (Aerospace, China) at 1,2,4,6,8,12 and 24 h of carrageenan injection. The percent inhibition of edema induced by carrageenan was calculated for each group using the following equation:

$$\%\ inhibition\ of\ edema = \frac{Vcontrol - Vtreated}{Vcontrol} \times 100$$

where, $V_{control}$ is the mean edema thickness of rats in control group and $V_{treated}$ is the edema thickness of each rat in test group.

RESULTS AND DISCUSSION

Ketorolac tromethamine is the salt of ketorolac, a weakly acidic drug which has low solubility in acidic medium and so it was precipitated from ketorolac tromethamine and further purified by recrystallization. The preparation of ketorolac was confirmed by determining melting point and IR spectra. The melting point of ketorolac tromethamine was 173ºC and of ketorolac was 161ºC.

Fourier transform infrared analysis

Figure 2 (a) and (b) exhibits IR spectra of Ketorolac tromethamine and Ketorolac acid in the frequency range from 4000-400cm^{-1}. The IR spectrum of Ketorolac tromethamine showed a characteristic band at 3347.82 cm^{-1} corresponding to N–H and O–H stretching vibrations. A band at 3061 cm^{-1} and 731 cm^{-1} may be attributed to aromatic C–H stretching and aromatic C–H bending vibrations, respectively. Aliphatic C–H stretching is indicated by bands at 2956 cm^{-1} and 2872.11 cm^{-1} but, aliphatic C–H bending vibration is observed at 1382 cm^{-1}. Carboxylic acid C=O stretching vibration is represented by a band observed at 1610 cm^{-1}. Bands at 1586 cm^{-1} and 1559 cm^{-1} are due to carbonyl C=O stretching vibrations, but a band at 896 cm^{-1} indicates the monosubstituted phenyl ring. While in IR data of Ketorolac acid a strong peak at 1720 cm^{-1} was due to carbonyl stretching and at 1612cm^{-1} due to aromatic C-C stretch. The alipathic C–H stretching and C–H bending vibrations are absent in Ketorolac confirming the precipitate obtained was Ketorolac acid [17].

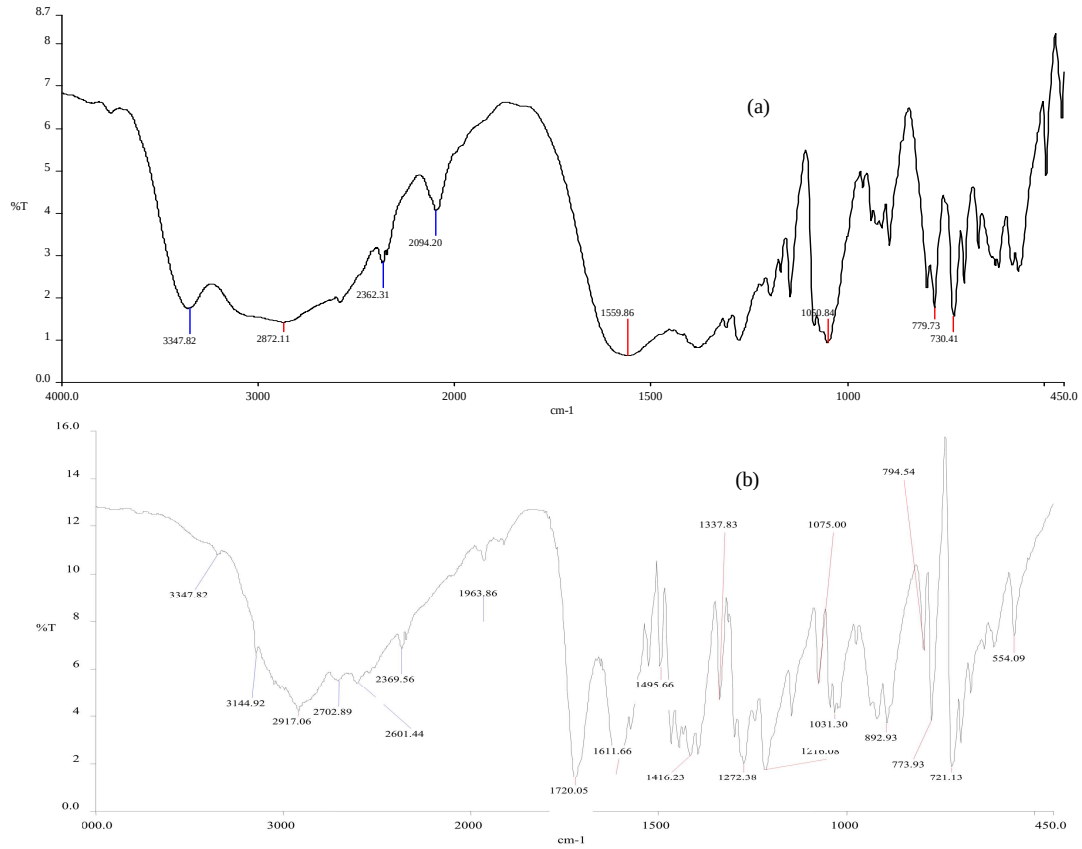

Fig. 2 FT-IR spectra of (a) Ketorolac tromethamine (b) Ketorolac acid

Validation of experimental design

The results (Table 1) of characterization of ketorolac-loaded solid lipid nanoparticles prepared using the experimental design were fitted into various polynomial models. It was observed that the response particle size (Y_1) fitted best into the quadratic response surface model after power transformation of the data while the response entrapment efficiency (Y_2) fitted best into the response surface quadratic model with backward elimination after inverse transformation of the data.

Table 1. Central Composite Design to study effect of formulation variables on particle size (Y_1) and % entrapment efficiency (Y_2)

Batch	Drug/lipid (X_1)	Conc. of soya lecithin (X_2) (%w/v)	Particle size (nm) (Y_1)	Entrapment Efficiency (%) (Y_2)
1	1 (+1)	2.5 (-1)	669	53.59
2	0.75 (0)	3.25 (0)	240.3	74.16
3	0.5 (-1)	2.5 (-1)	753	51.59
4	0.75 (0)	3.25 (0)	284.4	68.93
5	1 (+1)	3.25 (0)	374.1	65.17
6	0.5 (-1)	4.0 (+1)	336.9	67.72
7	0.75 (0)	3.25 (0)	279.2	70.5
8	0.75 (0)	2.5 (-1)	626	59.72
9	0.75 (0)	3.25 (0)	341.3	66.36
10	0.75 (0)	4.0 (+1)	300.8	68.87
11	1 (+1)	4.0 (+1)	259.9	71.74
12	0.5 (-1)	3.25 (0)	390.4	62.66
13	0.75 (0)	3.25 (0)	153	76.57

*****Values in paranthesis indicate coded values*

The polynomial models showing relationship between the independent variables and the response Y_1 and Y_2 are expressed by the following equations:

$$(Y_1)^1=270.86-29.55X_1-191.73X_2+1.75X_1X_2+83.36(X_1)^2+164.51(X_2)^2$$
$$1/Y_2=0.014-3.603E-004X_1-1.926E-003X_2+1.334E-003(X_1)^2+1.314E-003(X_2)^2$$

To estimate the significance of models they were subjected to ANOVA analysis. The results (Table 2) showed that the response surface model developed for the two response factors (Y_1 and Y_2) were significant ($p<0.05$) and adequate with insignificant lack of fit ($p> 0.05$). Further the 'adjusted R^2' were in reasonable agreement with 'predicted R^2'. The value of "adequate precision" was greater than 4 which is desirable and indicates an adequate signal.

Tab. 2. Statistical summary of the quadratic response surface model

Response Factor	Model							Lack of fit	
	F-value	Prob.>F	R^2			Adeq. Prec.	Std. dev.	F-value	Prob.>F
			Actual	Adj.	Pred.				
Y_1	18.55	<0.0007	0.9298	0.8797	0.7500	11.616	63.12	0.58	0.6573
Y_2	16.16	<0.0007	0.8899	0.8348	0.7035	11.561	7.736	0.85	0.5603

Table 3 represents the result of factor effects and P-values of responses Y_1 and Y_2. It can be explained from the data that the response Y_1 is significantly affected by the linear contribution of X_2 antagonistically while the quadratic contribution of X_2 exerted synergistic influence. The response Y_2 was found to be affected significantly by antagonistic linear contribution of X_2. However, the quadratic contributions of X_1 and X_2 affected the response Y_2 synergistically.

Table 3. Summary of factor effect and P-value of responses Y_1 and Y_2

Factor	Y_1		Y_2	
	Factor effects	P-value	Factor effects	P-value
X_1	-2149.0988	*0.2892*	-0.03344	0.2870
X_2	-2163. 6130	0.0001	-0.01775	0.0003
X_1X_2	+9.3333	0.9573	-	-
X_1^2	+1333.7103	0.0642	+0.02133	0.0210
X_2^2	+292.4567	0.0034	+0.002336	*0.0224*

Particle size analysis

Fig.3 portrays the 3-dimensional response surface plot showing the combined effect of drug/lipid ratio and soya lecithin concentration on particle size of ketorolac-loaded solid lipid nanoparticles. It can be elucidated from the graph that there exists a curvilinear relationship between particle size and concentration of soya lecithin. Further, the effect of soya lecithin concentration on particle size is more pronounced than the drug/lipid ratio. It can be observed that the increase in concentration of soya lecithin results in decrease in particle size which can be explained by the fact that soya lecithin, surfactant reduces the surface tension of the medium. Higher surfactant concentration resulted in decrease in particle size of solid lipid nanoparticles during homogenization.

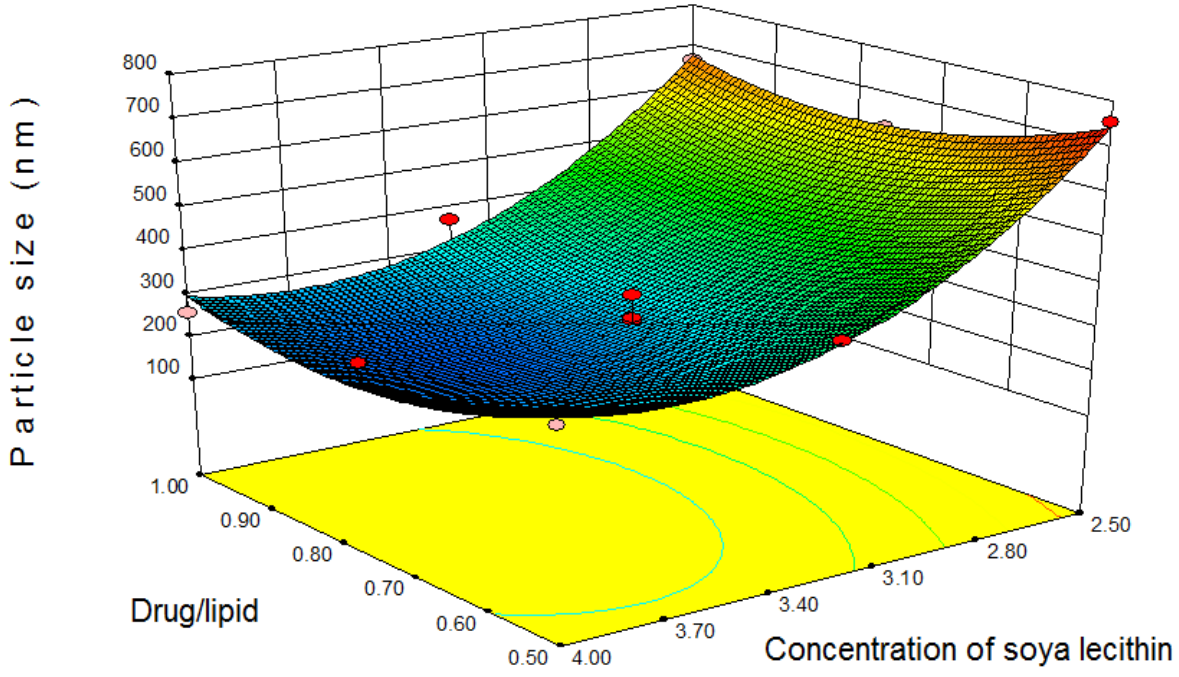

Fig.3. Response surface plot showing combined effect of drug/lipid(X_1) and concentration of soya lecithin(X_2) on particle size of nanoparticles

Entrapment efficiency

Fig.4 displays the curvilinear relationship between the entrapment efficiency of ketorolac-loaded solid lipid nanoparticles and concentration of soya lecithin. It can be inferred from the plot that the concentration of lipid has no significant effect on entrapment efficiency of SLN. Though it was expected that increasing the proportion of drug to lipid matrix will result in decrease in percent entrapment, but in the present study increasing proportion of ketorolac to lipid did not result in decrease in percent entrapment. Soya lecithin a surfactant also acts as a stabilizer of emulsion during the homogenization stage. Increasing the concentration of soya lecithin resulted in increase in percent entrapment which may be attributed to its stabilizing effect on the SLN during the homogenization stage which diminished the leaching of ketorolac form the lipid matrix. In this study, overall high entrapment efficiency indicated a good compatibility between ketorolac and lipid core of SLN.

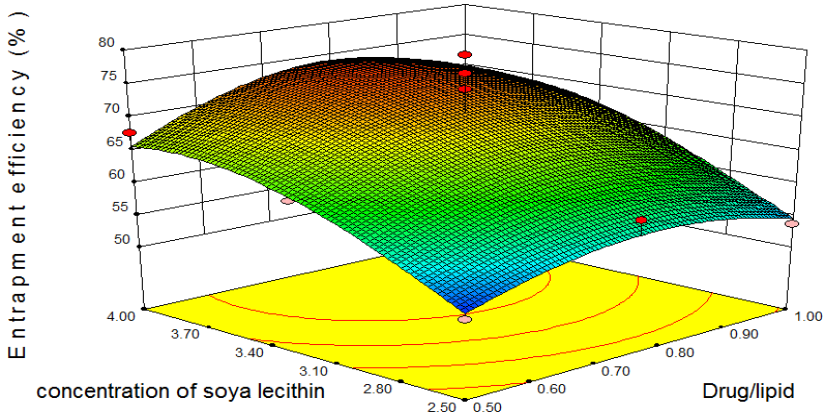

Fig.4. Response surface plot showing combined effect of drug/lipid(X_1) and concentration of soya lecithin(X_2) on entrapment efficiency of nanoparticles

A numerical optimization technique along with desirability approach was employed to develop an optimized formulation of ketorolac-loaded SLNs with desired responses. The constraints like maximizing entrapment efficiency and minimizing particle size were set as goals to locate the optimal settings of independent variables in the optimized formulation of SLN. The optimal formulation was predicted in the level of drug/lipid ratio 0.79 and soya lecithin concentration 3.73%w/v. To confirm the reliability of response surface model, the SLNs with the optimized factor levels was prepared and evaluated. The optimized batch of ketorolac-loaded SLN formulation was found to have particle size (Y_1) of 227.2 nm (predicted 213.24 nm) and entrapment efficiency (%) (Y_2) of 71.82% (predicted 73.48%). The lower values of % prediction error (3.24% for Y_1 and 2.25% for Y_2) confirmed the predictability and validity of the model.

In vitro drug release study

Figure 5 shows the comparative *in vitro* release profile of ketorolac from ketorolac conventional gel and ketorolac-loaded SLN gel. It can be inferred from the graph that the ketorolac-loaded SLN gel could prolong or retard the drug release by the fact that the drug molecules are entrapped in the solid lipid matrix. Comparing the drug release from conventional gel and ketorolac-loaded SLN gel, the release of ketorolac was slower from ketorolac-loaded SLN gel formulation. The percentage drug release at the end of 24 h in KSLNG formulation was found to be 55.06% whereas the percentage drug release at the end of 24 h in KCG was 86.14%.

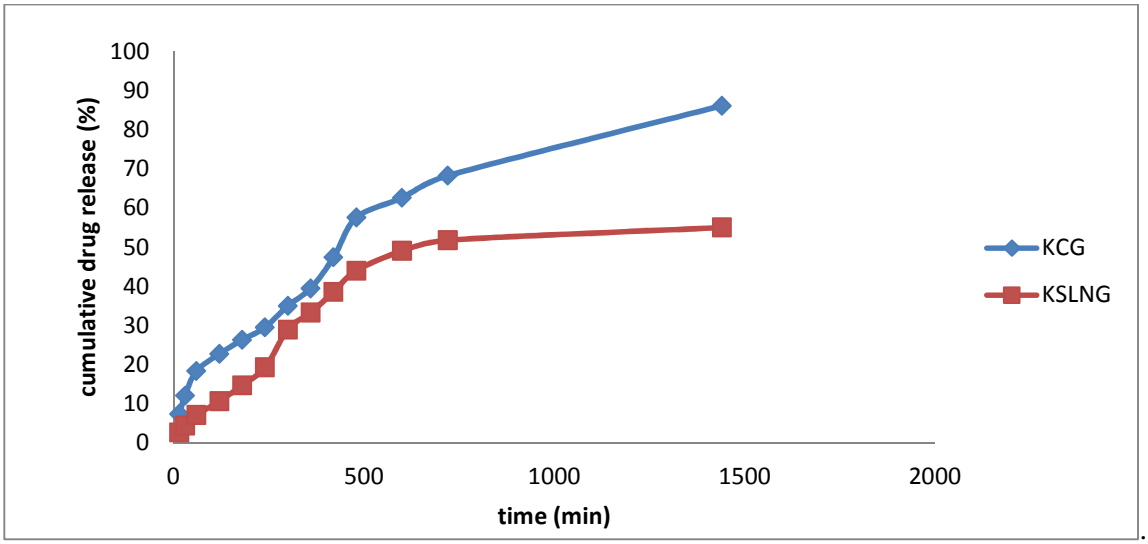

Fig. 5. Comparative drug release profile of ketorolac convention gel (KCG) and ketorolac-loaded SLN gel (KSLNG)

The release rate of Ketorolac-loaded SLN gel and ketorolac conventional gel were fitted into various kinetic models to estimate their release kinetics and mechanism of release. Table 4 shows the modeling and release kinetics of ketorolac from the two formulations. The release kinetics data was found to fit best into the Higuchi's square root release kinetics. Further the value of n (n<0.43) the release exponent of Korsmeyer-Peppas equation reveal that the drug is released from the two formulations by diffusion through the matrix.

Table 4. Mathematical modeling and release kinetics of Ketorolac from ketorolac conventional gel and ketorolac-loaded SLN gel

Formulation	Zero-order	First-order	Higuchi's square root	Korsmeyer-Peppas		Best fit model
	R^2	R^2	R^2	R^2	n	
KCG	0.884	0.67	0.968	0.849	0.18	Higuchi
KSLNG	0.741	0.562	0.904	0.861	0.19	Higuchi

KCG-ketorolac conventional gel, KSLNG-ketorolac-loaded SLN gel

In vivo anti-inflammatory activity

The *in vivo* performance of ketorolac-loaded SLN gel was carried out in carrageenan-induced rat paw edema model and was compared with the ketorolac conventional gel. Table 5 represents the percent inhibition in paw edema thickness at different time intervals for KSLNG and KCG gel. The formulation under study not only decreased the inflammation to the larger magnitude, but also sustained this magnitude. In ketorolac-loaded SLN gel formulation the maximum inhibition was observed at 6th h with higher value (78.9%), upto 8 h inhibition was maintained above 61%, and even after 24 h, 31.29% inhibition was observed (p<0.01). However, in case of ketorolac conventional gel inhibition was displayed at 2 h with magnitude of 81.36% and just after 6 h it scored below 33% (p<0.01).

The maintenance of inhibition upto 24 h, after achieving the maximum inhibition by KSLNG gel was significantly higher than that of the KCG gel. The reason could be the drug concentration in the blood, which was maintained for longer duration in case of KSLNG gel in comparison to KCG gel. These nano ranged lipid carriers stick into stratum corneum and dermis that allow drug release for long time and continue the anti-inflammatory action. It was closely observed that the KSLNG gel decreased edema effectively and sustained the anti-inflammatory effect in comparison to KCG gel.

Table 5. Percent inhibition in paw edema thickness at different time intervals

Treatment	% inhibition in paw edema thickness measured after						
	1h	**2h**	**4h**	**6h**	**8h**	**12h**	**24h**
KCG	45.85	81.36	52	33.01	24.72	10.98	5.31
KSLNG	20.66	34.67	47.07	78.9	61.85	37.05	31.29

KCG-ketorolac conventional gel, KSLNG-ketorolac-loaded SLN gel

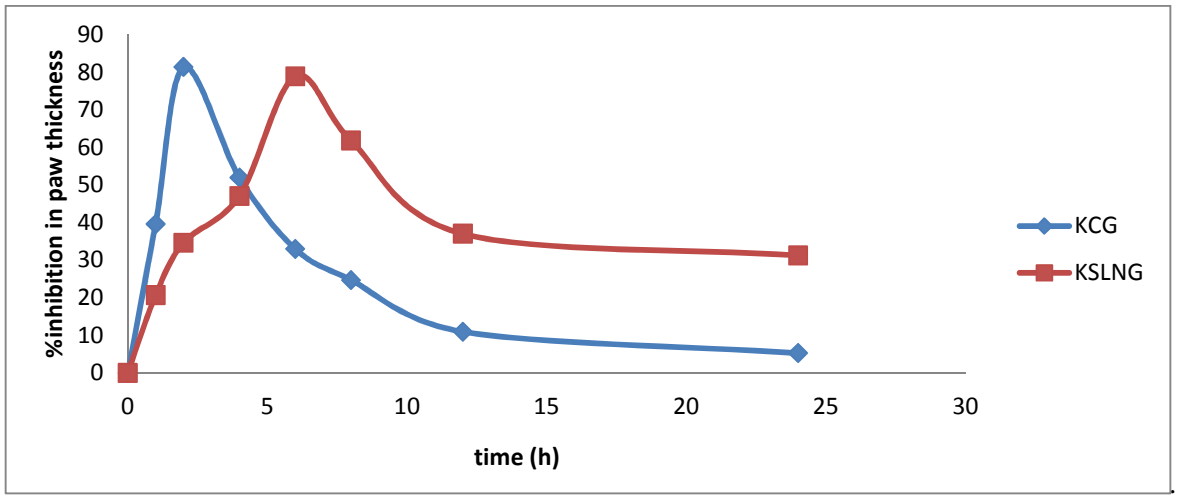

Fig.6. Anti-inflammatory activity of ketorolac-loaded SLN gel in comparison to ketorolac conventional gel after transdermal application in Carrageenan induced rat paw edema (n=4)

CONCLUSION

In the present study solid lipid nanoparticles of ketorolac were formulated using stearic acid and soya lecithin and were optimized employing central composite design. The optimized formulation had a particle size of 227.2 nm and entrapment efficiency of 71.82%. The optimized batch of SLN dispersion was formulated as gel and assessed for anti-inflammatory activity in carrageenan induced hindpaw edema in rats in comparison to ketorolac conventional gel. The ketorolac-loaded SLN gel possessed a sustained drug release over period of 24 h and the effect was more pronounced with the ketorolac conventional gel formulation. The anti-inflammatory activity of the ketorolac-loaded SLN gel was maintained for longer period of time due to slow release of the drug.

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