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Research Article

PREPARATION, OPTIMIZATION AND EVALUATION OF TRANSFEROSOMAL FORMULATION FOR ENHANCED TRANSDERMAL DELIVERY OF A COX-2 INHIBITOR

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ABSTRACT

Objective: The main aim of this study was to enhance the transdermal penetration of a poorly water-soluble NSAID, Piroxicam, by encapsulating it into a nanocarrier namely transferosomes.

Methods: Transferosomes were prepared using Rotary Evaporation and sonication method and optimized by design expert, version 7.0.2. Phospholipon 90G and sodium deoxycholate were used to prepare transferosomes. These formulations were characterized by Differential Scanning Calorimetry (DSC), Transmission electron microscopy (TEM), *in-vitro* dissolution, *ex-vivo* permeation studies and animal activity.

Results: On the basis of experimental designing transferosomes with 2% sodium deoxycholate and 0.125% piroxicam were selected for further studies. The transfersomal gel showed a significantly higher ($p<0.05$) cumulative drug permeation and flux than the drug solution and conventional gel. The pharmacodynamics study revealed that the transfersomal gel had better anti-inflammatory activity as compared to the conventional gel.

Conclusion: Thus, the study substantiated that the transfersomal gel can be used as a feasible alternative to the conventional formulations of piroxicam with advanced permeation characteristics for transdermal application.

Keywords: Piroxicam, Transdermal transfersomes, Permeation studies, *In- vivo* study.

INTRODUCTION

Nano vescicular piroxicam delivery systems have the ability to improve the pharmacokinetics and increase biodistribution of therapeutic agents to target organs, resulting in improved efficacy [1]. Secondly, drug toxicity is reduced as a consequence of preferential accumulation at target sites and lower concentration in healthy tissues.Hence nanovesicular systems have been promoted as a means of sustained or controlled release of drugs.

Rheumatoid arthritis (RA) is an inflammatory debilitating arthritis affecting large number of population [2]. Hence RA requires rapid onset of action for immediate pain relief as well long term treatment. In RA synovium gets inflamed leading to leaky vasculature. Hence drug carriers between 200-500 nm can be easily accumulated into the inflamed tissue.

A major obstacle to dermal and transdermal drug delivery is the permeation characteristics of the stratum corneum, which limits drug transport, making this route of administration frequently insufficient for medical use[3].Classical liposomes remain confined to the upper surface with little penetration into the stratum corneum. Hence a new class of liposomes also called as Transferosomes®have been developed. Transferosomes are promising nanocarriers for non invasive transdermal delivery.

Better drug delivery by transferosomes is due to the driving force provided by the osmotic gradient between outer and inner layer of stratum corneum [4], thus, they can pass through the intact skin spontaneously under the influence of the naturally occurring *in vivo* transcutaneous hydration gradient. These nanocarriers possesses an edge activator(EA) such as sodium deoxycholate, span 80 or tween 80 which provides stress dependant adaptability to these carriers so that they can easily squeeze between the pores of the stratum corneum. Also, transferosomes are colloidal carriers which are easily accumulated into the leaky synovial tissue which leads to peripheral targeting. Transferosomes also act as depot resulting in controlled drug delivery system.

Piroxicam, a non-steroidal anti-inflammatory drug (NSAID), are used in the treatment of various acute and chronic musculoskeletal disorders like rheumatoid arthritis, osteoarthritis etc., and also as potent analgesics. However, the use of piroxicam has been associated with a number of gastrointestinal disorders [5].Dermal delivery is an alternative route, but requires a formulation which ensures deep skin penetration. Thus the study encompasses the ability of lipid vesicles to deliver piroxicam across skin in order to evaluate its transdermal delivery potential as well as overcome its side effects.

MATERIALS AND METHODS

Materials

Commercial grade Piroxicam (PX) was a gift sample obtained from Ramdev chemicals, Mumbai. Phosphatidyl choline (Phospholipon 90G) was a gift sample from Lipoid, Germany. Sodium deoxycholate (SDC) was obtained from S.D Fine chemicals. All other chemicals were locally procured and solvents of merck grade were used. Albino Wistar rats used in the study were purchased from Haffkine's institute (Mumbai, India).

Methods

Differential calorimetry analysis (DSC)

Possible piroxicam-excipients interaction study was determined by DSC (Seiko Exstar SII DSC 6220). PX, SDC and lipid, physical mixture as well as optimized formulation were subjected to DSC analysis. DSC analysis was performed to ascertain the lack of potential interactions between the formulation components and to confirm the formation of transferosomes. The instrument was calibrated with indium standard. Accurately weighed samples were placed in open, flat bottom, aluminum sample pans. Thermograms were obtained by heating the sample at a constant rate of 10^0C/minute. A dry purge of nitrogen gas (20ml/min) was used for all runs. Samples were heated from 35C – 400C. Scans were obtained from the samples. The melting point and the peak maxima were observed in the DSC graphs.

Formulation of transferosomes (PX-TRS): Transferosomes can be prepared by

1. Rotary evaporation and sonication method.
2. Vortexing and sonication method

Rotary evaporation-sonication method was found to give more stable transferosomal formulation. Hence this technique was chosen for formulation development and batch processing. Initially lipids such as Phospholipon 90G and Phospholipon 90H were screened on

the basis of their film forming ability. Also four different edge activators such as tween-80, span 80, span 65 and SDC were evaluated on the basis of their entrapment efficiency. Hydrating agents such as water, phosphate saline buffer (PBS 6.4) and 7% ethanol were evaluated. Finally Phospholipon 90G, SDC and water were selected for further studies. Phospholipon 90G, PX and EA (surfactant) i.e SDC were dissolved in chloroform and methanol in a round bottom flask (RBF). The ratio of chloroform to methanol was 2:1 v/v. The solvent was evaporated and a thin film was formed using a rotary flash evaporator (PBU-6, Superfit, Mumbai, India). The film was hydrated using water as hydrating medium for 1 hr at room temperature which resulted in multilamelar vesicles. These were further size reduced by ultra sound cavitation using probe sonicator (Oscar, Japan) to form small unilamellar vesicles.

Optimization of processing parameters for Transferosomes

Initially, the effect of rotational speed of the rotovac and temperature of hydration on transferosomes was studied. Next, PX was added to the blank transferosomes. Being lipophilic PX got entrapped within the lipid bilayer. Hence, it was dissolved in methanol before film formation. The effect of hydration medium and hydration time on the entrapment of PX was studied. Further it was observed that, a stable bilayer could only accommodate certain amount of PX. Further increase in the amount of PX led to sedimentation. Also, the effect of these physical parameters remained constant irrespective of the ratios of lipids employed. The effect of sonication time was studied with respect to quality.

Table 1: It shows effect of rotation speed of the rotovac and hydration temperature on the trial batches

Ingredients	Quantity		
	T1(trial 1)	**T2(trial 2)**	**T3(trial 3)**
Phosphatidyl Choline (gm)	0.1	0.1	0,1
Edge activator (mg)	10	10	10
Chloroform: Methanol 2:1 v/v (ml)	20	20	20
Water (ml) up to	20	20	20
Effect of rotation speed of the Rotovac on the formulations			
60 rpm	Bumpy film with entrapped air bubbles		
80 rpm	Uniform Film		
100 rpm	Bumpy Film		
Effect of Time period for Ultracavitation on final formulations			
Time Clarification Precipitation			
2 min	+ no precipitate		
4 min	++ precipitates		
6 min	++ precipitates		

(+) good (++) very good

The above two observations were further corroborated by the values for entrapment efficiency of the formulation. Optimized process parameters were then used to process further batches. The above batches were subjected to experimental designing technique to study intricately the interactions of PX with the lipids and its effect on entrapment and particle size of the final formulation.

Experimental Designing

Here, a commercially available software program was used (Design Expert, Version 7.0.2, Stat-Ease Inc, Minneapolis, MN). The experimental design chosen was Response Surface, 2- factors, 3-level factorial; 9 formulations were formulated. Run order was kept in the randomize mode to protect against the effects of time related variables and also to satisfy the statistical requirement of independence of observations. Analysis of variance (ANOVA) and all statistical analyses were also performed using the same software. Calculation of the effects was performed; half- normal plots, response surface plots were plotted. Also ANOVA was used to treat the data, and for proper model selection. The F value was checked to see whether it is within the desired limits. The F value was calculated by comparing the treatment variance with the error variance. The factors considered were:

(1) Amount of Edge activator

(2) Amount of PX.

Factor A- Edge activator concentration: Surfactant concentration was varied to study the effect of surfactant concentration on particle size and stability as well as its interaction with the lipids.

Levels of factor A are shown in Table 2.

Table 2: It shows levels of factor A- edge activator concentration.

Level of factor (A)	Coded value	Concentration(mg)
Low	-1	20
Medium	0	40
High	1	60

Factor B- PX concentration:

PX concentration was varied to achieve maximum entrapment of the piroxicam and to adjust the drug loading and particle size. Levels of factor B are shown in Table 3.

Table 3: it shows levels of factor B- PX concentration

Level of factor (B)	Coded value	Concentration(mg)
Low	-1	15
Medium	0	25
High	1	35

Response: Average particle size and Piroxicam entrapment efficiency.

The formula of PX loaded transferosomes was based on 3^2factorial design where each of the two factors were considered at three levels. Thus as shown in Table 4, total 9 batches were prepared.

Table 4: Design matrix for Experimentation

Ingredients	Batches								
	TRS1	**TRS2**	**TRS3**	**TRS4**	**TRS5**	**TRS6**	**TRS7**	**TRS8**	**TRS9**
Piroxicam (mg)	15	15	15	25	25	25	35	35	35
Surfactant (mg)	20	20	20	40	40	40	60	60	60
Lipid (mg)	400	400	400	400	400	400	400	400	400
Water (as hydrating medium) in ml	20	20	20	20	20	20	20	20	20

Characterizations of transferosomal formulation

The vesicle size and distribution were determined by dynamic light scattering method Malvern zetasizer (Malvern Instruments Ltd.). Measurements were carried out at an angle of 90° at 25°C.

Dispersions were diluted with double distilled water to ensure that the light scattering intensity was within the instrument's sensitivity range. For the imagine analysis by TEM, copper grids having a thin layer of carbon was loaded with piroxicam loaded transferosomal dispersion and allowed to dry at room temperature. After the sample was dried thoroughly, the images were captured on a Philips Tecnai-20 Transmission Electron Microscope (Philips, Holland) with 0.27nm point resolution and accelerating voltage of 200 Kv. For zeta potential analysis, a proper amount of PX transfersomes were diluted appropriately and determined by Malvern particle size analyzer. For determination of entrapment efficiency the unentrapped drug was separated by centrifugation method. The amount of drug entrapped (Total amt. of drug- unentrapped drug) in the vesicles was then determined by disrupting the vesicles using methanol followed by filtration and amount of PX was quantified spectrophotometrically at 358 nm.

$$\text{Percentage entrapment} = \frac{\text{Amount of Entrapped PX}}{\text{Total PX added}} \times 100$$

***In vitro* piroxicam release from transferosomes**

In vitro piroxicam release was evaluated using Keshary chein diffusion cells. A cellophane dialysis membrane with molecular weight cut-off of 8000 Da (Hi-media) was hydrated with the receptor medium of phosphate buffer pH 7.4(PBS 7.4) overnight before being fastened between the donor and receptor compartments. The donor medium consisted of 2 ml of the transferosomal dispersions. The same was repeated with dispersion loaded gels. The receptor compartment was filled with 13 ml of PBS 7.4 and stirred with a magnetic bar. The available diffusion area was 2.61 cm^2. The temperature of the assay was controlled at 32 °C to mimic human skin. 2ml aliquots were withdrawn at fixed time intervals and immediately replaced with an equal volume of fresh buffer. All samples were analyzed for PX content by spectrophotometry at 358 nm. The experiment was done in triplicate.

***In vitro* skin permeation study**

Porcine ear skin from local slaughter house was used as a model membrane for the skin permeation study because of its similarity to human skin in lipid content and permeability. The skin samples were mounted on the keshary chein diffusion cell and the temperature was maintained at 37^0C. The dorsal surface of the skin was placed in contact with the donor chamber, which was filled with the transferosome formulation. The receptor chamber was filled with PBS 7.4 and stirred with a star-head Teflon magnetic bar driven by a synchronous motor. At time intervals of 0.5, 1, 2, 4, 8 and, 24 h, 1ml aliquot of receptor was withdrawn, and replaced by fresh medium. The concentration of drug in the samples was analyzed spectrometrically at 358 nm , and the cumulative amount was plotted against time. The steady state flux was determined as the slope of linear portion of the plot.

***Ex-vivo* lipid peroxidation studies**

Preparation of Liver Homogenate

• The albino wistar rats (weighing 100-150gm, overnight fasted before experiment) were sacrificed

• The liver was quickly removed and chilled in ice cold saline.

• After washing with ice cold saline, the liver was homogenized in 0.15 M KCl to get 10% liver homogenate.

Methodology

The method proposed by Ohkawa *et al*, 1979 [6] was used for estimation of inhibition of lipid peroxidation.

• Fresh 0.2 ml of liver homogenate was mixed with 150 mM of 0.1ml KCl and 0.4 ml of TRIS buffer. The test samples of 0.1 ml of plain PX, TRS gel solution were then added in various concentrations.

• *In vitro* lipid peroxidation was initiated by addition of 0.1 ml each of $FeSO_4$ (10μM) and 100 μM of test samples.

• After incubation for 1hr at 37^0C, reaction was terminated by addition of ThioBarbituric acid (TBA) 2 ml of reagent and boiled at 95^0C for 15 mins for development of colored complex.

• After cooling, the tubes were centrifuged at 4000 rpm for 10 mins. The absorbance of supernatant was determined colorimetrically at λ_{max} of 532 nm.

• Percentage inhibition of TBA reacting substances (TBARS) formation was calculated with respect to control in which no test sample was added. The inhibition of lipid peroxidation was determined by calculating the % decrease in the formation of TBARS and IC_{50} was calculated.

Calculation

The percent inhibition of lipid peroxidation of test/standard PX was calculated by following equation:

$$\% \text{ Inhibition} = \frac{(A_0 - A_1)}{A_0} \times 100$$

Where, A_0 is the absorbance of the control (blank) and A_1 is the absorbance of the test samples.

Preparation of transferosome enriched gel

Viscosity of the transferosomal dispersion (test sample) was low. Hence to achieve the desired rheological characteristics and texture for transdermal application, the optimized dispersion was converted into a gel. Various gelling agents like Carbopol Ultrez 10 and Carbopol 940 were evaluated for their gelling ability. Based on the compatibility with nanoparticulate dispersions, feel, aesthetic appeal and ease of spreadability Carbopol Ultrez 10 was selected as the gelling agent. Gel dosage forms of PX were prepared using a serial mixture of deionized water and glycerin in the ratio of 4.5:0.5 w/w as the vehicle and Carbopol Ultrex 10. Different concentrations of Ultrex 10 ranging from 0.5-1% w/w were used for gelling and the concentration giving the optimum viscosity was chosen for further studies. For the final formulation 0.75% w/w Ultrez 10 was selected and dispersed into the vehicle to give the total drug concentration of 0.125% w/w.

Triethanolamine was added to adjust the pH to 7, and then remaining vehicle was added to give a total weight of 20 g. Gel was dispersed thoroughly using an overhead stirrer at the speed of 800 rpm (Remi, Mumbai, India) for 3h.

***In vivo* studies (pharmacodynamics) by carrageenan paw edema test**

All studies were carried out in accordance with the principles of Laboratory Animal Care and the experimental protocols were reviewed and approved by the Institutional Animal Ethics Committee (Animal House Registration No. 25/1999/CPCSEA). The anti-inflammatory activity of the gel formulations was studied by carrageenan induced rat paw edema volume model. The rats weighing 150–180 g were randomly divided into 3 groups of six rats each. An identification mark was made at the ankle joint of each animal. PX-TRS gel and std conventional gel were applied on the subplantar region of the left hind paw of first, second, and third groups respectively. Fourth group was untreated and served as control. 1 h post transdermal application, paw edema was induced by subplantar injection of 0.1 ml of a 1% *w/v* freshly prepared carrageenan in normal saline into the left hind paw of each rat. The paw volume up to the ankle joint was measured before and at different time intervals after the carrageenan injection using graduated plethysmograph (INCO, India). All the test groups were compared with control group.

Percentage reduction in edema was calculated using the following formula [7].

$$\% \text{ Inhibition of paw edema} = \frac{(V_t - V_0)_{control} - (V_t - V_0)_{treated}}{(V_t - V_0)_{control}}$$

Where,

V_t is the paw volume at time't'

V_0 is the initial paw volume (before carrageenan treatment)

$(V_t - V_0)_{control}$ is edema produced in control group

$(V_t - V_0)_{treated}$ is edema produced in treated group

RESULTS

Drug- excipients interaction study

DSC detects drug-excipients interactions [8]. Thermal behavior of PX, Phospholipon 90 G, SDC alone and the physical mixture were studied using DSC. DSC thermogram of PX showed an endotherm at 207⁰C. Broad peaks were observed for both Phospholipon 90 G and SDC. The thermogram of the physical mixture was almost an overlap of each individual component, except for some slight differences as explained below. DSC thermogram of the physical mixture as shown in Fig. 1 showed broadening curve for the PX thermogram as compared to that of PX alone. This may be due to the melting of the lipid components and their interactions with PX. Partial incorporation of PX in the melted lipid is likely possible.

In the DSC of the optimized TRS5 batch overlay thermogram of PX loaded transferosomal dispersion as shown in Fig 1, shows absence of the melting endotherm of PX suggesting significant interaction of PX with the bilayer structure and hence its encapsulation into the nanocarrier system.

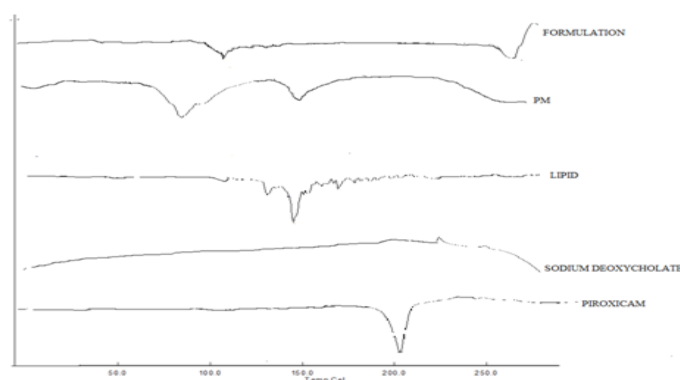

Fig 1: It shows overlay DSC spectrum of single loaded PX Transferosomes

Formulation Development

Selection of phospholipid (PL)

For the formation of a stable and uniform liposomal bilayer, presence of a suitable phospholipid is of utmost importance. Commercial grade of Phospholipon 90 H in powdered state yielded precipitates. This precipitates may be lipids coming out of the solution and hence could not be used as film former. When phospholipon 90G was used a uniform thin film was obtained. The drug entrapment and uniform film formation was attributed to the phase transition temperature of phospholipon 90G which is lower than 90H. Hence Phospholipon 90 G was selected as a film former.

Selection of edge activator

EAs are usually single chain edge activators, which when incorporated in the vesicular formulations destabilize the vesicles and enhance the deformability of the PL bilayer by decreasing the interfacial tension [9]. Four different edge activator under experimental were chosen for transferosome formulation. Entrapment efficiency was the main criteria for selection of edge activator. Tween 80 produced good dispersions however the entrapment was poor as compared to sodium deoxycholate which produced good and clear dispersions with higher entrapment efficiency.

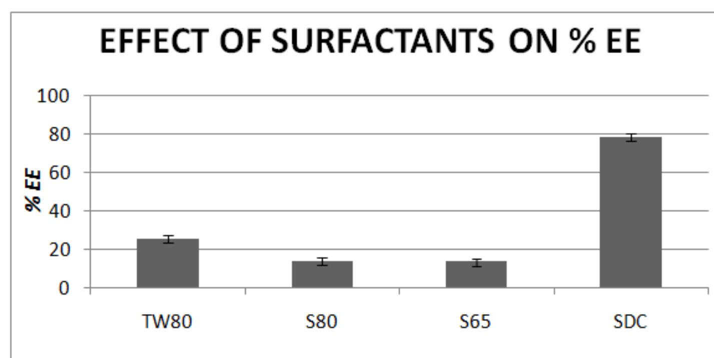

Fig. 2: It shows entrapment efficiency of transferosomal formulations with different edge activators [Data: Mean ± SD, n=3].

Selection of hydration medium

PBS 6.4, water and 7% ethanol were selected as hydration medium to investigate the effect of hydration media on PX-TRS quality. When in contact with excess buffer systems, SDC is able to form stable gels [10]. Ethanol as hydration media gave a clear solution leading to complete solubilisation of vesicles. Hence water was chosen aptly for hydration purpose.

Effect of drug added on trial batches

Entrapment efficiency was found to first increase with the increase of the drug while no sedimentation occurred till a certain amount; this was due to the fact that at first there was an abundance of phosphatidylcholine, and with the increase of the drug, the phosphatidylcholine amount decreased and at a optimum value it showed maximum entrapment [11]. The bilayer can only

incorporate certain amount of drug after which the stable liposomal bilayer starts sedimenting immediately upon formation. The threshold for PX was found to be 0.035 g per 0.4 gm of lipid employed.

Optimization of processing parameters for transferosomes

Rotational Speed of the Rotovac

Three different ratios of lipids were tried. Initially, the effect of rotational speed of the rotovac and time period of ultra sonication was studied. Lower speeds increased the time of contact of the film with the hot water contained in the water bath. At higher speeds, sufficient time was not available for the lipid to form film and proper liquid to gel transitions of the lipid could not occur. Both of this may have caused uneven distribution of heat leading to formation of uneven film. Based upon the observations cited in Table 1 and rpm of 80 was considered optimum for formation of batches.

Time period for Ultracavitation

The appearance of prepared transfersomes changed from turbid to clear as the time cycle increased. Time points set were 2 min, 4 min, and 6 min at a frequency of 70Mhz. simultaneously; encapsulation efficiency and particle size were gradually decreased with increase in time. During the preparation process, crude product was gradually homogenized to get unilamellar transferosomes with increasing time and cycles. The time period was optimized to 2 min giving optimum particle size and entrapment efficiency. Excessive sonication led to oxidation of phospholipids causing precipitation of the lipids. [12]

Effect of hydration temperature on transfersomes

As hydration had an influence on transfersomes formation, encapsulation efficiency of PX-TRS, which was obtained by hydration of transferosomes was affected by different temperatures. The hydration time of decreased with increasing hydration temperature [13]. When the temperature went too high, the stability of transfersomes was affected by the change of thermal stability of phospholipid material which reduced both drug loading ratio and encapsulation efficiency. Hence, the most appropriate temperature was found to be room temperature. Additionally, it was found to be stable at low temperature.

Experimental Design

The technique of 3^2 factorial design with 2 factors at 3 different levels affecting the particle size and entrapment efficiency was considered. All experiments were carried out in random order to nullify the effects of extraneous or nuisance variables. The results of the experimental design were analyzed using Design Expert software that provided considerable useful information and reaffirmed the utility of statistical design for conducting experiments. The selected independent variables like the concentrations of EA and concentration of PX significantly influenced the particle size and PX entrapment efficiency that is very much evident from the results in Table 4 which represents the various combinations of independent variables with its resultant affect on the dependant variable.

The experimental data were fitted into a quadratic polynomial model using the design-expert software and the equation in terms of the coded factors for the optimum particle size was found to be:

Particle size = $+140.57-45.67 * A+6.88 * B-8.93 * A * B+26.00 * A^2-13.35 * B^2$

In this case A, A^2, $B^2$are significant model terms, with their P-values less than 0.05. The value of the determination coefficient $R^2$was 0.9639, which implies that 96.39% of the variation in the responses was attributed to the independent variables. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. A ratio of 8.890 indicates an adequate signal. This model can be used to navigate the design space.

In case of the encapsulation efficiency too, experimental data were fitted into a quadratic polynomial model using the design-expert software and the equation in terms of the coded factors for the optimum entrapment efficiency was found to be:

Entrapment efficiency = $+70.18-6.73 * A-10.22 * B -1.27 * A * B -1.86* A^2 -30.72 * B^2$

In this case A, A^2, $B^2$are significant model terms, with their P-values less than 0.05. The value of the determination coefficient $R^2$was 0.9975, which implies that 99.75% of the variation in the responses was attributed to the independent variables. A ratio of 44.7 indicates an adequate signal. This model can be used to navigate the design space

Table 5: It shows design matrix and responses

Std. [Batch No.]	Run	Block	Factor A Amount of Edge activator [mg]	Factor B Amount of PX added [mg]	Response 1 Avg. Particle Size [nm]	Response 2 Entrapment efficiency [%]
TRS4	1	Block 1	-1.00	0.00	210	75.00
TRS8	2	Block 1	0.00	1.00	117	28.1
TRS9	3	Block 1	1.00	1.00	107	20.3
TRS6	4	Block 1	1.00	0.00	113.7	60.1
TRS7	5	Block 1	-1.00	1.00	178	35.6
TRS5	6	Block 1	0.00	0.00	150	71.7
TRS2	7	Block 1	0.00	-1.00	128	49.3
TRS3	8	Block 1	1.00	-1.00	115.3	42.9
TRS1	9	Block 1	-1.00	-1.00	222	53.1

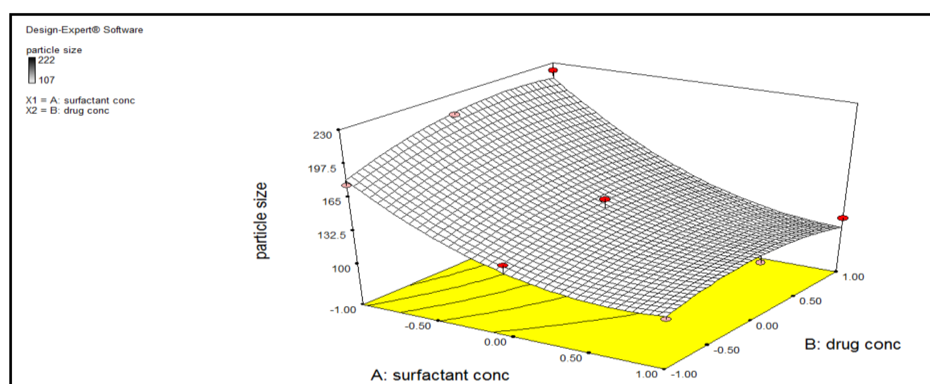

Fig. 3: It shows response surface plot for particle size

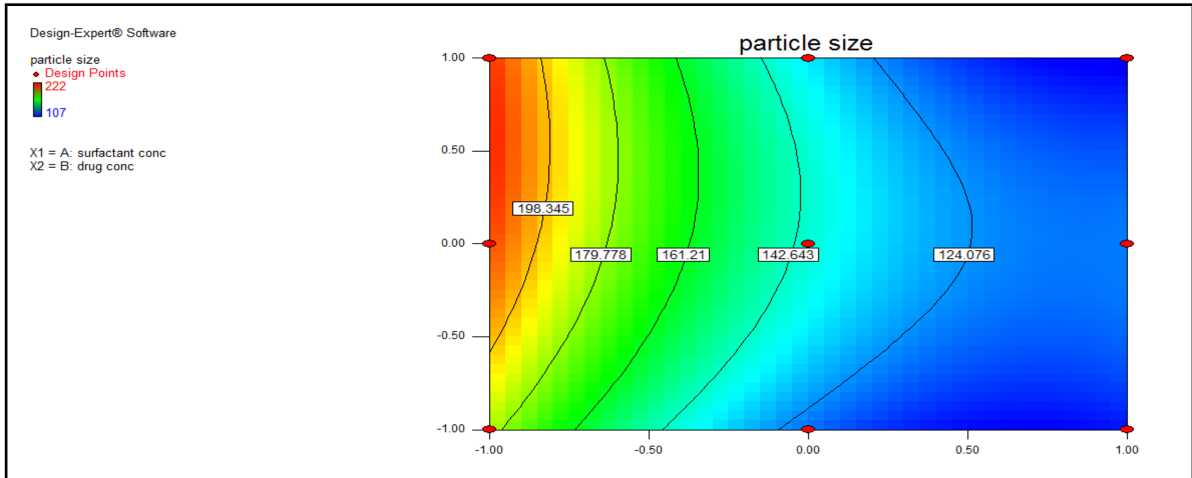

Fig. 4: It shows desirability plot for particle size

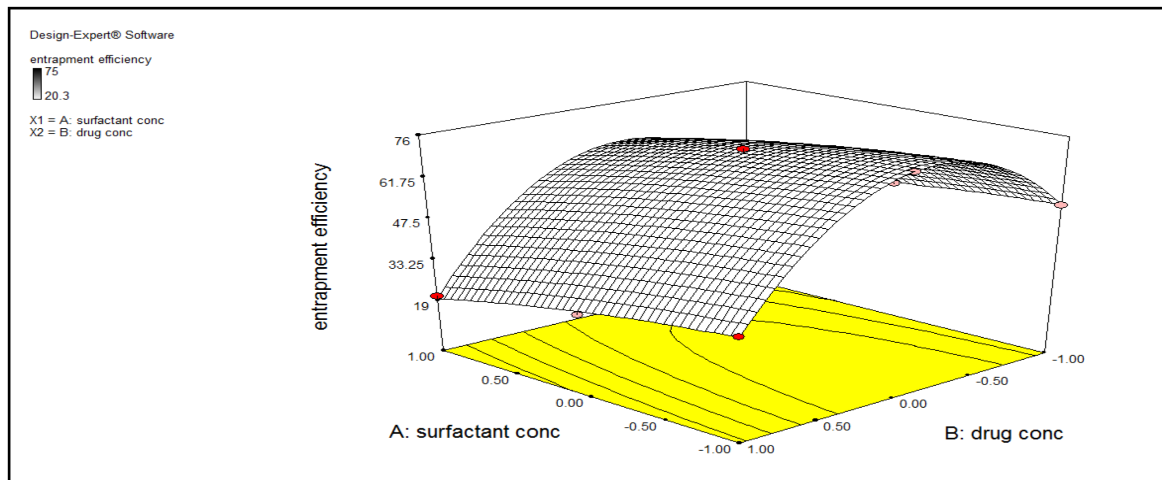

Fig. 5: It shows response surface plot for PX entrapment efficiency

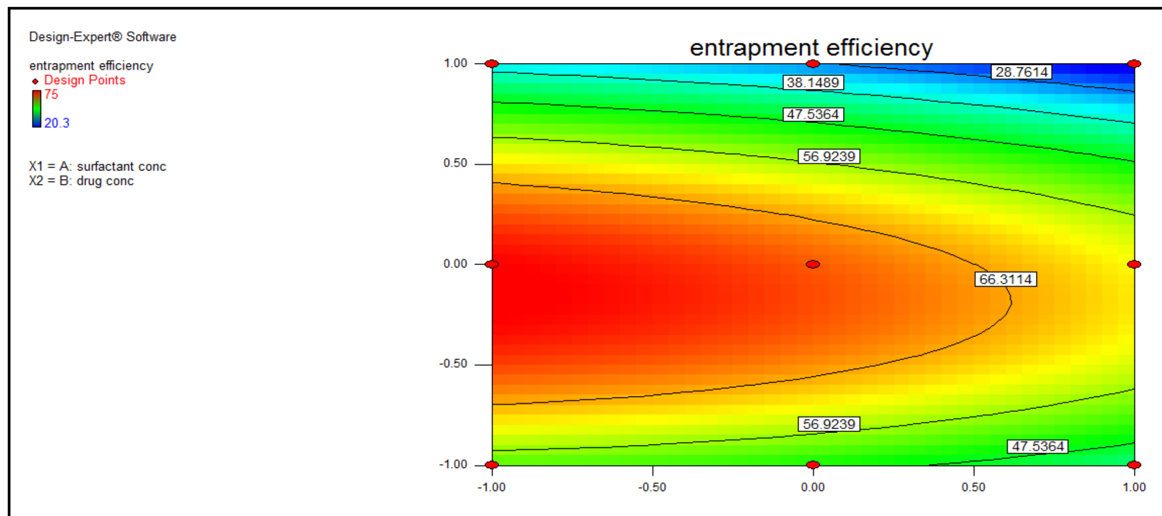

Fig. 6: It shows desirability plot for PX entrapment efficiency

Effect of amount of edge activators on transferosomes

Response plots as shown in Fig. 3and Fig. 5 indicate that an optimum amount of 40 mg for 20ml i.e. 2% of EA for total transferosomal dispersions generates particles of optimum size. Increasing or decreasing this amount causes changes in particle size. The entrapment efficiency is also highest when the amount of EA is 2%.

The initial increment in drug entrapment in the presence of low concentrations of EAs may be credited to the growth in vesicle size owing to the incorporation of more amount of drug. The decreased entrapment efficiency beyond a certain optimum concentration of EA can be ascribed to the pore formation/dissolution of PL bilayers in EA [14]. This may be due to the fact that at a certain concentration, surfactant molecule gets associated with the phospholipid bilayer, resulting in better partitioning of the drug. So above a 2% concentration of the surfactant, molecules may start forming micelles in a bilayer resulting in pore formation in vesicle membranes and complete conversion of vesicle membranes into mixed micelles. These mixed micelles were reported to have a lower drug carrying capacity and poor skin permeation due to their structural features [15]. Also particles greater than 200 nm are readily engulfed and taken up by the lymphatic system leading to lower bioavailibility of the drug. Moreover, transferosomes prepared with high EA concentrations exhibited smaller size than those with small EA concentrations. This reduction of the particle size diameter of elastic SDC transferosomes may be attributed to the increased flexibility and reduced surface tension of these vesicles.

From above data EA concentration had an influence on, not only the drug-loading ratio and encapsulation efficiency of transfersomes but also their structure and formation. Micelles were formed with excessive concentration of salt ions of sodium deoxycholate.

Effect of PX concentration on transferosomes

Addition of lipophilic drug within the liposomal barrier can, after a threshold level, disrupt the bilayer integrity and form mixed micelles, or can cause permeability changes in the bilayer membrane. Hence, the maximum amount of drug that can be entrapped to form stable bilayer vesicles was evaluated, which was found to be approximately 1/8 th the amount of lipid employed. Further, increasing the drug amount led to reduced entrapment efficiency and particle size.

The encapsulation of PX in transferosomes decreased the diameter of the transferosomes from addition of PX directly into lipid phase. These size modifications suggest that SDC can associate at liposome bilayer. This phenomenon can be understood since surface-active drugs as non-steroidal anti-inflammatory compounds are reported to self-associate and bind membranes causing partial disruption and solubilization, in a detergent-like fashion [16, 17].

Transmission Electron Microscopy

The morphology of PX transferosomes was as shown in fig.7. From the micrograph, it could be observed that unilamellar vesicles with an intact bilayer membrane were formed. The particle size of the vesicles was about 150 nm.

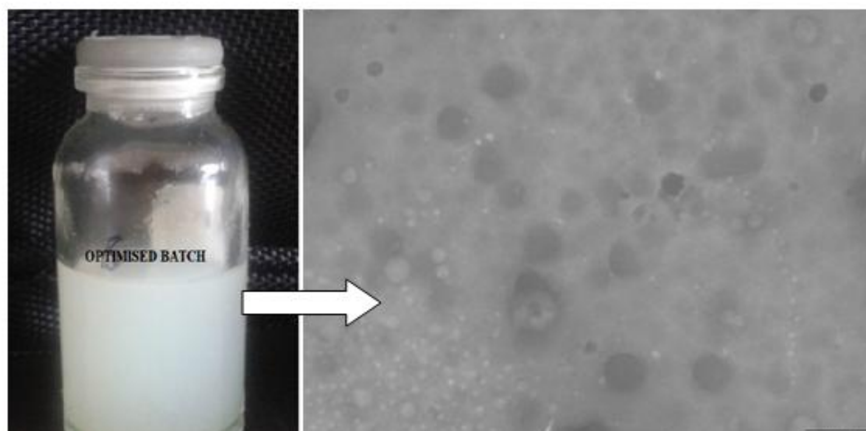

Fig. 7: It shows optimised batch TRS5 and TEM image of TRS5 (Bar: 1 unit = 200nm)

Zeta potential analysis

The zeta potential of optimized batch was found to be -22.7 mVwhich is in good agreement with literature due to the net charge of the lipid composition in the formulations. PC is a zwitterionic compound with an isoelectric point [pI] between 6 and 7. Under experimental conditions of pH 7.4, where the pH was higher than its pI, PC carried a net negative charge. The edge activator used was anionic edge activator, and the anion form of PX was also the predominant form at pH that pH. Therefore, a negative charge in all formulations was observed. Also the negatively charged liposome formulations strongly improved skin permeation of drugs in transdermal delivery [18]. The skin has also slight negative charge. Therefore, the negative zeta potential of the optimized transferosomal gel containing PX might influence improved PX permeation through porcine skin due to electrostatic repulsion between the same charge of the skin surface and the optimized gel. [19]

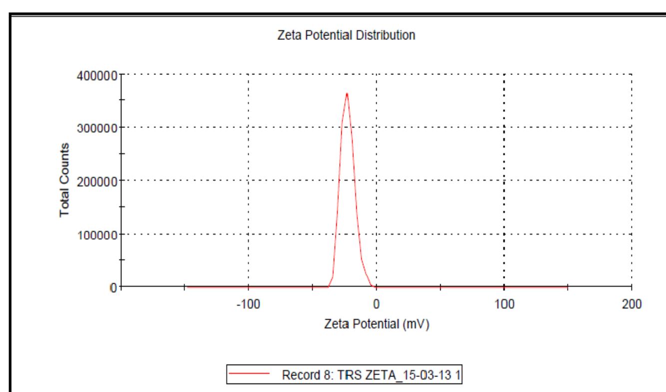

Fig. 8: It shows zeta potential analysis of optimized batch TRS5

In vitro diffusion studies

The diffusion studies were carried out in non-occlusive conditions to allow the driving force provided by the osmotic gradient. As expected, encapsulation of PX into transferosomes led to controlled release rate due to the well known reservoir effect of transferosomes, with the release profile being identical in all cases.

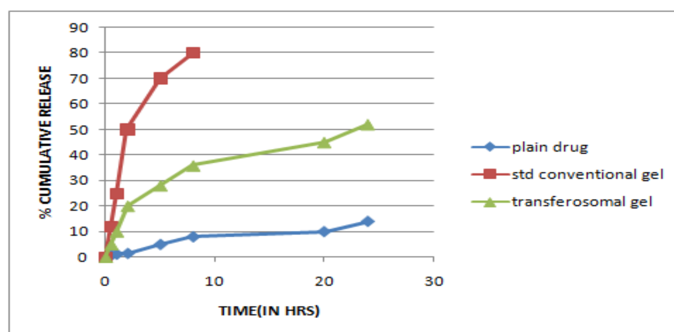

Fig. 9: It shows *in-vitro* release of PX from [A] Piroxicam Solution [B] Std. Conventional Gel [C] Piroxicam Loaded Transferosomal gel

The results indicate that, transferosomes release more PX in a sustained manner than do conventional gel, piroxicam release being related to entrapment efficiency and drug loading.

It was observed that the sustained effect in the order of transferosomal loaded gel > std conventional gel > plain piroxicam. While the lower solubility of the plain drug can account for its lower cumulative release.

Ex vivo skin permeation analysis:

The *ex vivo* permeation studies provide valuable information about the product behaviour *in vivo* since they indicate the amount of drug available for absorption. The action of transfersomes as penetration enhancer may predominantly be on the intercellular lipid of stratum corneum, raising the fluidity and weakness of stratum corneum. Ultradeformable character of transfersomes supports their passage through very fine pores in the skin under suitable osmotic gradient. Phospholipids have high affinity for biological membranes. Mixing of the phospholipid of the carrier system with the skin lipid of the intercellular layers may also contribute to the permeability of skin to lipid vesicles [20, 21]. The presence of unsaturated fatty acid in PC may be responsible for enhanced permeation. The packing nature of unsaturated fatty acids changed the fluidity of stratum corneum lipid structure and facilitated the permeation of the bioactive [22]. Topically applied lipid vesicles affect characteristics and integrity of the skin permeability barrier. In addition, they may extract the lipid from the skin or disrupt the order within and between the corneocyte upon binding to the keratin filament. Elastic vesicle can be used to transfer bioactive rapidly into the deeper layer of the stratum corneum, after which the bioactive can permeate into the viable epidermis. This is well supported by the study carried out by Gupta *et al* [23].

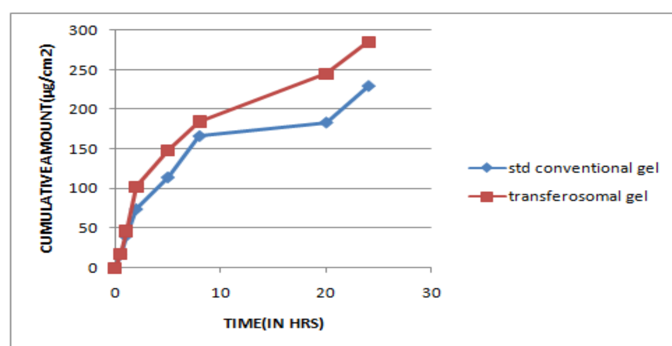

Fig. 10: It shows *ex vivo skin permeation analysis* of PX from [A] Std Conventional Gel [B] PX Loaded Transferosomal Gel.

The cumulative permeation amount in 24h was up to 52% for PX loaded transferosomal gel which was obviously higher than that of plain piroxicam and conventional gel. It can be corroborated that the presence of sodium deoxycholate contributed to deformation infiltration of transfersomes. Therefore, the PX transfersomes had good ability for transdermal permeation. Transdermal fluxes through the skin from the formulations were calculated by plotting the cumulative amount of PX permeating the skin against time and determining the slope of the linear portion of the curve by linear regression analysis.

The permeability coefficient [*k*p] was obtained by dividing the flux [J] by the initial PX concentration [C_o] in the donor phase:

$$kp = J / C_o$$

Table 6: It shows permeability coefficient and flux for the formulations assayed

Formulations	Permeability coefficient [x 10^{-3}] [cm h^{-1}]	Flux [µg h^{-1} cm^{-2}]
Std conventional gel	0.1486	7.43
Piroxicam loaded transferosomes	0.3096	14.48

The activity of penetration enhancers may be expressed in terms of an **enhancement ratio [ER]:**

$$ER = \frac{\text{Drug permeability coefficient [Treated]}}{\text{Drug permeability coefficient [Control]}}$$

The enhancement ratio was found to be 3 times more as compared to the conventional gel.

Ex vivo lipid peroxidation studies

Increased level of ROS has been implicated in the pathogenesis of RA. Excessive ROS production disturbs redox status, damages macromolecules, including DNA and can modulate expression of a variety of immune and inflammatory molecules leading to inflammatory processes, exacerbating inflammation and affecting tissue damage [24]. In inflammatory disease several chemokines are capable of triggering free radicals at the site of inflammation by the activated neutrophils [25]. These observations added another link to the relationship between ROS and chemotactic chemokines production in autoimmune diseases. Chemokines and oxidative stress in rheumatoid disease play an important role in recruiting and activating leukocytes and in enhancing oxidative stress in these diseases. Controlling the ROS in RA patients can be effective in preventing the cartilage destruction. Inhibition of free oxygen radical overproduction in RA by antioxidants and anti-inflammatory drugs can be considered as a useful supporting treatment regime for free radical pathologies. Selective COX-2 inhibitors are most promising therapeutic approach for RA.

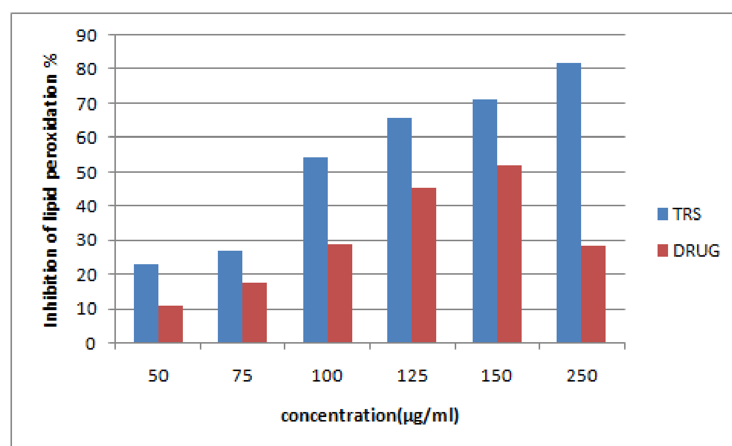

Fig 11: It shows lipid peroxidation inhibition percent of Piroxicam TRS, Data expressed as Mean± SEM

Overproduction of free radicals causes a chain reaction of peroxidation on the cell membrane's lipid that could lead to cell and tissue death. The inhibitory effect on ferrous ion–vitamin C system-induced lipid peroxidation in rat liver homogenate was used to assess the anti-lipid peroxidation activity of the different PX systems. PX loaded transferosomes elicited concentration dependent inhibition of $FeSO_4$ induced lipid peroxidation in rat liver homogenate. IC_{50} value is indicative of the concentration of the drug that is required to elicit 50% of the proposed activity. The IC_{50} value of TRS5 was 95.34 ± 12 µg/ml, whereas plain PX showed an IC_{50} value of 140.4. ± 33 µg/ml as shown in Fig. 12. Thus, TRS5 showed better inhibition of lipid peroxidation. In other words, almost double the quantity of plain piroxicam is required to elicit the same response as that produced by TRS5. (**P < 0.05 by one way annova)

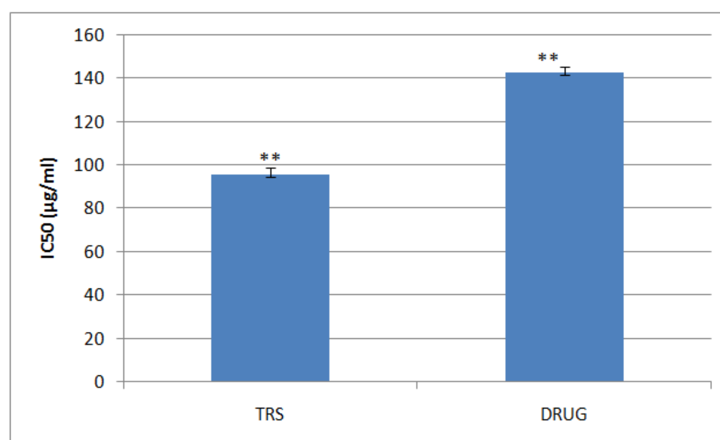

Fig. 12: It shows *ex vivo* lipid peroxidation of PX Transferosomes

Pharmacodynamics study

Carrageenan induced oedema [Fig. 13] in paw is attributed to two phases, the initial phase is the release of histamine and serotonin, and the second phase is the release of prostaglandins and lysosomal bodies, which are sensitive to most clinically effective anti-inflammatory drugs.

The fast inhibitory action of a gel formulation at the receptor sites may be a desirable feature for anti-inflammatory drugs to induce maximum pharmacological action without being prematurely wiped off from the applied site. Previously, Mei *et al* have reported that the formulations that had a small particle size showed higher acute anti-inflammatory activity [26].

Table 7: It shows percent reduction in edema produced by PX containing transferosomal gel (TRS5 gel), and std conventional (std gel) in carrageenan induced rat paw.

S. No	Treatment [n = 6]	0 hr	1 hr	2 hr	3hr	4 hr	6 hr	24 hr
1	Control	0.88 ± 0.0060	1.227± 0.0050	1.36± 0.0069	1.620± 0.0045	1.592 ± 0.0050	1.523 ± 0.0065	1.19± 0.0057
2	Standard [Marketed formulation]	0.88 ± 0.0060	1.179 ± 0.0059	1.71 ±0.0059	1.344± 0.0056	1.376± 0.0060	1.341± 0.0061	0.101± 0.006
3	TRS enriched gel	0.87 ± 0.0054	1.119 ± 0.0061	1.36 ± 0.0043	1.19 ± 0.0065	1.205 ± 0.0059	1.179 ± 0.0057	0.501± 0.0060

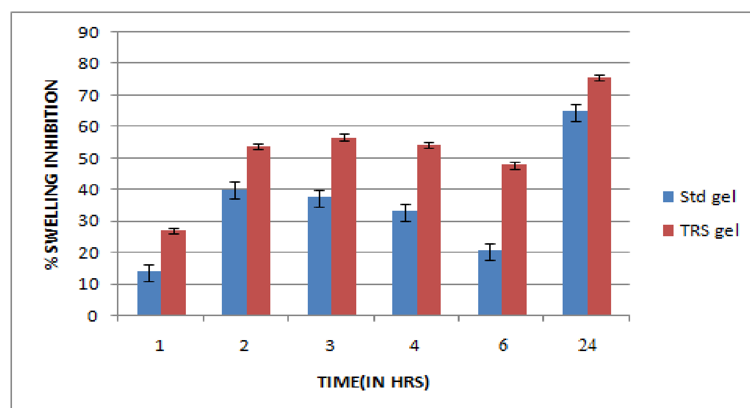

Fig. 13: It shows percent reduction in edema produced by PX containing transferosomal gel (TRS gel), and std conventional (std gel) in carrageenan induced rat paw.

Application of formulation TRS5 and the conventional gel produced, mean percentage inhibition of edema after the carrageenan insult and the values were 74% and 60.46%, respectively of the initial amount, after 24 h. This may be due to the reduced percentage of PX release from conventional PX gel, which was not enough to control edema effectively for multiple hours. There is a significant difference between the tested groups and the control as determined by a one way ANOVA with $p < 0.05$

CONCLUSIONS

The results of the present study indicate that the piroxicam transfersomal gel formulated by using sodium deoxycholate, soya phosphatidyl choline and carbopol can be used to enhance skin delivery of PX because of excellent release and permeation of the drug. The carrageenan induced paw edema test showed that the transfersomal gel formulation has better anti-inflammatory activity because of the increased permeation of deformable vesicles into the skin. These transferosomal gel studies have shown a 3 fold increase in permeation of the drug making it an ideal candidate for delivery through transdermal route. Thus, the developed transfersomal formulation may prove to be a promising carrier for piroxicam and other similar drugs, especially due to their simple production and ease of scale-up.

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