Academic Sciences

International Journal of Current Pharmaceutical Research

ISSN- 0975-7066 Vol 6, Issue 4, 2014

Research Article

TRANSETHOSOMES AND ETHOSOMES FOR ENHANCED TRANSDERMAL DELIVERY OF KETOROLAC TROMETHAMINE: A COMPARATIVE ASSESSMENT

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Received: 26 September 2014, Revised and Accepted: 07 October 2014

ABSTRACT

The aim of this investigation was to formulate, evaluate and compare the transdermal potential of novel vesicular carriers: transethosomes and ethosomes. Transethosomes (TELs) and ethosomes (Els) were prepared by cold method and were characterized for particle size, entrapment efficiency, transmission electron microscopy (TEM), zeta potential, elasticity measurement, *in-vitro* drug release, *ex-vivo* permeation studies and *in-vivo* study. Transethosomal and ethosomal formulation showed particle size of 180 ± 70 nm and134 ± 65 nm. Transethosomes showed higher drug entrapment (80.08 ± 4.5%) than ethosomes (70.79 ± 5.6%). Both the formulation showed good zeta potential indicating good stability. The elasticity of transethosomal vesicles was found to be 3-fold higher than the ethosomal vesicles. The transdermal flux of transethosomal gel was 47.43± 0.2 µg/cm^2/h and was found to give 3 fold increase in release as compared to ethosomal gel which gave 2 fold higher release of 40.38 ± 2.50µg/cm^2/h as compared to the hydroethanolic solution with a release of 17.333± 0.15µg/cm^2/h. Hence, the results suggested transethosomes to be a more efficient carrier system as compared to ethosomes for transdermal delivery of ketorolac tromethamine.

Keywords: Ketorolac tromethamine, Transdermal delivery, Transethosomes, ethosomes, Permeation studies.

INTRODUCTION

Ketorolac tromethamine is a non-steroidal anti-inflammatory drug (NSAID) and analgesic. It is used in treatment of OA and acute pain including pain after surgery. Its oral route has been associated with the number of gastrointestinal disorders [1]. Thus transdermal is the only alternate route for delivering ketorolac tromethamine so as to overcome its side effects.

Low permeability of skin limits number of drugs which can be delivered in this manner and is the major disadvantage of transdermal delivery [2]. Conventional liposomes do not penetrate deep into the skin but remains confined to the upper layer of the stratum corneum [2,3]. Hence, new classes of ultradeformable lipid carriers such as transfersomes and ethosomes were developed to enhance transdermal delivery of drugs. These carriers are extremely flexible and can squeeze through pores smaller than its own diameter. Due to its elasticity they can easily penetrate the skin and deliver the drugs encapsulated within the carrier. Transfersomes are elastic carriers containing edge activators such as Tween 20, Tween 60, Tween 80, Span 60, Span 65, Span 80, dipotassium glycyrrhizinate, sodium cholate or sodium deoxycholate to enhance transdermal delivery of the drug. Edge activators destabilizes the lipid bilayer of transfersomes and increases its flexibility [4,5]. Ethosomes are ultradeformable carrier containing high concentration of ethanol. Enhanced skin permeation of drugs incorporated in these carriers is due to the interdigitation effect of ethanol on the lipid bilayer of the carriers and increase the fluidity of lipids of the stratum corneum [6]. Ethosomes and transfersomes are excellent carriers for transdermal drug delivery as it contains the high concentration of ethanol and edge activators respectively. Both ethanol and edge activators act as a penetration enhancer. Hence a carrier which consists both these penetration enhancer will further accentuate the transdermal delivery of the drug. It will enhance transdermal permeation of drugs to the dermal layer through the stratum corneum due to its ultradeformable nature. Transethosomes are elastic vesicles which contains high concentration of ethanol along with edge activator [7].

MATERIALS AND METHODS

Materials

Ketorolac Tromethamine was a generous gift sample from Dr. Reddy's, Mumbai. Phospholipon 90G [Phosphatidylcholine (PC)] was a gift sample obtained from Lipoid (Ludwigshafen, Germany). Sodium deoxycholate (SDC) was obtained from S. D. Fine chemicals. All other chemicals used were of analytical grade. After getting approval from an ethical committee, Albino wistar rats were obtained from Bharat Serum and Vaccines Pvt. Ltd. (Mumbai, India).

Preparation of ethosomes

ELs were prepared by cold method as previously reported [8]. Composition of ELs formulation is shown in table 1. 3% w/v of Phospholipon 90G was dissolved in ethanol in a conical flask with constant stirring at 700 rpm. To this alcoholic solution, 20% propylene glycol was added. The temperature of this alcoholic mixture was maintained at 30 ^{0}C. 0.2%w/v of ketorolac tromethamine was dissolved in water and heated to 30 ^{0}C in a separate vessel. This aqueous phase was then added to the alcoholic phase slowly in a fine stream with constant stirring using a mechanical stirrer (Remi, Mumbai) at 700 rpm in a closed vessel. Stirring was continued for additional 5 min. The system was kept at 30°C throughout the preparation. Finally preparation was sonicated using probe sonicator (Oscar, Japan) for 4 mins.

Table 1: It shows composition of elastic vesicles in 1 ml dispersion

Ingredients (mg)	TELs	ELs
Phospholipon 90G	30	30
Ethanol (µl)	300	300
Sodium deoxycholate	3	-
Propylene Glycol (µl)	-	200
Ketorolac tromethamine	2.5	2.5
Water (µl)	700	500

Preparation of transethosomes

Same procedure was used to prepare TELs as stated above, which were prepared by adding 0.3% w/v edge activator instead of 20% propylene glycol. Composition of TELs formulation is shown in table 1. Els and TELs were kept at room temperature until used.

Preparation of gel

The formulations had low viscosity. In order to achieve the desired rheological characteristics and texture for transdermal application,

the formulation was converted into a gel. Gelling agents like Carbopol Ultrez 10 and Carbopol 940 were used to evaluate their ability to gel the formulations. Based on the compatibility with the vesicular formulation, appearance, feel, aesthetic appeal and ease of spreadability, Carbopol Ultrez 10 was selected as the gelling agent. Gels of different concentrations using Ultrez 10 ranging from 0.5-1% w/w were prepared. 0.75% w/w of Ultrez 10 was selected to prepare gels of ethosomal and transethosomal formulations. 0.75% w/w of Ultrez 10 was sprinkled into the water and soaked for 30 mins. The vesicular formulation was added to this swollen gel to give the total drug concentration of 0.3%w/w. Triethanolamine was added to adjust the pH to 7, and then remaining water was added to give a total weight of 10 g. Gel was thoroughly dispersed using an overhead stirrer at the speed of 800 rpm (Remi, Mumbai, India) for 3h.

Physicochemical characterization

Morphology of the vesicles was examined using TEM. The formulations were negatively stained with 2% w/v aqueous solution of phosphotungstic acid on a carbon-coated copper grid. The grid was examined under transmission electron microscope (Philips CM 200) with resolution of 2.4A° at accelerating voltage of 200 kV. The particle size and zeta potential of freshly prepared TELs and Els were determined by nanoparticle tracking analysis (NTA 2.3) using Nanosight NS500 with automated sample introduction, computer controlled motorized stage with CCD camera and red (638 nm) laser [9]. Drug entrapment efficiency of the formulations was determined by ultracentrifugation method [8]. The vesicles were separated by ultracentrifugation at 15,000 rpm for 60 minutes at a temperature of 4ºC. The sediment and supernatant liquid was separated, amount of drug in the sediment was determined by disrupting the vesicles using methanol and amount of drug was quantified spectrophotometrically at 322 nm. Entrapment efficiency was determined by the following equation:

$$\% \text{ Entrapment efficiency} = \frac{\text{Amount of entrapped drug}}{\text{Total amount of drug added}} \times 100$$

Elasticity measurement

The elasticity of the prepared vesicles was measured by extrusion method as previously reported [10]. The vesicles were extruded through a filter membrane of pore diameter of 50 nm for 5 min by applying a pressure of 0.5 bars. The quantity of vesicle dispersion extruded in 5 min is measured. The elasticity of the vesicles was calculated by the following Eq.:

$$E = J \times (r_v/r_p)^2$$

Where; E is the elasticity index of the vesicle membrane, J is the rate of penetration through a membrane filter i. e., the weight of suspension extruded in 5 min, r_v is vesicle size after extrusion, and r_p is pore size of the membrane.

***Invitro* drug release**

Invitro drug release was evaluated using Franz diffusion cell. A cellophane dialysis membrane with molecular weight cut-off of 12,000 daltons (Hi-media) was hydrated with phosphate buffer saline pH 7.4 (PBS 7.4) overnight. Vesicular formulation of 2 ml or 1 gm of gel equivalent to 5 mg of ketorolac tromethamine was placed in the donor compartment. The receptor compartment was filled with 13 ml of PBS 7.4 and stirred with a magnetic bar at 100 rpm and the temperature of the system was maintained at 32 ± 1°C to mimic human skin. The available diffusion area was 2.61 cm^2. 1 ml aliquot was withdrawn at predetermined time intervals and was immediately replaced with an equal volume of fresh buffer. All samples were analyzed for ketorolac tromethamine content by UV spectrophotometry at 322 nm.

***Exvivo skin* permeation study**

Porcine ear skin obtained from a slaughter house was used as a model membrane for the skin permeation study because of its similarity with human skin in lipid content and permeability. The skin sample was mounted between the donor and receptor compartments of the diffusion cell. The receptor compartment was filled with 2 ml of TELs and Els suspensions or 1 gm of TELs gel and Els gel equivalent to 5 mg of ketorolac tromethamine. The receptor chamber was filled with 13 ml of PBS 7.4 and stirred with a magnetic bar at 100 rpm and the temperature was maintained at 32 ± 1°C. 1 ml of aliquot from the receptor compartment was withdrawn at predetermined time intervals and was replaced with equal volume of fresh PBS. The concentration of the drug in the samples was analyzed spectrometrically by UV, and the cumulative amount of drug was plotted as a function of time.

Permeation data analysis

The flux (J,µg cm^{-2} hr^{-1}) was calculated from the slope of linear portion of the plot divided by the skin surface area [11]. The steady state permeability coefficient (Kp) of the drug through porcine skin was calculated by using the following equation:

$$Kp = J/Co$$

Where; J is the flux and Co is the concentration of ketorolac tromethamine in the gel. The penetration enhancing activity of the enhancer may be calculated in terms of enhancement ratio (ER), using the following equation:

$$ER = \frac{\text{Drug permeability coefficient (Treated)}}{\text{Drug permeability coefficient (Control)}}$$

Skin deposition studies

At the end of 24 hrs of the permeation experiment, the surface of the skin was washed five times with 5 ml of 30% methanol to remove excess ketorolac tromethamine from the surface of the skin. The skin was cut into small pieces which were further homogenized with 5 ml of 30% methanol. The resulting solution was then centrifuged for 10 min at 5,000 rpm, the supernatent was then separated to determine the ketorolac tromethamine content by UV spectrophotometer at 322 nm [12].

Evaluation of bilayer fluidity of stratum corneum (SC) using FTIR spectroscopy

Stratum corneum (SC) was treated with TELs and ELs formulations for 12 hrs and it was cut into small circular discs which were placed in 20 ml of 7.0% w/v of sodium chloride solution. SC disc was washed, blotted dry, and then air dried for 2 hrs. SC was kept under vacuum in a desiccator for 15 min to remove any traces of the formulations. Fourier transform infra-red (FTIR) spectra was recorded for ethosomal treated and untreated (control) in frequency range of 400 to 4000 cm^{-1} (Shimadzu, Japan) using KBr as the substrate [13].

Vesicle Skin Interaction Studies by Light Microscopy

An ultra structural change in the skin upon exposure to the TELs and ELs formulation with ketorolac tromethamine was carried out by preliminary histopathological evaluation. After 6 hrs of application of the preparation, skin was removed from the diffusion cell and stored in 10% formalin solution in PBS 7.4 followed by ethanol dehydration. It was then treated with antimedia and embedded in paraffin for fixing. Control skin section was prepared by the same procedure but without application of the formulation. Sections of 5 µm thickness were cut from each skin piece and stained with hematoxylin and eosin. These samples were then observed under light microscope (Leica, DMLB, Herrbrug, Switzerland) and compared with control sample which was treated with PBS 7.4 for any histological changes in stratum corneum, epidermis and dermis [14].

***In-vivo* anti inflammatory activity**

The anti-inflammatory activity of the gel was carried out by carrageenan induced paw edema method [15] to compare the activity of marketed gel and ketorolac tromethamine loaded TELs and Els gels. After approval from ethical committee**,** male albino rats of Wister strain weighing about 150-200 gms were randomly divided into 4 groups of six rats each. TELs gel, Els gel and marketed 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). Percentage reduction in edema was calculated using the following formula

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

Where; Vt is the paw volume at time't'

V_0 is the initial paw volume (before carrageenan treatment), (Vt – V_0) control is edema produced in control group, (Vt – V_0) treated is edema produced in treated group

RESULTS AND DISCUSSION

Physicochemical characterization

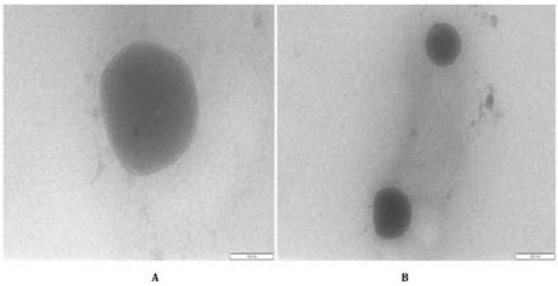

Fig. 1: It shows TEM images of A) TELs and B) ELs

Fig. 1 shows TEM image of TELs and Els. TEM images showed that the TELs and Els are spherical shaped vesicles. Visualization by TEM showed that TELs and ELs are unilamellar vesicular structure, and this confirms the existence of vesicular structure at the higher concentration of ethanol and edge activator.

Table 2: It shows physicochemical properties of the vesicular formulations

Formulation	Size (nm)	Zeta potential (mv)	% Entrapment efficiency n=3
ELs	134±65	-40.76±12.4	70.79±5.6
TELs	180±70	-46.19±13.3	82.08±4.5

Table 2 shows the particle sizes of ELs and TEls. No significant differences were observed in the vesicle sizes of ELs and TEls in the presence of an edge activator. Standard deviations were in the range of 65-70 nm which suggested better polydispersity of the formulation. Ethanol causes a modification in net charge of the system and confers it with some degree of stearic stabilization that may lead to decrease in mean vesicle size [16]. Zeta potential of the prepared vesicles is shown in table 2. Zeta potential of vesicles showed negative values, which may be due to the presence of edge activator or permeation enhancer [17, 18]. The charge of the vesicles is an important parameter that can influence both vesicular properties such as stability, as well as skin-vesicle interactions. % drug entrapment of TELs and Els formulations ranged from 70.79% to 82.08% as shown in table 2. Increase in drug encapsulation may be due to the presence of sodium deoxycholate in the bilayer, which "solubilise" and "hold" ketorolac tromethamine in the lipid bilayer and hence enhances the encapsulation efficiency for the TELs as compared to Els [19]. Increase in ethanol concentration increases the drug entrapment due to increased fluidity of the vesicular membrane. Further increase in ethanol concentration decreases the drug entrapment as vesicle membrane became leakier [20].

Elasticity measurement

The elasticity of transethosomal vesicle membrane (19.28 ± 0.50) was found to be 3-fold higher than the elasticity of ethosomal vesicle membrane (6.04 ± 0.8). Elasticity of the lipid bilayers is an important factor for permeation enhancing effect of the lipid vesicles. Penetration of lipid vesicles through skin is related to the elasticity of the vesicle membrane. Increasing the concentration of the edge activator above a certain concentration in vesicle membranes brings no advantage in terms of transcutaneous permeation efficiency. Only an optimum ratio of lipid and edge activator would enhance bilayer flexibility of the vesicular membranes [21]. Ethanol has been reported to increase the flexibility of the lipid bilayers in the vesicles [22]. Edge activators changes the packing characteristics of the lipids in the carrier bilayer, hence resulting in more flexible carrier. Hence, enhances the skin delivery of various drugs [21].

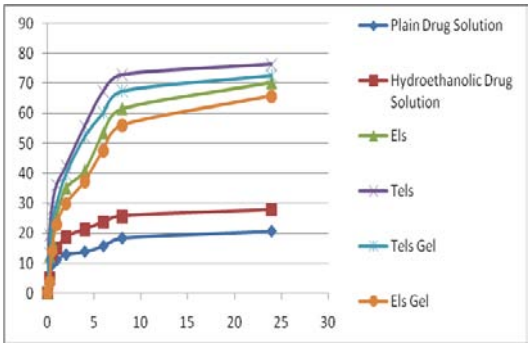

Fig. 2: It shows *in-vitro* release of ketorolac tromethamine from (A) Plain drug solution (B) Hydroethanolic drug solution (C) ELs dispersion (D) TELs dispersion (E) TELs gel (F) ELs gel

In-vitro diffusion studies

Encapsulation of ketorolac tromethamine into TELs and ELs led to significant prolongation of the release across the artificial membrane in comparison with the hydroethanolic and plain drug solution. It was observed from Fig. 2, the sustained release was in the order: TELs dispersion > TELs gel > ELs dispersion > ELs gel > hydroethanolic drug solution > Plain drug solution. The result indicated that. TELs dispersion release more drug than Els dispersion, the drug release was due to higher entrapment efficiency of the latter. Higher permeation of TELs may be due to a combination of both ethanol and edge activator effect. The release profile of ketorolac tromethamine loaded TELs and Els enriched gel indicated slow release as compared to TELs and Els dispersion respectively. This may be due the fact that the drug is diffusing from the TELs and Els carriers first which is then followed by diffusion from the gel matrix resulting in sustained release effects.

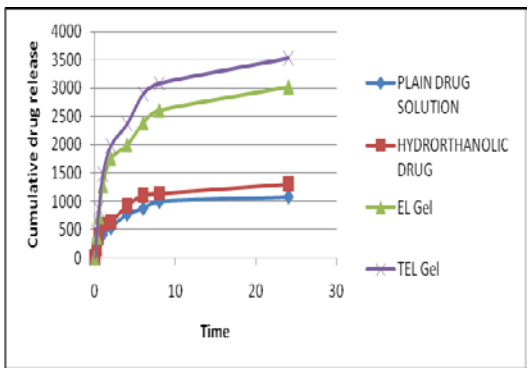

Fig. 3: It shows *exvivo* release profile of ketorolac tromethamine from (A) Plain drug solution (B) Hydroethanolic drug solution (C) ELs gel (D) TELs gel

Ex-vivo skin permeation analysis

Ethanol has been reported to possess permeation enhancement properties. Permeation of TELs and Els are much enhanced as compared to simple hydroethanolic drug solution. It has been suggested that ethanol provides the vesicles with soft flexible characteristics, which allow them to easily penetrate into deeper layers of the skin [23]. Synergistic mechanism is observed between phospholipid vesicles, ethanol and skin lipids. Ethanol fluidizes the stratum corneum lipids resulting into enhanced drug penetration. On the other hand ethanol interacts with lipid vesicles increasing the lipid fluidity making them more flexible. These flexible vesicles squeeze themselves intact through the disturbed SC to the deeper layers of skin with consequent release of the drug by the fusion of ethosomal vesicles with skin lipids [20].

Several studies have reported the penetration enhancing effect of liposomal formulation containing edge activator (transfersomes) compared to conventional liposomes [24]. Propylene Glycol also interacts with the SC keratins without significantly altering SC lipid organization [25]. From Fig. 3 the order of permeation profile obtained was TELs gel > Els gel > hydroethanolic drug solution > plain drug solution. On comparison of TELs gel, ELs gel, hydroethanolic drug solution and plain drug solution, higher flux and higher release were observed for the former because of the synergistic mechanisms of ethanol, phospholipid vesicles, edge activator and skin lipids interaction, which promoted the passage of ketorolac tromethamine through porcine skin. The percent cumulative permeation in 24h was found to be 70.593% and 60.3868% for transethosomal and ethosomal gel which was found to be higher than that of hydroethanolic and plain drug gel.

Table 3: It shows permeability coefficient, flux and ER for the formulations

Formulations	Permeability coefficient [x 10-3] [cm h-1]	Flux [µg h-1 cm-2]	ER
Transethosomal gel	9.486	47.432	3.150
Ethosomal gel	8.076	40.383	2.682
Hydroethanolic drug solution	3.466	17.333	1.151
Plain drug solution	3.010	15.053	-

The enhancement ratio of the Transethosomal gel was found to be 3 fold and ethosomal gel was found to be 2 fold higher as compared to the hydroethanolic solution.

Drug deposition studies

The amount of drug retained in the skin from TELs and ELs formulation and control formulations at the end of 24 h permeation studies is depicted in Fig. 4. Higher skin deposition of TELs as compared to Els was due to the combined effect of phospholipids, edge activator and ethanol on skin and thus providing a mode for sustained delivery of drug for a greater period of time. [26]

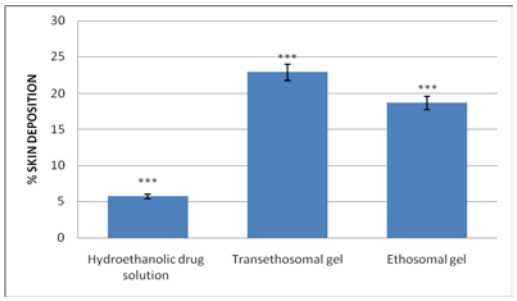

Fig. 4: It shows % skin deposition of ketorolac tromethamine of TELs gel, Els gel and hydroethanolic drug solution

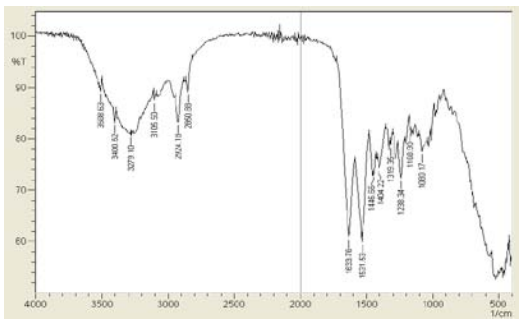

Fig. 5a: It shows FTIR of untreated skin

Bilayer fluidity of stratum corneum (SC) using using FTIR spectroscopy

FTIR spectroscopy is a non-invasive technique for characterization of SC at a molecular level. FTIR spectrum of untreated SC (control) in Fig. 5a showed various peaks due to molecular vibration of proteins and lipids present in the SC. The absorption bands in the wave number of 3000 to 2700 cm^{-1} were seen in untreated SC.

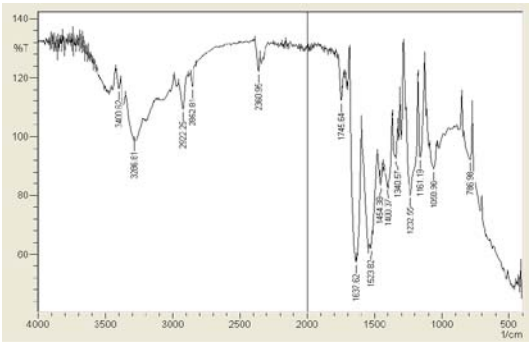

Fig. 5b: It shows FTIR of TELs treated skin

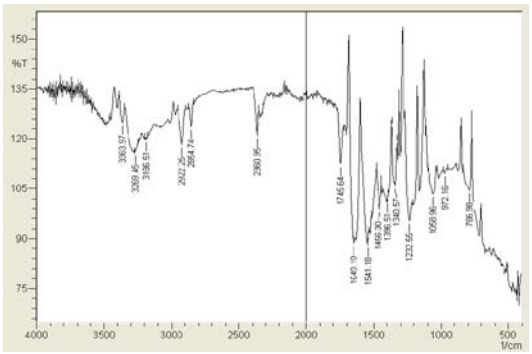

Fig. 5c: It shows FTIR of ELs treated skin

These absorption bonds were due to the C-H stretching of the alkyl groups present in both proteins and lipids. The bands at 2978.09 cm^{-1} and 2880 cm^{-1} were due to the asymmetric -CH_2 and symmetric -CH_2 vibrations of long chain hydrocarbons of lipids respectively. The bands at 2949.16 cm^{-1} and 2870.08 cm^{-1} were due to the asymmetric and symmetric CH_3 vibrations respectively. These narrow bands

were attributed to the long alkyl chains of fatty acids, ceramides and cholesterol which are the major components of the SC lipids. The two strong bands namely1649.14 cm^{-1} and 1544.98 cm^{-1} were due to the amide I [C=O] and amide II [C-N] stretching vibrations of SC proteins. The amide I band consisting of components bands, represented various secondary structure of keratin. The skin band for CH_2 scissoring mode was also present in the wave number region of 1470-1450 cm^{-1}. The band for C=O stretching from fatty acids were present at 1786.08 cm^{-1} [27].

There was clear difference in the FTIR spectra of untreated, TELs and ELs treated skin with prominent decrease in asymmetric and symmetric CH- stretching of peak height and area [fig. 5b, 5c]. The rate limiting step for transdermal drug delivery is the lipophilic part of the SC in which the lipids are tightly packed as bilayers due to high degree of hydrogen bonding. The amide I group of ceramide is hydrogen bonded to amide II group of another ceramide forming a tight network of hydrogen bonding at the head of ceramides. This hydrogen bonding imparts stability and strength to the lipid bilayers and thus gives barrier property to SC. When skin was treated with TELs and ELs formulations, ceramides loosen because of competitive hydrogen bonding leading to breaking of hydrogen bond networks at the head of ceramides due to penetration of TELs and Els into the lipid bilayers of SC. Treatment with TELs and ELs formulations resulted in either double or single peak (fig. 5b and 5c) which suggested breaking of hydrogen bonds by TELs and Els [28].

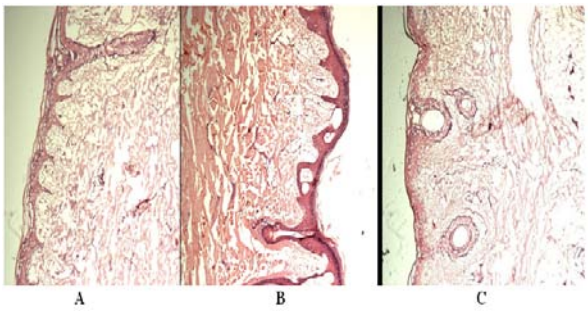

Fig. 6: It shows photomicrographs of histoarchitectural structure of A) Untreated skin B) TELs treated skin C) ELs treated skin

Vesicle-Skin interaction studies by light microscopy

Histological examination of porcine ear skin which was treated with TELs, ELs and control PBS 7.4 for 6 hrs was observed for any morphological changes in skin using light microscopy. Micrographs of control and treated samples are illustrated in Fig. 6a, 6b and 6c. The photomicrographs of PBS 7.4 treated porcine skin (control) showed normal skin with uniform layer of SC with well-defined epidermal and dermal layers.

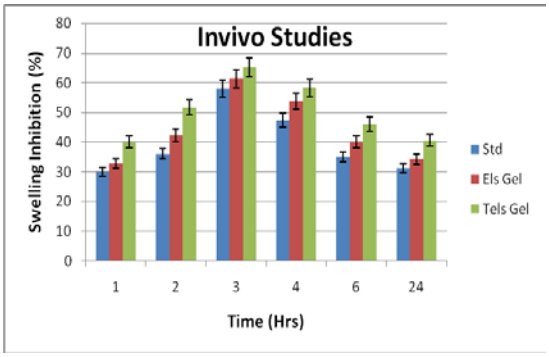

Fig. 7: It shows percent (%) swelling inhibition produced by ketorolac tromethamine loaded TELs gel, Els gel and std marketed gel in carrageenan induced rat paw

While TELs and Els treated skin showed definite changes as compared to the control. The treated sections showed a disruption of SC organization, confirming the penetration enhancing capacity of the carriers. Also, thickness and appearance of the SC were found to be changed in comparison to the control porcine skin. No apparent changes in dermal region were observed.

Pharmacodynamics study

Application of TELs gel, Els gel and the marketed gel resulted in 65%, 61% and 58% inhibition of edema after inducing carrageenan at 3 hrs as shown in Fig. 8. After 3 hrs there was decrease in percent swelling inhibition. There is a significant difference between the tested groups and the control as determined by a one way ANOVA with $P < 0.05$.

CONCLUSION

The results obtained from this study indicates, new phospholipid carrier transethosomes which consists of high concentration of ethanol and edge activator enhances the permeation of ketorolac tromethamine due to its enhanced penetration as compared to ethosomes, hydroethanolic drug solution and plain drug solution. *In-vivo* studies showed better anti-inflammatory activity for both TELs and Els due elastic nature of the carriers as compared to the marketed formulation. Hence, the elastic formulation TELs was found to be more effective as compared to the Els as it contains both ethanol and edge activator which further enhances its transdermal permeation.

ACKNOWLEDGEMENT

Authors are thankful to Dr. Reddy's Laboratory (Mumbai, India) for the gift sample of Ketorolac tromethamine. Further, we extend our thanks to Lipoid (Germany) for kind gift of Phospolipon 90G used in this study.

CONFLICT OF INTERESTS

Declared None

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