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RESEARCH ARTICLE

Pharmacokinetic and pharmacodynamic studies of etodolac loaded vesicular gels on rats by transdermal delivery

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Abstract

Background The present study includes the development of liposomal and ethosomal gels for transdermal delivery to overcome the side effects associated with oral route.

Methods The liposomes and ethosomes were prepared by 3^2 factorial design using film hydration and cold methods, respectively. Different concentrations of liposomal (ETO-LG) and ethosomal (ETO-EG) gels were prepared at 1%, 2 and 3% (*w*/*v*) using carbopol 940 NF. 1%w/v ETO-LG & ETO-EG were optimized upon rheological studies of prepared gels. The optimized gels were further characterized for various physicochemical properties and biophysical studies using FTIR, pharmacokinetic (PK) and pharmacodynamic (PD) studies. The pharmacodynamic activity was performed using carrageenan paw oedema model. The prepared vesicular gels were compared with 45% *v*/v ethanolic ETO-solution and marketed gel PROXYM® in all the characteristic parameters.

Results The pharmacokinetic study reveals that the half life of etodolac in ETO-EG was 1.56 folds whereas ETO-LG showed 1.31 folds higher than PROXYM®. The mean residence time (MRT) of etodolac in ETO-EG and ETO-LG is increased in 1.57 and 1.25 folds, respectively, when compared to PROXYM®. The ETO-EG showed higher percentage reduction in oedema (81.67%) compared to other test products.

Conclusion The pharmacokinetic and pharmacodynamic studies indicated that the vesicular gels show better results compared to PROXYM®. The correlation coefficient value between PK and PD was found to be 0.9635.

Keywords Etodolac · Pharmacokinetic · Pharmacodynamic · Correlation coefficient · Transdermal delivery

Abbreviations

PK	Pharmacokinetic
PD	Pharmacodynamic
HSPC	Hydrogenated soya Phosphotidylcholine
RBF	Round bottom flask
ETO-LG	Etodolac liposomal gel
ETO-EG	Etodolac ethosomal gel
RIE	Reduction in oedema

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Background

Etodolac is a selective non-steroidal anti-inflammatory (NSAIDS) cyclo-oxygenase-2 inhibitor. It has analgesic, anti-pyretic and anti-inflammatory activities and chemically knows as 2-{1, 8-diethyl-1H, 3H, 4H, 9H-pyrano [3, 4- b] indol-1-yl} acetic acid] [1, 2]. On oral delivery, daily dose of etodolac is around 200 mg to 600 mg [3]. On oral administration, etodolac causes the gastro-intestinal (GI) disturbance and it should be contraindicated in patients who suffer with peptic ulcer (due to severe GI bleeding), renal failure and heart diseases [4]. To conquer these problems, the current research

aims to develop an ideal transdermal drug delivery system that succeeds in ensuring the stable plasma concentration of etodolac over a prolonged period of time through non-invasive technique via transdermal route with reduced dosing frequency. However, the most difficult aspect of transdermal delivery system is to overcome the barrier (stratum corneum) against foreign substance. The vesicular systems are promising tool for transdermal delivery to enhance the drug therapeutic efficacy [5]. The vesicular gels (liposomes and ethosomes) have the ability to alter the pharmacokinetic and pharmacodynamic parameters of drugs by modulating skin properties [6]. The liposomes are nano sized bilayered vesicles made up of lipid with or without cholesterol whereas ethosomes are made up of lipid and 45% *v*/v ethanol. The liposomes and ethosomes are not alternative to each other. Their entire physico chemical properties and drug permeability mechanisms are quite different from each other [7]. There are reports in demonstrating the efficiency of etodolac loaded niosomal gel for topical delivery and proved that the niosomal gels shows better PK and PD properties compared to other test products [8]. The current study focuses on development of the etodolac loaded liposomes and ethosomes by 3^2 experimental design and incorporating the optimized formulations into the gel base (carbopol 940 NF). The newly developed formulations are evaluated for their bioavailability because in vitro testing cannot predict the in vivo performance. The selected products were subjected for PK and PD analysis to assess the potential and efficacy of etodolac vesicular gels [9].

The pharmacokinetic studies were carried out to describe the time course of the drug concentration in the blood in mathematical terms. Two important applications of these studies are:

1. The performance of pharmaceutical dosage forms can be evaluated in terms of the rate and amount of the drug they deliver into the systemic circulation.
2. The dosage regimen of a product can be adjusted to maintain therapeutically effective drug concentration in blood without any toxic effect [10, 11].

Materials

HSPC was purchased from Lipoid Pvt. Ltd. Germany, Cholesterol purchased from sigma Aldrich Pvt. Ltd. India, Etodolac and Aceclofenac are the gift samples from Emucure Pvt. Ltd., Pune, India. Acetonitrile, methanol and acetic acid HPLC grade solvents were purchased from M/s. Qualigens Fine Chemicals. Carrageenan purchased from Sigma Aldrich, India. All the materials used in this were analytical grade.

Preparation of vesicular systems (liposomes and ethosomes) and their gels

Preparation of vesicular systems

Liposomes were prepared by 3^2 factorial design using film hydration method. In this method, required amounts of etodolac, phospholipid (HSPC), cholesterol and stearic acid were simultaneously dissolved in 1:3 ratios of methanol and chloroform in round bottom flask (RBF-250 ml) and subjected to rotary evaporation at 50 °C and a speed of 120 rpm. This was continued till a dried film formed around the walls of RBF. The prepared dry film containing RBF was kept a side, overnight, in a vacuum desiccator, to stabilize lipid film and to remove organic traces. The phosphate buffer pH 7.4 was added to the RBF containing the lipid dry film. This causes hydration of the lipid film and formation of the liposomes takes place. The formed liposomes were subjected to probe sonication for 3 min to get desired particle size. The temperature of liposomal dispersion was brought to room temperature (25 °C). Then liposomal dispersion was filled into 10 CC clear vials, followed nitrogen sparging and sealed by fluorotech coated bromobutyl stoppers (West Pharma Pvt. Ltd) and stored at 2–8 °C [12].

Whereas ethosomes were prepared through cold method by taking the accurately weighed amounts of HSPC, cholesterol, stearic acid and drug in various percentages (15%, 30% and 45% *v*/v) of ethanol at 30 °C by vigorous stirring using Heidolph homogenizer. The phosphate buffer pH 7.4 was heated up to 30 °C in a separate vessel. Then lipid solution was added to phosphate buffer pH 7.4 followed by stirring for 45 min, ethosomes are appeared as milky dispersion. The temperature of ethosomal dispersion was brought to room temperature (25 °C). The prepared ethosomes subjected to probe sonication for 3 min to get desired particles size. Then liposomal dispersion was filled into 10 CC clear vials, followed nitrogen sparging and sealed by flurorotech coated bromobutyl stoppers (West Pharma Pvt. Ltd) and stored at 2–8 °C [13].

The prepared vesicular systems (liposomes and ethosomes) were characterized for various physicochemical properties such as percent drug content, pH, particle size, polydispersibility index (PDI), zeta potential, % EE and 100% in vitro drug release studies. The optimization of liposomes and ethosomes was done by statistical optimization using overlay and desirability plots. The optimized liposomes and ethosomes were further selected for preparation of gels using carbopol 940 NF. Based on rheological studies, ETO-LG and ETO-EG gels were optimized and subjected for pharmacokinetic and pharmacodynamic studies on rats in comparison with PROXYM®.

Procedure for gel preparation

The liposomes and ethosomes dispersions were subjected for ultra centrifugation technique to remove the unentrapped drug. The obtained liposomal pellet was resuspended in pH 7.4 phosphate buffer whereas ethosomal pellet was resuspended in 45% *v*/v of ethanolic pH 7.4 phosphate buffer respectively. The required amount of carbopol 940 NF was added to the respective liposomal and ethosomal dispersions and kept a side for soaking. Then prepared solutions are subjected to stirring with the aid of glass rod. The final concentrations of ETO-LG and ETO-EG were obtained 0.55% and 0.66% *w*/*v* of etodolac, respectively. Triethanolamine was used to change the pH of gel between 6 and 7 [14].

The three different concentrations (1%, 2% and 3% *w*/*v*) of liposomal and ethosomal gels were prepared. The viscosity studies of the test products were conducted at 25 ± 0.1 °C using Brookfield viscometer (model LV-DV III) for all prepared gels. Required quantity of gel sample was placed on the viscometer plate and analyzed for its viscosity (η), shear stress (τ) and shear rate at various speeds and at 100 rpm, likewise viscosity was test for all prepared gels. The rheogram of test products were analyzed at rotational speeds of 0.5, 1.0, 2.0, 2.5, 4.0, 5.0, 10, 20, 50 and 100 rpm using spindle (≠4). The resulting shear stress (τ) was noted [15]. The gels were optimized based on the rheology. The optimized ETO-LG and ETO-EG were further characterized for percent drug content, pH, particle size, zeta potential, %EE, spreadability, occlusion factor, comparative permeation studies (in vitro and ex vivo), FTIR, pharmacokinetic and pharmacodynamics.

Evaluation of vesicular systems and their gels

Percent drug content (%)

The vesicular systems and their gels were subjected to percent drug content analysis. The required quantity of suspension as well as gel was measured and it was lysised with required volume (9 ml) of methanol and further it was diluted with (10 ml) 7.4 pH phosphate buffer and the samples were analysed spectrophotometrically at 226 nm [16].

Size distribution, zeta potential (ζ) and PDI

The particle size and zeta potential of test products was done by Malvern particle size analyzer (Nano ZS series). The vesicles and gels were diluted with distilled water (1:100) and the samples were taken in the cuvette. The cuvette was placed inside the sample holder of the instrument (Malvern Nano ZS90, Malvern, UK) for measurement of size. The principle of photon correlation spectroscopy was used for determining the hydrodynamic diameter of the vesicles via Brownian motion. The observations of vesicle size were recorded at 90° light scattering angle and at 25 °C. The ζ was measured based on the mobility of vesicles [16].

Percent entrapment efficiency (%EE)

The %EE of vesicles was determined by ultracentrifugation method. Required volume of the vesicular dispersion was centrifuged at 20,000 rpm for 3 h at a temperature of 4 °C (Remi cooling centrifuge CPR-30). The supernatant solution containing unentrapped drug was withdrawn and measured the concentration by UV spectrophotometer at 226 nm against the pH 7.4 phosphate buffer.

The amount of drug unentrapped in liposomes and ethosomes were determined by the Eq. 1 [17].

$$\%\ \text{Entrapment Efficacy (\%EE)} = \frac{(C_d - C)}{C_d} \times 100 \qquad (1)$$

Where C_d is total drug concentration and C is unentrapped drug concentration.

The % EE of test gels was determined by ultra dialysis method. The free drug was removed from the gels by ultra dialysis using dialysis membrane. The dialyzed formulation was lysised with required quantity of methanol and it was further diluted with pH 7.4 phosphate buffer. The samples were analysed spectrophotometrically at 226 nm.

In vitro and ex vivo drug permeation

The vesicular systems and their gels were subjected to 100% in vitro drug release using vertical Franz diffusion cell having 28 ml capacity, with an effective diffusional area of 4.52 cm^2. The cellulose dialyzing membrane (Membra–Cel MD 34–14, cut-off 14 KD) was placed between the donor and receptor compartments. The procedure was same for both vesicular systems and their gels. The dose equivalent to 8 mg of test formulation was placed in the donor compartment. The pH 7.4 phosphate buffer was used as a medium in receptor to ensure sink condition. The whole system maintained at 32 °C ± 0.5 °C and stirred on a magnetic stirrer at 100 rpm. The donor compartment was separated from the receptor compartment by cellulose dialyzing membrane (Membra–Cel MD 34–14, cut-off 14KD), which was overnight soaked in the receptor medium. The samples were collected at predetermined time intervals (1, 2, 4, 6, 8, 10, 12, and 24 h) and 1 ml of aliquots were withdrawn from the sampling port and replaced with an equal volume of fresh buffer to maintain constant volume. The samples were analysed spectrophotometrically at 226 nm. The test products were subjected to order of drug release and drug release mechanism [18]. In case of ex vivo studies instead of using dialysis membrane, the excised rat skin was mounted on the receptor compartment with the stratum corneum side

facing upwards into the donor compartment. The remaining procedure was same as in vitro drug release studies.

Determination of spreadability

The spreadability of gel products were determined using wooden block and glass slide apparatus. Required quantity of test products (approximately 1 g of gel) was spread on measuring diameter around (20*20 cm^2) of horizontal glass slides which in turn placed on measuring pan. Another slide was placed over this sample which was attached to fixed load weight 10 g and a after 5 min weight was removed on upper slide and measures the spreadability in terms of time in seconds taken up by two slides to slip off from gel. Spreadability of gel was calculated by using Eq. 2.

$$\text{Spreadability (S)} = \text{m} \times \frac{l}{t} \quad (2)$$

In which S is the spreadability, m is the weight (g) tied on the upper plate; l is the length (cm) of the glass plates, and t is the time taken for spreading the gel [14, 19].

Determination of occlusion factor

The occlusive property of gel products was evaluated by in vitro occlusion test. The 100 ml beaker was filled 50 ml of water and covered with a filter paper (Whatman number 6, cut-off size: 3 μm, USA) on the upper surface of which 100 mg of the test products was evenly distributed. The beakers were subsequently stored at 32 ± 0.5 °C for 48 h in order to mimic the temperature of skin surface. The beaker without sample was used as control and covered with filter paper. Evaporation of water through the filter paper was measured and used to calculate the occlusion factor "F" at 6, 12, 24 and 48 h using the below given Eq. 3.

$$\text{Occlusion factor (f)} = \text{A} - \frac{\text{B}}{\text{A}} \times 100 \quad (3)$$

Where A stands for the water flux through the filter paper without sample (control) and B is the water flux through the paper with sample. An F value of 0 means no occlusive effect compared to the reference, while an F value of 100 means maximum occlusivity [14, 19].

FTIR studies

After completing ex vivo permeation experimental studies, treated skin samples were subjected for biophysical studies using Attenuated total reflection (ATR) technique. The IR spectra of samples were recorded on a Bruker FTIR spectrophotometer equipped with opus software. The IR spectra of treated skin samples were recorded in the range of 400 to 4000 cm^{-1}.

Pharmacokinetic study

Animals

In the current study, Wistar male rats, weighing around 200 to 250 g were used. They were kept in cages in the laboratory and were acclimatized to the laboratory surroundings and room temperature (25 ± 3 °C) for a period of 1 week before experiment was performed [20, 21]. They were housed in cages with free access to food and water. All the procedures for kinetic and dynamic activities of etodolac were carried out as per the guidelines of Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA). The study protocol was approved by the Institutional Animal Ethics Committee, A.U. College of Pharmaceutical Sciences, Andhra University (Reg.No.516/01/a/CPCSEA), Andhra Pradesh, India.

Experimental design

The design and procedure mentioned in the literature was adopted for the pharmacodynamic studies of rat models [22–24]. The animals were divided into four groups, each group containing four animals. Group I was treated with 45% *v*/v ethanolic ETO-Solution, group II was treated with ETO-LG, group III was treated with ETO-EG and group IV was treated with PROXYM®. The dose equivalent to 8 mg was taken from all test products. The test products were applied on dorsal site of abdominal skin of the rats. A porous gauge dressing and non-irritating tape (3 M transpore) was attached to the skin on top of the application.

Sample collection

The blood samples (300 μL) were collected from rats' retro-orbital sinus into micro centrifuge tubes containing dipotassium ethylene diaminetetraacetic acid. Samples were collected at predetermined time intervals of 0, 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12 and 24 h. Samples were collected at zero time were the blank samples. Plasma was immediately separated by ultracentrifugation under refrigeration (5 °C) at 5000 rpm for 15 min, and each plasma sample was collected and stored at −20 °C.

Analytical method

The plasma concentration of etodolac was analyzed by modified RP-HPLC method using C-18 column (Gemini TM 5 μm, 250 × 4.6 mm). The ratio of 55:45 of acetonitrile and

pH 4.0 phosphate buffer was used as mobile phase. It was sonicated for about 30 min before use and filtered through 0.45 μ PVDF membrane filter and delivered at a flow rate of 1 ml/min. The column temperature was maintained at 25 °C and injection volume was taken as 20 μl. Etodolac was detected at 225 nm in short retention time of 5.13 min. In the current study, the method was validated according to the standard validation procedures [25, 26]. The linearity was shown to range between 0.25–2.5 μg/ml. The coefficients of variation for the intra-day and inter-day precision were < 2%. The limit of quantification (LOQ) for etodolac was 0.25 μg/ml and a signal-to-noise ratio of 3:1.

Pharmacokinetic and statistical analysis

The plasma concentration versus time curves of each test products were statistically analysed using Prism 5.0 software trial version (Graph padInc, CA, USA). The pharmacokinetic parameters were calculated using non-compartmental approach using PK solver software. The maximum plasma concentration (C_{max}) and maximum time to reach peak plasma concentration (T_{max}) were determined from the individual plasma concentration Vs. time curves. The first-order elimination rate constant (K_{el}) was estimated by linear regression of the terminal data points. The terminal elimination half-life ($t_{1/2}$) was calculated as $0.693/K_{el}$. The area under serum concentration Vs. time curve ($AUC_{0-\infty}$) and area under first moment time curve ($AUMC_{0-\infty}$,) were calculated using log linear trapezoidal rule. Mean residence time (MRT_{0-t}) was calculated as $AUMC_{0-\infty}/AUC_{0-\infty}$.

The differences found between pharmacokinetic parameters were statistically evaluated by t test. All the values were compared with PROXYM® and all the values were expressed as their mean ± SD. Differences were considered to be significant at a level of $P < 0.05$.

Pharmacodynamic studies

The anti-inflammatory activity of etodolac was evaluated by carrageenan induced rat paw oedema model. The oedema was induced by injecting 0.1 ml of carrageenan (1% solution in saline) into sub-plantar tissue of the right paw. The animals were divided into five groups, each group containing four animals. Group I served as control, group II was treated with 45% *v*/v ethanolic ETO Solution, group III was treated with ETO-LG, group IV was treated with ETO-EG and group V treated with PROXYM®. The entire animal's right paw was marked before starting the experiment. After 2 h of carrageenan injection, test product was applied on the right paw. The paw oedema was measured by changing paw volume over various time points using plethysmometer (INCO, India). The paw oedema volume was determined once just before and every hour during the 7 h. The reduction in oedema volume was recorded at various time points such as 0, 1, 2, 3,4,5,6 and 7 h and % RIE (reduction in oedema) was calculated by following equation Eq. 4 [27–29].

$$\%\text{Reduction in oedema (RIE)} = \frac{\text{oedema(control)} - \text{oedema (treated group)}}{\text{oedema(control)}} \times 100 \quad (4)$$

Correlation between $AUC_{0\text{-}24h}$ and reduction in the paw volume

A graph was plotted by taking 2 Y- axis's. The $AUC_{0\text{-}24h}$ (μg. hrs/ml) values on left side Y-axis and reduction in paw volume (ml) were in right side Y-axis. The Correlation Coefficient (r) value was calculated.

Results

The present work reveals that ethosomal formulation shows better results compared to other test products. The percent drug content of prepared liposomes and ethosomes were found in the range of 96 ± 1.1% to 102 ± 0.9%. The liposomal particle size were found in the range of 186 ± 1.1 nm to 363 ± 1.6 nm and the PDI was in the range of 0.102 ± 0.8 to 0.321 ± 0.6 whereas ethosomal particle size was found in the range of 170 ± 1.2 nm to 303 ± 1.3 nm and PDI was in the range of 0.112 ± 1.1 to 0.214 ± 0.8 respectively. The zeta potential values of liposomes and ethosomes were found to be in the range of −12.3 ± 0.8 mV to −36.5 ± 1.1 mV and − 16.3 ± 1.1 mV to −48.3 ± 1.4 mV respectively. The %EE varied from 34 ± 1.1% to 71.5 ± 1.2% and 32.9 ± 0.8% to 78.5 ± 1.1% for liposomes and ethosomes, respectively. The in vitro drug release from liposomal formulations was found in the range of 67.5 ± 0.8% to 86.6 ± 1.2%, whereas ethosomal formulations were found in the range of 52.2 ± 0.9% to 99.4 ± 1.2% respectively. The obtained results indicated that the selected independent variables influenced the physicochemical parameters.

The 1%*w*/*v* ETO-LG and 1%w/v ETO-EG gels were optimized based on their average low viscosity values (1822.62 ± 22.4 Cps and 1662.42 ± 31.2Cps) respectively. From the viscosity result, viscosity versus shear rate follows pseudo-plastic flow and the results are shown in Fig. 1.

The percentage drug content was found in the range of 91.8 ± 1.2 to 99.8 ± 0.8% for ETO-LG, ETO-EG, PROXYM® and 45% *v*/v ethanolic ETO-Solution respectively. The pH of test formulations (ETO-LG, ETO-EG, PROXYM® and 45% v/v ethanolic ETO-Solution) was found as 7.4 and no significant difference was found in the pH among test products. The mean vesicle sizes of test products ETO-LG, ETO-EG, PROXYM® and 45% v/v ethanolic

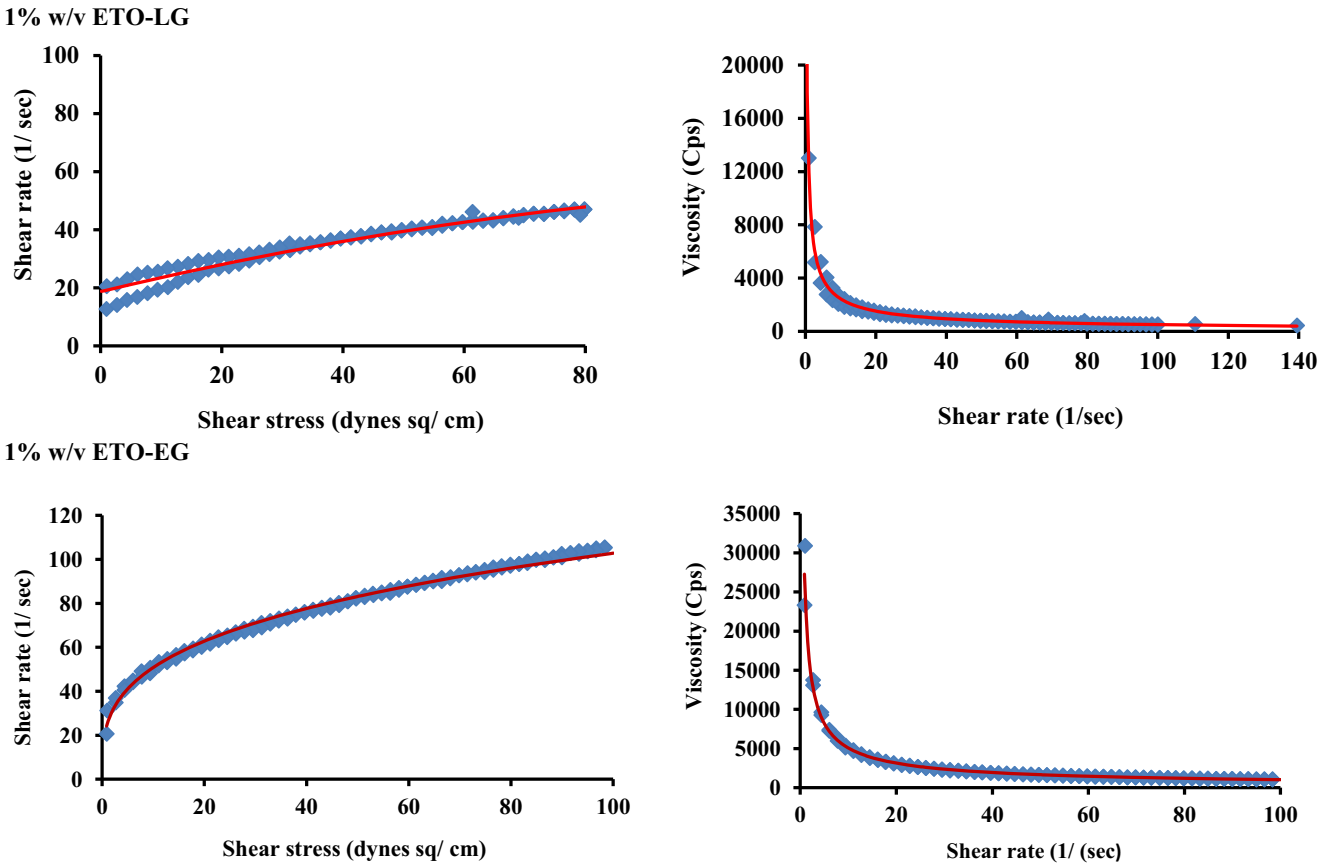

Fig. 1 Viscosity profiles of 1% w/v ETO-LG and ETO-EG

ETO-Solution were found as 201 ± 1.1 nm, 178 ± 1.3 nm, 841 ± 0.9 nm and 18 ± 1.2 nm and PDI was found in the range of 0.08 ± 0.1 to 0.712 ± 0.8 respectively.

The zeta potential values of the formulations ETO-LG, ETO-EG, PROXYM® and 45% v/v ethanolic ETO-Solution were found to be −24.5 ± 1.1 mV, −28.1 ± 0.8 mV, −12.6 ± 1.2 mV and − 16.1 ± 1.6 mV respectively. The zeta potential values of the gels of ETO-LG and ETO-EG were significantly decreased as compared to the vesicular dispersion phase. The zeta potential values influence the shelf life of gels that can affect the pharmacokinetic properties of gels. The %EE of ETO-LG and ETO-EG were found to be 70.1 ± 1.1% and 77.8 ± 0.9% respectively. It indicated that carbopol 940 NF gel base does not affect the %EE of ETO-LG and ETO-EG. The spreadability of test products was found to be 61.6 ± 1.1%, 66.8 ± 1.2% and 58.1 ± 0.8% for ETO-LG, ETO-EG and PROXYM® respectively. The percentage spread by weight of ETO-EG was more (66.8 ± 1.2%) due to its low viscosity. The ETO-EG has low spreading time compared to ETO-LG. The occlusion studies of test products showed values of 72.8 ± 1.3%, 77.2 ± 0.6% and 67.2 ± 1.5% for ETO-LG, ETO-EG and PROXYM® with in 24 h respectively.

The in vitro permeation studies were conducted by Franz diffusion cells using a dialysis membrane. The in vitro permeation was found to be 44.2 ± 1.6%, 76.4 ± 1.3%, 91.2 ± 1.7%, and 63.4 ± 1.3% for 45% *v*/v ethanolic ETO-solution, ETO-LG, ETO-EG and PROXYM® respectively. The ETO-LG shows controlled and long lasting release profile compared to ETO-EG. This might be due to the effect of amount of cholesterol and rigidity of vesicles modulates the release pattern from ETO-LG whereas the ETO-EG slightly improved the permeation compared to ETO-LG. The synergetic effect of ethanol and malleable bilayered lipid vesicles promotes better permeation compared to ETO-LG. This assumption was concluded with the following conclusions. 1) In case of ETO-LG, the rigidity of the vesicles was imparted due to the presence of lipid and cholesterol in the composition. 2) In case of ETO-EG presence of ethanol making the vesicles more soft and malleable in nature. Ethanol also acts as a permeation enhancer [30]. Hence, the presence of ethanol in ETO-EG may cause the high flux, high permeability and high permeation enhancement ratio.

The ex vivo cumulative percent release was found that 36.2 ± 1.8%, 68.9 ± 1.2%, 83.3 ± 1.5% and 56.4 ± 1.8% for 45% v/v of ethanolic ETO-Solution, ETO-LG, ETO-EG and PROXYM® respectively. The ex vivo studies reveal that, ex vivo drug permeation of test products was decreased as compared to in vitro drug release. The ETO-EG shows maximum transdermal flux as compared to other test products, so the permeation rate was more from vesicular gels as compared

to PROXYM®. It was observed that permeation of vesicular gels was decreased, compared to vesicular dispersions, which is explained by increased viscosity of formulation due to carbopol 940 NF that retards the release of vesicles from its cohesive mass.

From the biophysical studies, FTIR technique confirmed that permeation profiles of test products against rat skin. The FTIR spectrum of untreated rat skin showed the C-H symmetric stretching peaks at 2851.75 cm-^{1} and C-H asymmetric stretching peaks at 2921.09 cm-^{1}. Amide-I band showed a strong peak at 1643.99 cm-^{1} due to C=O stretching vibrations, whereas amide II band showed peak at 1548.04 cm-^{1} due to N-H bending vibrations and ester band at 1743.4 cm-^{1} respectively. Slight changes were observed in characteristic peaks (C-H symmetric stretching, C-H asymmetric stretching, amide I, amide II and ester) of 45% *v*/v ethanolic ETO-Solution and PROXYM® treated skins. The FTIR spectrum of ETO-LG and ETO-EG treated skin did not show the symmetric C-H stretching and asymmetric C-H stretching bands. The absence of symmetric and asymmetric C-H stretching bands indicate that disruption of corneocytes which are enriched with keratin lipid portions in the skin or denaturation of lipids in the stratum corneum of skin.

The FTIR study provides an insight into the effect of test products on the biophysical properties of the rat skin. A typical FTIR spectrum of rat skin showed separate lipid and protein peaks. The biophysical study of rat skin lipid peaks caused by C-H stretching vibrations would be helpful in identifying the influence of the dosage forms proposed in the study. The absorbance of stratum corneum lipids occur near 2851 and 2920 cm^{-1} symmetric and asymmetric C-H stretching vibrations, respectively. The change in the amount of stratum corneum lipids have been correlated with C-H stretching absorbance intensity. The stratum corneum lipid extraction leads to a decrease in the C-H stretching absorbance intensity [31, 32]. Biophysical technical information correlated with ex vivo permeation profiles of test products, i.e., ETO-LG and ETO-EG showed a higher permeability rate compared to 45% *v*/v ethanolic ETO-Solution and PROXYM®. The FTIR studies of ex vivo test products are shown in Table 1 and Fig. 2.

Pharmacokinetic study (Fig. 3)

The pharmacokinetic study was conducted on Wistar rats. The C_{max} values of 45% *v*/v ethanolic ETO-Solution, ETO-LG, ETO-EG and PROXYM® were found to be 0.751 ± 0.13 μg/ml, 3.30 ± 0.01 μg/ml, 3.5 ± 0.10 μg/ml and 2.73 ± 0.20 μg/ml, respectively. The mean C_{max} value of ETO-EG showed higher value whereas 45% v/v ethanolic ETO-Solution was shown lowest C_{max} in all test products. The results are represented in Table 2. ANOVA of the test products indicated that the significant variation was observed among them ($p < 0.0001$).

On oral delivery, etodolac showed T_{max} value of 2 h [33]. In the current research, etodolac in the form of (ETO-LG and ETO-EG) showed up to 4 h. The PROXYM® was also showed the same result. It indicated that gels, which are applied on to the skin showed steady-state plasma concentration, thus it achieves drug release in controlled manner. On oral delivery, etodolac showed C_{max} 29 mg/l but in the current research on transdermal delivery, it was shown as 3.30 ± 0.4 μg/ml and 3.51 ± 0.1 μg/ml for ETO-LG and ETO-EG respectively. It indicated that vesicular gels reduced the unwanted concentration of etodolac by transdermal route and it leads to reduce the toxicity effect of etodolac in stomach. This statement was supported with previous reports mentioned in the literature [34]. The T_{max} values of test gels was shown 4 h whereas the 45% *v*/v ethanolic ETO-Solution shown 10 ± 0.02 h to reach maximum concentration respectively. The T_{max} results are given in Table 2. The $AUC_{0-\infty}$ values of test products; 45% *v*/v ethanolic ETO-Solution, ETO-LG, ETO-EG and PROXYM® were found to be 12.14 ± 0.6 μg.hr./ml, 61.16 ± 1.0 μg.hr./ml, 85.16 ± 0.1 μg.hr./ml and 33.6 ± 0.2 μg.hr./ml respectively. The ETO-EG showed highest mean $AUC_{0-\infty}$ value over all test products. ANOVA of the $AUC_{0-\infty}$ values of the different formulations indicated that the difference is significant ($p < 0.05$).

Table 1 FTIR Spectrums of ex vivo skin permeation of test products

Test products	C-H symmetric stretching [cm^{-1}]	C-H asymmetric stretching [cm^{-1}]	Amide I cm^{-1}	Amide II[cm^{-1}]	Ester [cm^{-1}]
Untreated skin	2851.75	2921.09	1643.99	1548.04	1743.54
45% v/v ethanolic ETO-Solution treated skin	2833.61	2911.87	1635.76	1534.65	1731.68
ETO-LG treated skin	–	2891.37	1637.04	1527.37	1637.04
ETO-EG treated skin	–	–	1522.42	1421.66	1619.25
PROXYM® treated skin	2843	2922.1	1638	1541.3	1736.2

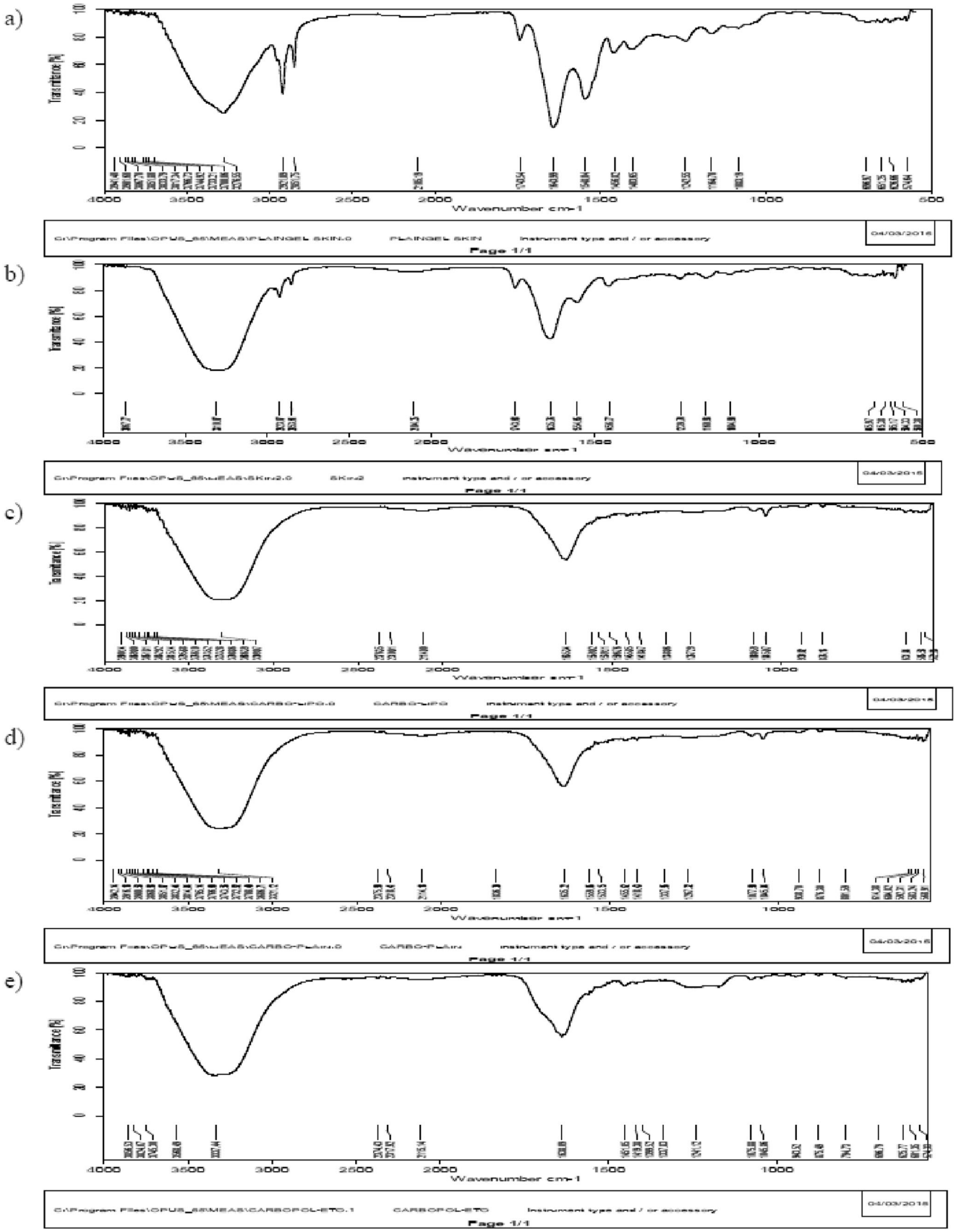

Fig. 2 FTIR spectras of (**a**) untreated skin, (**b**) 45% v/v ethanolic ETO-Solution treated skin (**c**) ETO-LG treated skin (**d**) ETO-EG treated skin (**e**) PROXYM® treated skin

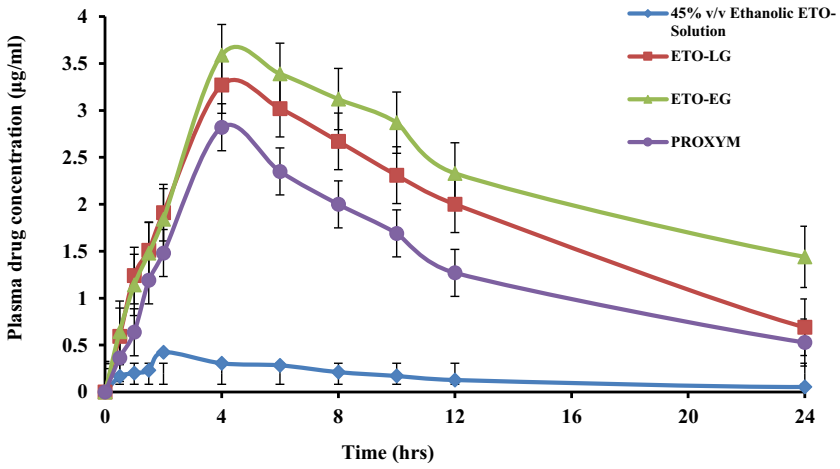

Fig. 3 Comparison of average plasma drug concentration of test products in individual rats

The $AUMC_{0-\infty}$ values of test products; 45% *v*/v ethanolic ETO-Solution, ETO-LG, ETO-EG and PROXYM® were found to be 154.4 ± 1.2 µg.hr./ml, 1074 ± 0.9 µg.hr./ml, 1858.2 ± 0.6 µg.hr./ml and 546.5 ± 0.7 µg.hr./ml respectively. The mean $AUMC_{0-\infty}$ of the 45% *v*/v ethanolic ETO-Solution was the lowest whereas the ETO-EG showed the highest, results are represented in Table 2**.** ANOVA of the $AUMC_{0-\infty}$ values of test products indicated that the difference among them is significant ($p < 0.0001$). The elimination rate constant (K_{el}) of test products were found to be 0.1123 ± 0.1 h^{-1}, 0.0619 ± 0.02 h^{-1}, 0.0547 ± 0.9 h^{-1} and 0.0817 ± 0.3 h^{-1}, 45% *v*/v ethanolic ETO-Solution, ETO-LG, ETO-EG and PROXYM® respectively. The ETO-EG shown the lowest elimination rate compared to other test products. The K_{el} of vesicular gels values further reflect the longer blood circulating nature compared with rapidly clearing nature of 45% v/v ethanolic ETO-Solution and PROXYM®. The half life of test products were found to be 6.18 ± 0.2 h, 11.02 ± 0.4 h, 13.03 ± 0.9 h and8.35 ± 0.6 h of 45% v/v ethanolic ETO-Solution, ETO-LG, ETO-EG and PROXYM® respectively. The mean half life value of ETO-EG was highest when compared to other test products.

The mean residence time (MRT) of test products were found to be 12.70 ± 0.2 h, 16.89 ± 0.47 h, 21.14 ± 0.70 h and 13.44 ± 0.70 h of 45% v/v ethanolic ETO-Solution, ETO-LG, ETO-EG and PROXYM® respectively. The results are showed in Table 2**.** The MRT of etodolac in ETO-EG and ETO-LG were found to be 1.57 and 1.25 folds increased when compared with PROXYM®.ANOVA of the MRT values of test products indicated that the difference among them is significant ($p < 0.0001$). The relative percent bioavailability (F_{rel}) was found to be 0.82, 1.57 and 2.1 for 45% *v*/v ethanolic ETO-Solution, ETO-LG and ETO-LG respectively. This might be attributed to the combined effect of lipid and cholesterol in the case of liposomes and to the synergetic effect of lipid and ethanol in the case of ethosomes. The ETO-LG and ETO-EG alter the rat skin structural properties, by interaction with

Table 2 Pharmacokinetic parameters of all test products in rats

Pharmacokinetic parameters	45%v/v ethanolic ETO-Solution	ETO-LG	ETO-EG	PROXYM®	*p* value	Remarks
T_{max} (Hrs)	7 ± 0.01	4 ± 0.00	4 ± 0.00	4 ± 0.00	<0.001	S
C_{max} (µg/ml)	0.75 ± 0.3	3.30 ± 0.4	3.51 ± 0.1	2.73 ± 0.2	<0.001	S
K_{el} (hr^{-1})	0.112 ± 0.1	0.061 ± 0.2	0.054 ± 0.9	0.081 ± 0.6	<0.001	S
$t_{½}$ (hrs)	6.18 ± 0.4	11.02 ± 0.1	13.03 ± 0.1	8.35 ± 0.3	<0.001	S
AUC_{0-24h} (µg.hrs/ml)	10.76 ± 0.5	45.90 ± 0.3	57.15 ± 1.8	33.6 ± 0.7	<0.001	S
$AUC_{0-\infty}$ (µg.hrs/ml)	12.14 ± 0.6	61.61 ± 0.2	85.16 ± 2.6	39.11 ± 1.6	<0.001	S
$AUMC_{0-\infty}$ (µg.hrs/ml)	154.4 ± 0.5	1074.3 ± 0.9	1858.2 ± 0.9	546.5 ± 0.6	<0.001	S
MRT (hrs)	12.70 ± 0.2	16.89 ± 0.7	21.14 ± 0.7	13.44 ± 0.7	<0.001	S
F_{rel} (%)	0.82 ± 0.6	1.57 ± 0.1	2.1 ± 0.3	–	<0.001	S

the skin's hydrophilic portion, and this leads to increased membrane fluidity. This causes enhanced permeation.

Pharmacodynamic activity

The anti-inflammatory activity was studied by using carrageenan induced rat paw oedema volume model. This activity increased in value from the first hour to the 4th hr., reached its peak in the 4th hr. (ETO-LG was slightly higher in the 5th hr), and from that time point onwards, decreased in value. The results are shown in Table 3 and Fig. 4.

The percentage reduction in oedema (%RIE) of test products showed 46.97 ± 1.23%, 70.02 ± 1.98% and 38.67 ± 0.38% for ETO-LG, ETO-EG and PROXYM® respectively. The %RIE of 45% v/v ethanolic ETO-Solution was observed only 44.18 ± 0.17% up to 4 h. After 4 h the activity was decreased. The vesicular gels were showed better %RIE compared other test products. After 4 h the anti-inflammatory activity kept decreasing up to 8th hr. The ETO-EG showed better anti-inflammatory activity over ETO-LG and the results are shown in Table 4 and Fig. 5.

The vesicular gels showed better % RIE as compared to (45% v/v ethanolic ETO-Solution and PROXYM®). It was also observed that the percentage reduction was observed at 4 h in the pharmacodynamic studies and the T_{max} value came out to be 4 h in the pharmacokinetic studies for the vesicular gels (at the 5thhr, ETO-LG inhibition was slightly more) as well as for the PROXYM®. The physicochemical properties of vesicular gels alter the dynamic properties of other test products. Finally, all these properties were stated that vesicular gels were advanced in reducing inflammation, when they are applied transdermally. These results were well correlated with in vitro, ex vivo permeation and pharmacokinetics. Analysis of % RIE data by ANOVA showed that there was significant difference ($P < 0.05$) among the different values. The anti-inflammatory activity results of present research work correlated with previous literature reports [35].

Correlation between AUC $_{(0\text{-}24h)}$ and reduction in the paw volume

A good correlation was observed in between AUC $_{0\text{-}24h}$ and reduction in the paw volume. The Correlation Coefficient (r) value was found to be 0.9635. The relationship between the PK and PD are shown in Fig. 6.

Discussions

The liposomes and ethosomes were successfully developed by following standard procedures using 3^2 factorial design and characterized for several parameters which reflect the robustness of the formulations. The best formulated liposomes and ethosomes were incorporated into gels using carbopol 940 NF as a gel base. Three different gels each, with different ratios of drug containing vesicles: base, were prepared for liposomes and for ethosomes. These gels were also characterized by in vitro and ex vivo studies. The selected formulations were subjected to pharmacokinetic and pharmacodynamic studies.

The current work stated that, ethosomal formulation showed better results compared to other test products. The percent drug content studies indicated that the etodolac was uniformly distributed in the vesicular dispersions. The particle size of ethosomes shows lowest size distribution and highest encapsulation efficiency as compared to liposomes. The particle size was decreased by increasing the lipid and %*v*/v ethanol concentration. The highest ethanol concentration, affects the lipid membrane thickness. This result was probably due to the interpenetrating hydrocarbon chains in the lipid.

Apart from ethosomes, the cholesterol concentration is critical for development of liposomes. The particle size was increased with increasing the concentration of lipid and cholesterol. The lower level of lipid and cholesterol produces higher encapsulation efficiency. While increasing the concentration of lipid and cholesterol, the particles size was increased whereas encapsulation efficiency was reduced. The both vesicular systems are attained their size in nanometer range with

Table 3 Reduction of paw volume in test products

Test products	Paw volume (ml)							
	0 min	1 h	2 h	3 h	4 h	5 h	6 h	7 h
Control	0 ± 0.0	2.63 ± 0.35	2.63 ± 0.35	2.63 ± 0.35	2.63 ± 0.35	2.63 ± 0.35	2.63 ± 0.35	2.63 ± 0.35
45% v/v ethanolic ETO-Solution	0 ± 0.0	2.67 ± 0.21	2.8 ± 0.18	3.0 ± 0.29	3.2 ± 0.25	3.5 ± 0.13	2.8 ± 0.36	2.8 ± 0.12
ETO-LG	0 ± 0.0	3.20 ± 0.22	3.3 ± 0.14	3.6 ± 0.31	3.8 ± 0.64	4.1 ± 0.47	3.9 ± 0.87	3.9 ± 0.65
ETO-EG	0 ± 0.0	3.6 ± 0.36	3.9 ± 0.41	4.3 ± 0.58	4.5 ± 0.24	4.4 ± 0.59	4.1 ± 0.11	4.1 ± 0.74
PROXYM®	0 ± 0.0	2.75 ± 0.15	2.86 ± 0.21	3.1 ± 0.19	3.5 ± 0.13	3.4 ± 0.12	2.9 ± 0.35	2.9 ± 0.19

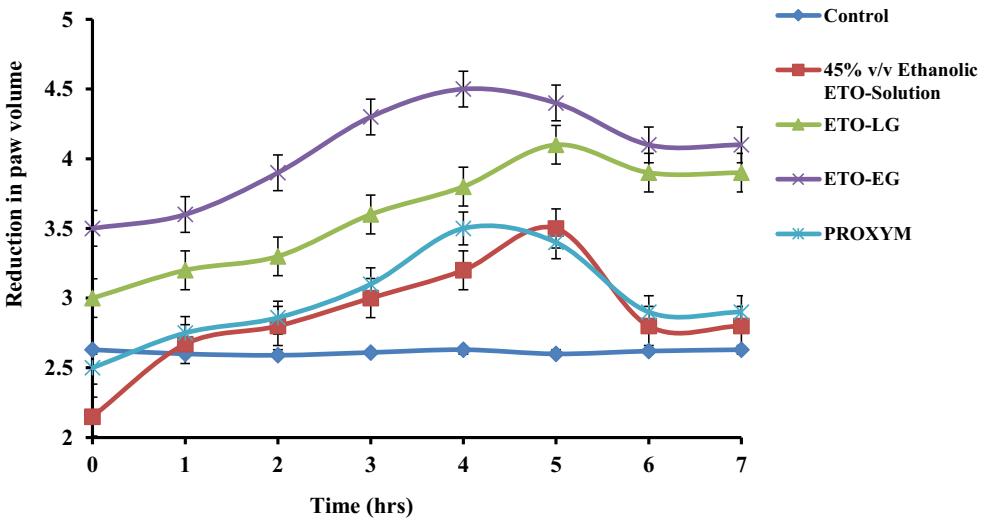

Fig. 4 Reduction in paw volume of tested products

low polydispersity indicates uniform distribution of dispersed phase in a dispersed medium. It was strongly affected by selected variables. The results indicated a profound effect of HSPC, cholesterol and % v/v ethanol on the particle size and %EE of etodolac. The zeta potential is essential parameter which influences the stability of colloidal dispersions. For any liquid dosage form surface charge is essential for its stability. Vesicular systems exhibited higher zeta potential value of −36.5 and − 48.3 mV for liposomes and ethosomes respectively. The values of zeta potential showed that vesicles had sufficient charge to inhibit aggregation of vesicles due to electric repulsion [16].

The in vitro drug release studies by diffusion indicates that vesicular drug release is influenced by various physicochemical factors such as amount of cholesterol, lipid composition, lamellarity, dispersion medium and preparation method. In case of liposomes, cholesterol makes the lipid bilayers more rigid and retards the release of the drug. When the combined effect of lipid and cholesterol was studied, it was observed that, at the lowest level of these components, the percent drug release was the maximum. It indicated that cholesterol concentration in liposomes decides its membrane fluidity, which in turn influences the rate of drug release. Cholesterol influences the lipid membrane modulating, membrane fluidity, vesicle elasticity and permeability. In case of ethosomes, the superior release may be due to the combined effect of ethanol and phospholipid. The presence of ethanol causes vesicles to be soft and malleable. So they can easily pass through the diffusion membrane. The combined effect of these components indicated that, ethanol enhaces the permeation of ethosomal vesicles through the membrane. The experimental design was successfully used in the development of etodolac loaded liposomes and ethosomes. The response surface methodology and contour plots used to interpret the predicted and observed response for liposomal and ethosomes formulations. The developed formulations and the predictable formulations were matched practically and the percent relative error was found below 5% for both liposomes and ethosomes.

From the figures shown in Fig. 1, ETO-LG and ETO-EG viscosity was decreased up to the end of the lag phase; after the lag phase, a steady state was observed with a constant shear rate. The optimized ETO-EG shown the lower viscosity compared to ETO-LG. It may be due to the presence of ethanol in its composition, which reduces the viscosity of the gel. The viscosity studies results are correlated with previous research work on done on lidocaine hydrochloride liposomal gels using chaitosan. [15].

The highest % EE was observed in case of gels, the lipophilicity of etodolac caused this high % EE in vesicular dispersions into gels. The concentration of carbopol 940 NF influenced the electrical double layer of the dispersion systems which leads to decrease the zeta potential of the gels compared to their dispersion state. The viscosity alters the zeta potential of ETO-LG and ETO-EG. The spreadability indicated that gels are easily spreadable by small amount of shear. The highest spreadability for ETO-EG was found that 66.8% indicating spreadability of ETO-EG was good as compared to the PROXYM® (58%). The prepared vesicular gels and PROXYM® showed good homogeneity with lack of lumps.

Table 4 Percentage reduction in oedema of test products

Time (hrs)	45% *v*/v ethanolic ETO-Solution	ETO-LG	ETO-EG	PROXYM®
1	28.17 ± 0.12	26.09 ± 0.48	42.34 ± 0.76	20.89 ± 0.42
2	35.52 ± 0.14	31.65 ± 0.76	56.12 ± 1.58	27.98 ± 0.51
3	38.19 ± 0.19	39.35 ± 0.58	61.89 ± 0.84	31.49 ± 0.53
4	44.18 ± 0.17	46.97 ± 1.23	70.02 ± 1.98	38.67 ± 0.38
8	31.16 ± 0.16	33.14 ± 1.72	55.67 ± 0.58	31.59 ± 0.17

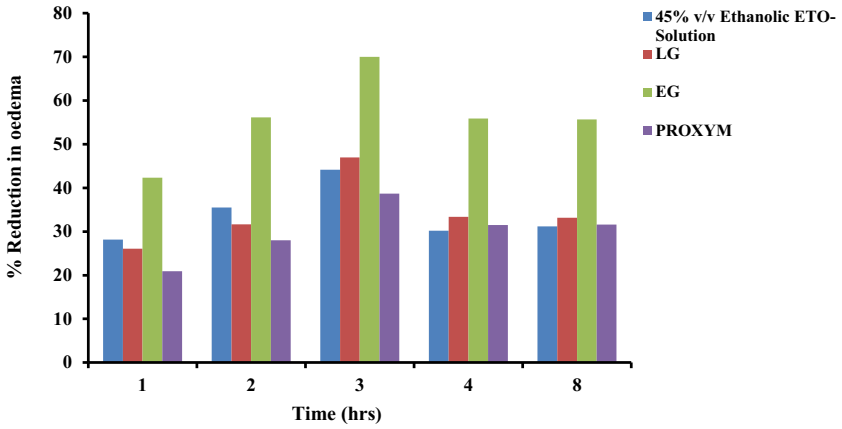

Fig. 5 Percentage reduction in oedema of test products

The vesicular gels were much clear and transparent as compared to PROXYM®. The therapeutic efficiency of the formulation also depends upon its spreading value and patient compliance also depends on the spreading efficiency of gel. Generally liposomes and ethosomes are posing adhesive nature due to their small size. They tend to adheres to the surface of the skin. The occlusive factor (F) depends on lipid concentration and the vesicles size. The smaller size of ETO-LG and ETO-EG shows higher occlusive factor (F) compared to PROXYM®. The occlusive factor enhanced skin hydration and can promote permeation/penetration of drugs into the multilayers of skin by reducing corneocytes packing and widening intercorneocyte gaps.

The ETO-LG shows controlled permeation as compared to other test products. The liposomal vesicles are high rigid in nature, unable to across stratum corneum but localized drug concentration within the skin layers. The liposomal vesicles increase fluidity by interacting with dried stratum corneum lipids, which promotes accumulation of drug (localization of drug) within skin layers, due to this reason, ETO-LG achieved long skin residency time and controlled local anti-inflammatory activity. The overall, ex vivo permeation data clearly indicated that ETO-EG shows effective drug delivery into the skin. In the present study, ethosomal system disrupted the intracellular lipids of stratum corneum and resulted in enhancing the permeation of etodolac when compared to other test products. Ethanol is absorbed into the skin membrane, where it acts as a chemical penetration enhancer. The synergetic effect of lipid and ethanol enhanced permeation by extracting skin lipids and proteins. The intercalation action of ethosomes increased thermodynamic activity of etodolac which leads to increase the transdermal flux compared to test products except 45% *v*/v of ethanolic ETO-Solution. The flux is proportional to gradient of thermodynamic activity rather than concentration of drug. The presence of ethanol suggesting that pronounced effect on the stratum corneum. This may be because of varying influence of ethanol on the hydration of skin and biophysical properties of the stratum corneum. In the current study, ethosomes shows excessive dehydration of the skin, which leads to have counterproductive effects to the permeation enhancement of etodolac. It was practically correlated with biophysical studies of FTIR.

The in vitro and ex vivo permeation studies may be interpreted in the following way. The 45% *v*/v ethanolic ETO-Solution shows very low percentage of permeation. In the form of 45% v/v ethanolic ETO-Solution, the drug was

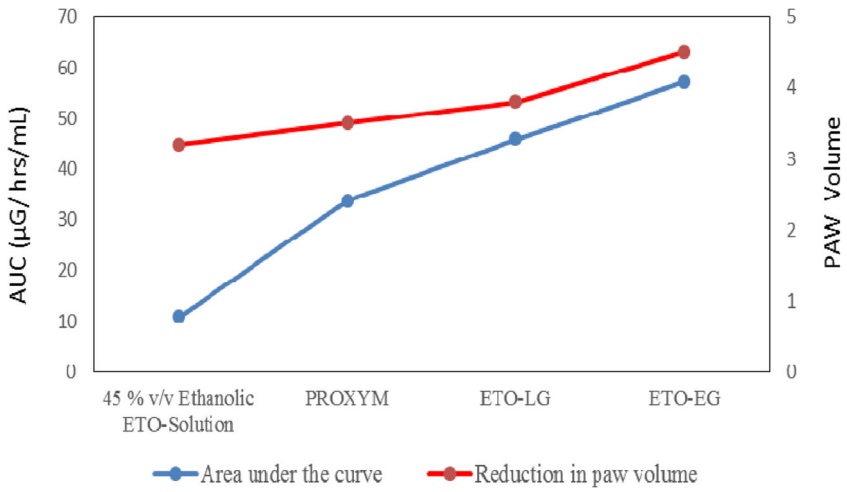

Fig. 6 Correlation of PK and PD of tested formulations

available in the solubilized form. It was observed that etodolac solubility does not affect the permeation rate across semi permeable membrane/delipidized rat skin [31]. This may due to low thermodynamic activity of etodolac from 45% v/v ethanolic ETO-Solution. The flux is proportional to the gradient of thermodynamic activity rather than concentration, in case of ETO-EG, the enhanced permeation may be the influence of ethanol on hydration level of the skin and biophysical properties of the stratum corneum. The in vitro/ ex vivo permeation studies of test products were found to be significant ($p < 0.05$). After ex vivo experiment, delipidized rat skin and untreated rat skin was subjected to FTIR.

The C_{max} of ETO-EG is highest compared to other test products, due to the malleability and fluidity of the vesicle movement which is imparted by ethanol. It indicated that the vesicles succeeded in causing enhanced permeation. The highest $AUC_{0-\infty}$ value of ETO-EG indicated that highest amount of etodolac was absorbed in the systemic circulation. The $AUC_{0-\infty}$ of ETO-EG was 2.5 folds increased compared with other test products. The $AUMC_{0-\infty}$ of the ETO-EG was increased 3.4 folds, when compared with PROXYM®. It indicated that, ETO-EG gave the maximum relative bioavailability among all test products. The highest K_{el} value of the 45% *v*/v ethanolic ETO-Solution indicated that, clearance of drug from blood is more rapid when compared to that of the vesicular gels or that of PROXYM®. The t½ of etodolac in ETO-EG was 1.56 folds higher whereas ETO-LG showed 1.31 folds higher, when compared with PROXYM®. The ETO-EG contains ethanol as a penetration enhancer, which makes the phospholipid membrane malleable and retains the drug in the core of the vesicles untill complete release of the drug. The MRT of etodolac in ETO-EG and ETO-LG were found to be 1.57 and 1.25 folds increased when compared with PROXYM®. The ANOVA of the MRT values of other test products indicated that difference among them is significant ($p < 0.0001$). Increased MRT values of etodolac from vesicular gel formulations indicated that the effective plasma concentrations were maintained for longer time when compared to PROXYM®.

The ETO-EG showed better anti-inflammatory activity over ETO-LG. The vesicular gels showed better oedema inhibition as compared to 45% v/v ethanolic ETO-Solution and PROXYM®. This may be attributed to the reason that the vesicular gels having better retention of the drug in the stratum corneum, permeation enhancement, long circulation time (because of reduced elimination of the drug) and controlled release of drug. It was observed that the inhibition slowly progressed, reached a peak in 4 hrs and then slowly decreased, but was present to a high degree up to 8 h. The paw volume of ethanolic ETO-solution was 2.8 ml in the 6th hr. and for PROXYM® was found to be 2.9 ml. The advantage of holding the percent inhibition at a high level for a maximum time of 8 h from the vesicular gels must be due to the controlled release of the drug from the vesicular gels. It was also observed that the peak was observed at 4 h in the pharmacodynamics studies and for the vesicular gels (For the liposomal gels the 5th hr. inhibition was slightly more) as well as for the PROXYM®.

Conclusion

In the present work, etodolac loaded vesicular systems were successfully developed by 3^2 factorial design. Both liposomes and ethosomes have shown different results as it indicated; both systems are not alternatives to each other. This statement was proved by their pharmacokinetic and pharmacodynamic studies. The ethosomal formulation shows superior properties compared to liposomal and other test products. Thus it can be concluded that the prepared vesicular gels offer a better choice for the delivery of NSAID through skin in order to overcome the gastro intestinal side effects which are associated with oral administration.

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Compliance with ethical standards

Conflict of interest Author declares no conflict of interest.

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