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Research Article

Systematic Development of Transethosomal Gel System of Piroxicam: Formulation Optimization, *In Vitro* Evaluation, and *Ex Vivo* Assessment

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Abstract. Piroxicam is used in the treatment of rheumatoid arthritis, osteoarthritis, and other inflammatory diseases. Upon oral administration, it is reported to cause ulcerative colitis, gastrointestinal irritation, edema and peptic ulcer. Hence, an alternative delivery system has been designed in the form of transethosome. The present study describes the preparation, optimization, characterization, and *ex vivo* study of piroxicam-loaded transethosomal gel using the central composite design. On the basis of the prescreening study, the concentration of lipids and ethanol was kept in the range of 2–4% *w/v* and 0–40% *v/v*, respectively. Formulation was optimized by measuring drug retention in the skin, drug permeation, entrapment efficiency, and vesicle size. Optimized formulation was incorporated in hydrogel and compared with other analogous vesicular (liposomes, ethosomes, and transfersomes) gels for the aforementioned responses. Among the various lipids used, soya phosphatidylcholine (SPL 70) and ethanol in various percentages were found to affect drug retention in the skin, drug permeation, vesicle size, and entrapment efficiency. The optimized batch of transethosome has shown 392.730 $\mu g \, cm^{-2}$ drug retention in the skin, 44.312 $\mu g \, cm^{-2} \, h^{-1}$ drug permeation, 68.434% entrapment efficiency, and 655.369 nm vesicle size, respectively. It was observed that the developed transethosomes were found superior in all the responses as compared to other vesicular formulations with improved stability and highest elasticity. Similar observations were noted with its gel formulation.

KEY WORDS: elastic vesicles; entrapment efficiency; ethosome; experimental design; liposome; permeability; transethosome; transfersome.

INTRODUCTION

Piroxicam is one of the most potent nonsteroidal anti-inflammatory drugs that are widely used in the treatment of rheumatoid arthritis, osteoarthritis, and other inflammatory diseases. Piroxicam administered through the oral route causes multiple adverse effects like ulcerative colitis, gastrointestinal irritation, edema and peptic ulcer. (1–3). On the other hand, when it is administered through the parenteral route, it exhibits intense pain and inflammation at the injection site. Since patients suffering from arthritis have to take it on a daily basis, whereas administration of this drug through the parenteral route causes discomfort to the patients at the injection site. Thus, the present work endeavors to develop an alternative drug delivery system, which effectively delivers this drugs

with improved patient compliance. The topical route for drug delivery is considered to be one of the promising approaches for such drugs as it bypasses the hepatic first-pass metabolism and provides local action of the drug in the targeted area. Besides enhancement of the therapeutic efficacy of the drug, it also alleviates the adverse effects of the drug through systemic circulation (1).

Despite the promising advantages of topical delivery systems, conventional topical systems face the challenge concerning their penetration through the deeper layers of the skin. Hence, in order to achieve localized action of the drugs for an effective treatment, vesicular drug delivery systems like liposomes and niosomes are highly suitable. However, liposomes and niosomes are unable to penetrate deep into skin owing to their less flexible nature. In order to overcome these limitations, formulation of elastic vesicles has been attempted in the past few years. These elastic vesicles are of two types *viz*. transfersomes are composed of an edge activator and lipid, which are popularly known as transfersomes, and ethosomes are composed of lipid and ethanol (1–6). These help in minimizing the washout of the drug through the blood, which is a common problem observed when drugs are given through the topical route using penetration enhancers, ionotophoresis, or electrophoresis mechanisms. Besides these advantages, elastic vesicles have the ability to bypass the

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stratum corneum as well as the capillary bed of the skin and deposit drug in the deeper skin layers (7,8).

Transfersomes increase the elasticity of vesicles by redistribution of the edge activator and lipid in their milieu, whereas ethosomes act by fluidizing the lipids of both the skin as well as the vesicles (2–5). In the present study, an attempt has been made for formulating transethosomes as novel vesicles containing both ethanol along with lipids, which mimic the properties of both transfersomes and ethosomes (9).

Systematic optimization of pharmaceutical products using experimental designs helps in selecting the best formulation under a given set of conditions using less number of experiments, followed by enormous saving of resources like time, effort, and money. Among the various designs available for the purpose, the central composite design (CCD) has extensively been employed in optimization practice for identifying the best formulation (10–12). In CCD, the factors are studied in all possible combinations, as it is considered to be efficient in estimating the influence of individual variables (main effects) and their interaction effects. Besides, this design has the added advantage of determining the quadratic response effects, which are not estimated using the factorial design (FD) at two levels. Thus, a 3^2-CCD was employed for optimizing the two factors at three levels for systematic optimization of the prepared vesicles (13–15).

The present study, therefore, addresses the formulation of transethosomes wherein the developed carrier system has been successfully applied to formulate the gel for the topical delivery of piroxicam. Systematic optimization of the formulation variables that could affect the formulation properties of transethosomes has been screened and optimized with the help of a selected experimental design.

METHODS

Material

Piroxicam was a gift from Apex Healthcare Ltd, Ankleshwar, India. Soya phosphatidylcholine 70 (SPL 70) had been procured from Nabros Pharma Pvt Ltd, Ahmedabad, India. Sephadex G-50 (medium) was procured from M/s Sigma-Aldrich, St. Louis, USA. Span 80 has been procured from Loba Chemie Pvt Ltd, Mumbai, India. Ethanol was purchased from Lobache, Mumbai, India. All the other chemicals used for formulation development were of analytical grade. Double distilled water was used throughout the study.

Preliminary Screening Studies

Preliminary screening studies were carried out to identify suitable levels for the formulation variables for optimization studies using the CCD. A total of 20 formulations were prepared (Table I) and evaluated for entrapment efficiency and vesicle size as the responses in the preliminary screening study.

Formulation of Transethosomes and Other Vesicles

The thin film hydration method was used for the preparation of the vesicles (2,16,17). A series of lipids (*i.e.*, soya phosphatidylcholine and egg phosphatidylcholine) and edge activators (*i.e.*, Tween 20, 40, and 80 and Span 20, 60, and 80) were employed for the formulation of the vesicles. Among these, soya phosphatidylcholine 70% (SPL 70) and Span 80 have been selected as the lipid and edge activator, respectively, for the formulation of vesicles. The lipid, edge activator, and drug (piroxicam) were weighed accurately and dissolved in chloroform/methanol (2:1 *v/v*). This solution was evaporated on a rotary evaporator (Eyela SB-2100) at 50°C under reduced pressure. The thin film obtained was hydrated with pH 7.4 phosphate buffer saline with or without ethanol. The suspension was kept overnight for complete hydration of the vesicles. Placebo formulations of the same composition without drug were also prepared (18–23). The batch composition of the prepared vesicles for the prescreening study is shown in Table II. Nine different formulations have been prepared as per the CCD given in Table III.

In order to compare the potential of newly developed transethosomes, the other vesicle systems (liposomes, transfersomes, and ethosomes) were also prepared. The method for the preparation of liposomes, transfersomes, and ethosomes was similar to that of transethosomes. The amount of piroxicam, *i.e.*, 500 mg of piroxicam per 100 ml of the formulation, was fixed in all the formulations, and the amount of Span 80 was fixed according to the ratio of SPL 70 and Span 80 (5,24,25). The composition for these formulations is shown in Table IV.

Systematic Optimization Studies Using the Experimental Design

Initial screening trials were carried out for the evaluation of the formulation parameters, which may influence the properties of transethosomes. Various factors like the concentration of the drug, percentage of the edge activator (Span 80), percentage of lipid (soya phosphatidylcholine 70), and percentage of ethanol were identified as the critical formulation parameters for the target product with acceptable formulation characteristics and stability. The results of the initial screening studies suggested that the percentage of soya phosphatidylcholine and the percentage of ethanol in the formulation were selected as the main factors that could affect drug retention in the skin, drug permeation, entrapment efficiency, and vesicle size. Based on the number of factors and their levels, the CCD was used to evaluate the effect of formulation parameters affecting the physical properties of the developed transethosomes. A CCD for two factors at three levels was carried out to optimize the varied response, *i.e.*, drug retention in the skin, drug permeation at 6 h ($Q_{6\ h}$), entrapment efficiency, and vesicle size. Design-Expert® software ver. 8.0 (Stat-Ease, Minneapolis, USA) was used to employ this design. The concentration of the drug and edge activator was kept the same for all the experiments. A total of 13 experiments were prepared as per the trial runs suggested by the design including the five replicates of center point formulation at 0, 0 level. Table III shows the design matrix containing the experimental runs and levels of the independent factors employed for the design (26).

Drug Entrapment Efficiency

The minicolumn centrifugation method was used for studying entrapment efficiency. Vesicle suspension loaded with 0.2 ml of the drug was placed in the column containing

Table I. Prescreening Study

S. no.	Formulation code	Soya phospholipid (SPL) *w/v* (%)	Span 80 (mg) SPL/S80 = 85:15 *w/w*	Varying concentration of ethanol in pH 7.4 PBS (20 ml) (%)
1	P1	2	70.58	0
2	P2	2	70.58	10
3	P3	2	70.58	20
4	P4	2	70.58	30
5	P5	2	70.58	40
6	P6	3	105.9	0
7	P7	3	105.9	10
8	P8	3	105.9	20
9	P9	3	105.9	30
10	P10	3	105.9	40
11	P11	4	141.16	0
12	P12	4	141.16	10
13	P13	4	141.16	20
14	P14	4	141.16	30
15	P15	4	141.16	40
16	P16	5	176.5	0
17	P17	5	176.5	10
18	P18	5	176.5	20
19	P19	5	176.5	30
20	P20	5	176.5	40

Sephadex G-50, and the column was centrifuged at 2000 rpm. The column was allowed to elute. The eluted vesicles were collected and observed under a microscope for the absence of any crystal. The column was eluted four times with 0.2 ml distilled water. These eluted vesicles were digested, ruptured with methanol, and analyzed spectrophotometrically at 365 nm (5,8,27). Equation 1 showed the formula for entrapment efficiency.

$$\text{Percentage drug entrapment} = \frac{\text{Amount of entraped drug}}{\text{Total amount of drug}} \times 100 \quad (1)$$

Evaluation of Vesicle Size

Vesicle size and size distribution study was done by Beckman Coulter using dynamic light scattering (8,28).

Ex Vivo Permeation Study

Fresh porcine skin from ear pinna was used for conducting the permeation studies. Ears were collected from a local slaughterhouse and stored at −20°C till further use. Skin was separated out from the ears and hair was removed.

Table II. Batch Composition of Transethosomal Formulation

S. no.	Component	Range (unit)
1	Soya phospholipid 70% (SPL 70)	2–5 (% *w/v*)
2	Span 80	85:15 (SPL 70/Span 80)
3	Drug	100 (mg)
4	Ethanol	0–40 (% *v/v*)

Afterwards, the skin was separated from adipose tissue and other fatty tissues. The skin was then washed with saline and kept in pH 7.4 phosphate buffer saline for further use (16,20,29).

The permeation study was conducted in phosphate buffer saline (PBS) of pH 7.4, which was maintained at $37 \pm 0.2°C$ in a Franz diffusion cell (FDC) having a surface area of 3.14 cm^2 and a receptor compartment of 30 ml capacity. The diffusion medium was maintained at a stirring speed of 100 ± 4 rpm (30,31). Porcine skin was fixed in the base of the donor compartment of FDC, which acted as the diffusion barrier between the donor and receptor compartments. Formulation (1 ml) was kept in the donor compartment over the porcine skin, and the diffusion study was carried out for 6 h. The sample (1 ml) was withdrawn at predetermined time intervals and the withdrawn volume was replaced with fresh buffer. Withdrawn samples were filtered through a 0.20-μm membrane filter and analyzed by a UV spectrophotometer (31,32).

Drug Retention in Skin Studies

In order to carry out the study on drug retention in the skin, the skin was washed with pH 7.4 PBS several times followed by blotting with tissue paper to remove any formulation that adhered on its surface. The skin was cut into small pieces and kept in methanol for 24 h to extract the drug deposited in the skin. The processed skin was sonicated for 20 m and centrifuged, and the supernatant was taken. The withdrawn supernatant was filtered and analyzed using a UV spectrophotometer and the amount of drug was calculated (5,33).

Search for the Optimum Formulation and Validation Studies

The search for the optimum region was carried out by brute-force methodology employing feasibility and grid

Table III. Factor Levels and Response Data for the CCD Study

Run	Factor A (SPL 70) (X1)	Factor B (ethanol) (%) (X2)	Skin retention (μg cm^{-2}) (Y1)	Drug permeation at 6 h (μg cm^{-2}) (Y2)	Drug entrapment efficiency (%) (Y3)	Vesicle size d (90) (nm) (Y4)
1	−1	0	229.17	61.86	55.18	687.30
2	+1	−1	124.53	55.54	54.40	687.00
3	+1	0	149.30	139.66	63.40	678.80
4	0	0	303.95	42.07	64.00	699.60
5	+1	+1	105.80	69.78	60.30	714.10
6	0	+1	280.70	43.64	74.36	682.30
7	−1	−1	456.72	41.56	50.50	705.20
8	0	−1	201.46	40.80	61.09	693.40
9	−1	+1	496.10	42.78	70.36	675.80

Independent factors				Design level	
Uncoded		Coded		Uncoded	Coded
Soya phospholipid (SPL 70) (% w/v)		A		2	−1
				3	0
				4	+1
Ethanol (% v/v)		B		0	−1
				20	0
				40	+1

Variables for central composite design

search, and the optimized formulation was selected from the design space in the overlay plot. The feasibility search was conducted by using the polynomial model equations generated for each of the responses as per the criteria—highest drug retention in the skin, highest skin permeation, higher entrapment efficiency, and small vesicle size. Further, the formulations were screened during the grid search. The criterion for the selection of optimized formulation was based on responses in order of preference of maximum drug retention in the skin, drug permeation at 6 h ($Q_{6\ h}$), entrapment, and minimal vesicle size. Graphical optimization was carried through optimization of the independent variables.

Six formulations were selected as the validation checkpoints and their observed as well as predicted responses were compared. Formulations were prepared as per the procedure described in the "Formulation of Transethosomes and Other Vesicles" section and evaluated for vesicle size, entrapment efficiency, drug permeation, and drug retention in the skin. The observed results were compared with the predicted results, and the percentage error was calculated to check the deviation within the range of ±10%. Table V illustrates the

Table IV. Composition of Liposome, Transfersome, Ethosome, and Transethosome

Formulation	SPL 70 (% w/v)	Ethanol (% v/v)	Span 80 (SPL/S80 = 85:15)
Liposome[a]	2.2	0	0
Transfersome[a]	2.2	0	77.64
Ethosome[a]	2.2	36	0
Transethosome (OP)	2.2	36	77.64

OP optimized formulation
[a] The formula composition of liposome, transfersome, and ethosome is based on the optimized batch that has been obtained from CCD (which is discussed in the "RESULTS AND DISCUSSION" section)

composition of the checkpoint formulations prepared for the validation studies.

Characterization Studies

Transmission Electron Microscopy

Vesicle morphology of optimized formulation of transethosomes was done by using transmission electron microscopy (TEM). The sample was placed on a carbon-coated copper grid to form a thin film on the grid. Phosphotungstic acid (PTA) solution (1% v/v) was placed on the grid. Excess solvent was drained off with a filter paper and examined using TEM (8).

Degree of Deformability

Elasticity evaluation of transethosomes was compared with conventional liposomes, transfersomes, and ethosomes. The vesicles were passed through 50 nm polycarbonate membrane fitted on an extruder. Vesicles before and after extrusion were analyzed for vesicle size, elasticity, and deformability index. Vesicle size was analyzed during dynamic light scattering (DLS). Elasticity was calculated by dividing the vesicle size after extrusion with mesh size through which this was passed. Deformability index was calculated by dividing vesicle size before and after extrusion (5).

Stability Study

Stability study was conducted by keeping the optimized formulation at refrigerated conditions (5 ± 3°C) and 25 ± 2°C for 28 days (34). These vesicles were evaluated for entrapment efficiency at an interval of 7 days for 28 days (5,8).

Table V. Composition of Formulations Selected for Validation Studies

Formulation code	Levels		SPL (% *w/v*)	Ethanol (% *v/v*)	Span 80 (mg) (SPL/S80 = 85:15)
	SPL	Ethanol			
OP	−0.8	0.80	2.20	36.00	77.64
V1	−0.32	0.44	2.68	28.80	94.59
V2	−1	+1	2.00	40.00	70.59
V3	−0.60	−1	2.40	0	84.71
V4	−0.08	0.6	2.92	32.00	103.06
V5	0.2	−0.8	3.20	4.00	112.94

OP optimized transethosome

Preparation of Secondary Vehicle/Gel

The vesicles were made rheologically acceptable by incorporating them into Carbopol 934 gel (1% *w/w*). The optimized transethosomes, transfersomes, ethosomes, and conventional liposomes were incorporated into the gel to form optimized transethosomal gel (OTG), ethosomal gel (EG), transfersomal gel (TG), and liposomal gel (LG), respectively. For the preparation of hydrophilic Carbopol gel, vesicle dispersion equivalent to 0.5% *w/w* drug was taken and incorporated into 10% *w/w* Carbopol 934 hydrogel. The final composition of the gel used was 1% *w/w* Carbopol 934 hydrogel equivalent to form 0.5% *w/w* drug. Triethanolamine was used in the gel to maintain pH and consistency. Table VI shows the composition of the hydrogel.

Characterization of the Vesicular Gel

Rheological Study of Gel Formulation

The rheological behavior of the gel and the property of the fluid, to check whether it is Newtonian or non-Newtonian, was determined by using a rheometer. The viscosity of the optimized formulation and marketed formulation was determined at 30°C with a cup and bob rheometer using approximately 15 g of the sample. Viscosity was determined at various shear rates by putting the gel to various torque values. Measurement of each sample was done over a range of $2–100 \text{ s}^{-1}$ of the shear rate.

Texture Analysis of Gel Formulation

Various gel characteristics, *i.e.*, firmness and stickiness, of the formulations were determined by employing spreadability rig that was fitted on the Texture Analyzer (Stable Micro Systems Ltd., Godalming, Surrey GU7 1YL, UK). It consists of two probes: an upper probe and a lower probe.

Table VI. Composition of Topical Hydrogel

Component	Topical hydrogel formulation
Vesicle dispersion	Equivalent to 0.5% *w/w* of drug
Carbopol 934	Equivalent to 1% *w/w*
Triethanolamine	q. s.
Distilled water	q. s.

Formulation was added into the lower cone of the instrument and pressed to remove any entrapped air. Excess formulation was scraped off to leave a flat test area. The formulation was allowed to equilibrate for 30 min at room temperature in a closed environment before initiating the test. Before testing, the upper probe was calibrated against the lower cone probe in such a way that the starting point can be kept at the same height for each test. During testing, the instrument was set to compression mode with a test speed of 3 mm/s. The upper cone probe was allowed to approach and penetrate the sample up to a depth of 2 mm above the sample holder surface. Thus, the probe moved a distance of 23 mm from its starting point. The following test conditions were delineated in the software program of the instrument: mode, measure force in compression; option, return to start; pretest speed, N/A; test speed, 3.0 mm/s; posttest speed, 10.0 mm/s; distance, 23 mm; trigger type, button; tare mode, auto; and data acquisition rate, 200 pps.

The proposed method was based on the measurement of the force, *i.e.*, penetration and detachment force in grams and work, *i.e.*, work of shear and adhesion (g s) done by the upper cone to penetrate and remove from the test formulation present in the lower cone (35).

Ex Vivo Drug Permeation Studies of Gel Formulation

Transethosomal gel was compared with liposomal, transfersomal, ethosomal, and marketed gel for drug permeation studies for 24 h. The drug permeation study for transethosomal gel was conducted in the same way as given for vesicles in the "Entrapment Efficiency, Drug Retention in the Skin, and Drug Permeation Study" section. One gram of the gel was applied in the donor compartment.

Analysis of Release Mechanism

Various release models like zero order, first order, Higuchi, and Hixson Crowell were fitted to optimized transethosomal (OTG) and other gel formulations, *i.e.*, LG, TG, EG, and marketed gel (MG), respectively, to ensure drug release mechanism.

Drug Retention in the Skin of Gel Formulation

Transethosomal gel was compared with liposomal, transfersomal, ethosomal, and marketed gel concerning drug retention in the skin. The study of drug retention in the skin
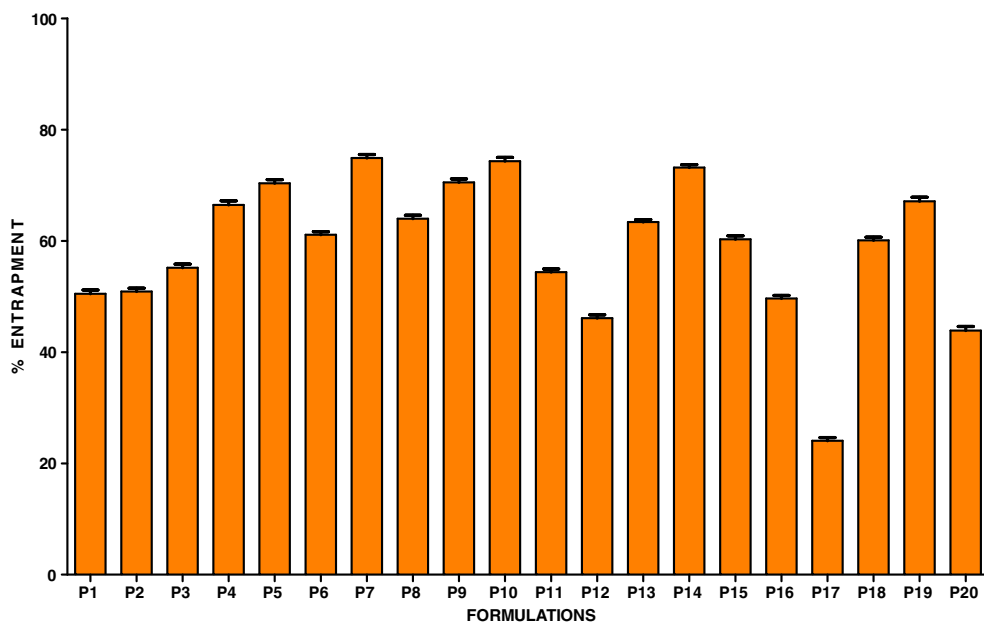
Fig. 1. Percentage entrapment of elastic vesicles

for transethosomal gel was conducted in the same way as given earlier in this paper.

RESULTS AND DISCUSSION

Preliminary Screening Study

The preliminary screening study was carried out to select the levels of the factors investigated for the experimental design. A total of 20 formulations were prepared and the levels were decided on the basis of entrapment efficiency and vesicle size. Figure 1 represents the entrapment efficiency and Fig. 2 represents the vesicle size of all the formulations. From the results of entrapment efficiency, it has been observed that P10 formulation showed maximum entrapment efficiency in the range between 24.10 and 74.36%. It is pertinent to add here that P7 formulation has almost similar entrapment efficiency but larger vesicle size (728 nm) as compared to P10 (649.2 nm). Entrapment efficiency in formulations prepared with 5% *w/v* soya phospholipid (*i.e.*, P16–P20) was more or less the same as compared to formulations prepared with 2, 3, and 4% *w/v* soya phospholipid.

All the formulations showed good uniformity in vesicle size as the polydispersity index (PDI) was found in the range between 0.261 and 0.385. P6 formulation showed the maximum vesicle size, while P4 (551.7 nm) showed the smallest vesicle size (Fig. 2). Vesicle size was
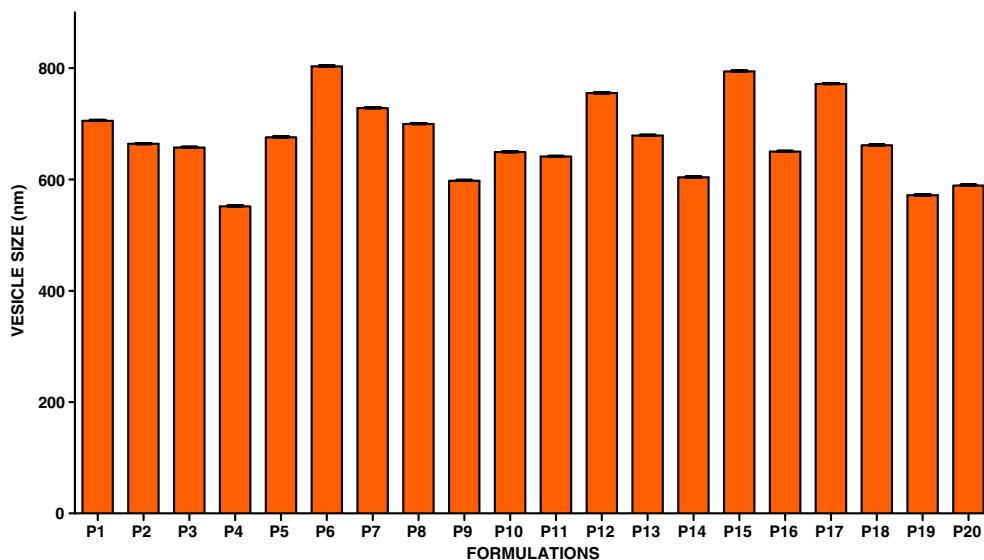

Fig. 2. Vesicle size in nanometers of elastic vesicles gel different formulations

Table VII. Summary of ANOVA of the Central Composite Design for Formulated Transethosome

Response variables	Regression parameters		Observed *P* value*
	R^2	$F_{cal.}$	
Skin retention	0.56	6.28	0.0100
Drug permeation at 6 h	0.74	7.92	0.0058
Drug entrapment efficiency	0.81	6.27	0.016
Vesicle size *d* (90)	0.57	4.01	0.045

*Indicates statistical significance at $P < 0.05$

obtained in the range of 551.7–803.4 nm. Little variation was observed with formulations containing 5% *w/v* soya phospholipid as compared to formulations prepared with 2, 3, and 4% *w/v* soya phospholipid. Thus, considering entrapment efficiency and vesicle size, the levels for soya phospholipid were fixed at 2–4% and ethanol at 0–40% for further studies using the experiment design.

Response Surface Analysis

A total of 13 experiments were carried out to study the effect of ethanol and SPL 70 on drug retention in the skin, drug permeation at 6 h ($Q_{6\,h}$), entrapment efficiency, and vesicle size, respectively. Response data for all experimental runs of CCD is illustrated in Table III.

The values of responses Y1 (drug retention in the skin), Y2 (drug permeation at 6 h), Y3 (entrapment efficiency), and Y4 (vesicle size) range from 105.8 to 496.1 $\mu g\ cm^{-2}$, 40.8 to 139.66 $\mu g\ cm^{-2}\ h^{-1}$, 50.5 to 74.36%, and 675.8 to 714.1 nm, respectively. The ratio of maximum to minimum for Y1, Y2, Y3, and Y4 is 4.68, 3.42, 1.47, and 1.05, respectively. This obviates the power transformation of the response data. Analysis of variance (ANOVA) was applied to determine the statistical significance and the magnitude of the main effects of each variable and their interactions. The regression model obtained was used to generate the counter plots for independent factors. The ANOVA table confirms the adequacy of the model (*i.e.*, $F < 0.05$) as shown in Table VII. It also identifies the significant factors that affect responses Y1, Y2, Y3, and Y4 of the developed transethosome. For drug retention in the skin (Y1), the percentage of SPL 70 was
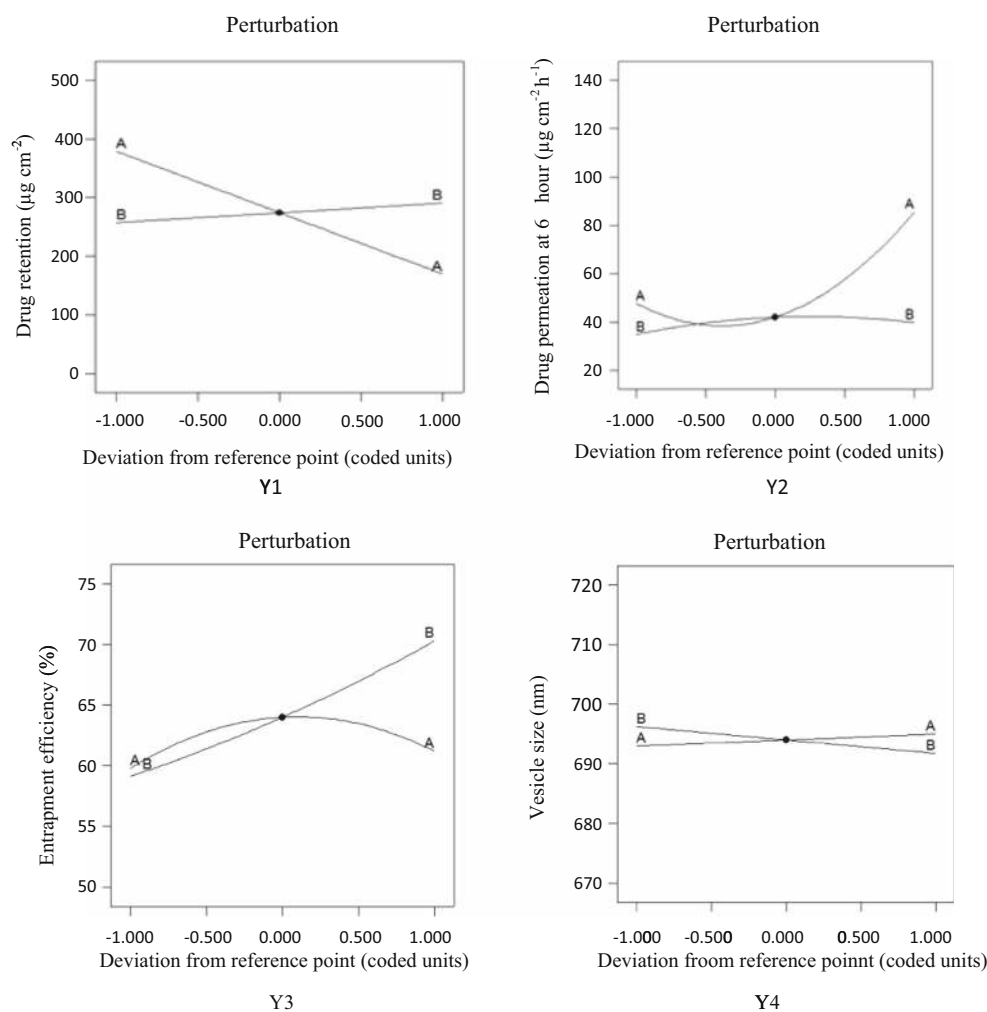

Fig. 3. Perturbation graph for the effect of individual factors on response Y1 (drug retention), Y2 (drug permeation at 6 h), Y3 (entrapment efficiency), and Y4 (vesicle size *d* (90))
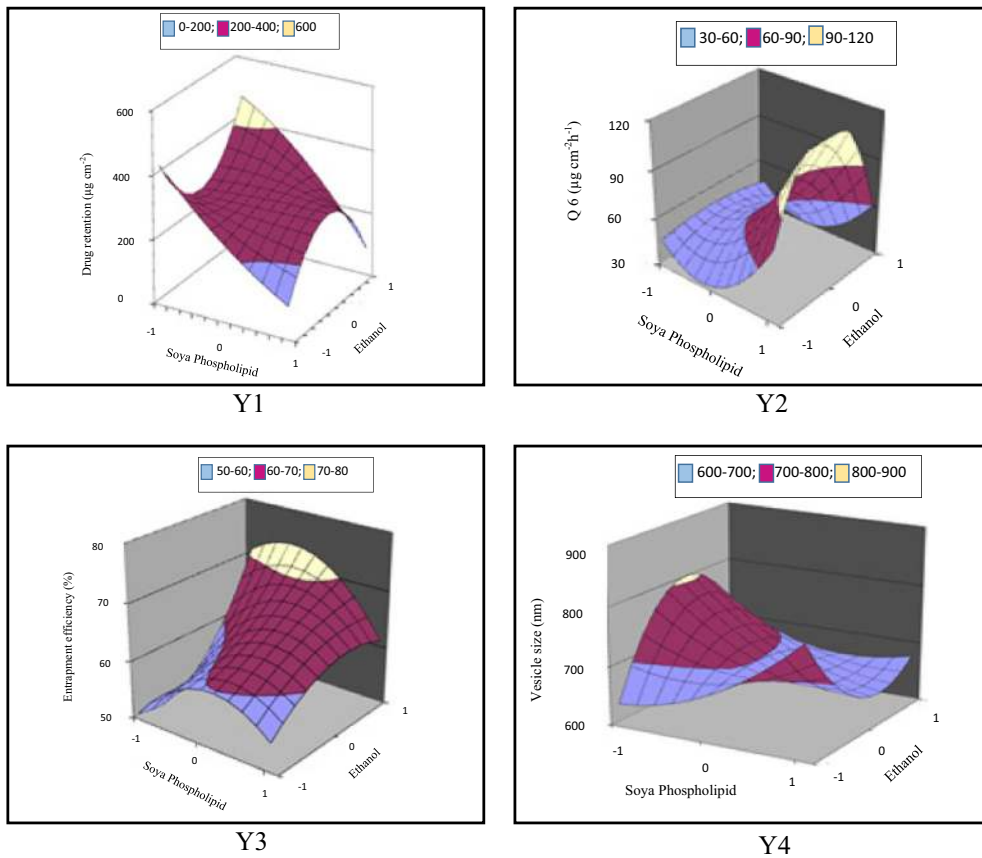
Fig. 4. Response surface graphs depicting the effect of individual factors on responses, *i.e.*, Y1 (drug retention), Y2 (drug permeation at 6 h), Y3 (entrapment efficiency), and Y4 (vesicle size *d* (90)) as per the central composite design

identified as a significant model term. Drug retention in the skin of the vesicles also depends on the ratio of ethanol, but the observed effect was very less. However, drug permeation and entrapment efficiency were significantly dependent on the percentage of SPL 70 as well as the percentage of ethanol. Vesicle size was less affected by both independent factors.

The final mathematical model in terms of coded factors as determined by the Design-Expert software is shown below in Eqs. (2–5) for responses Y1, Y2, Y3, and Y4, respectively.

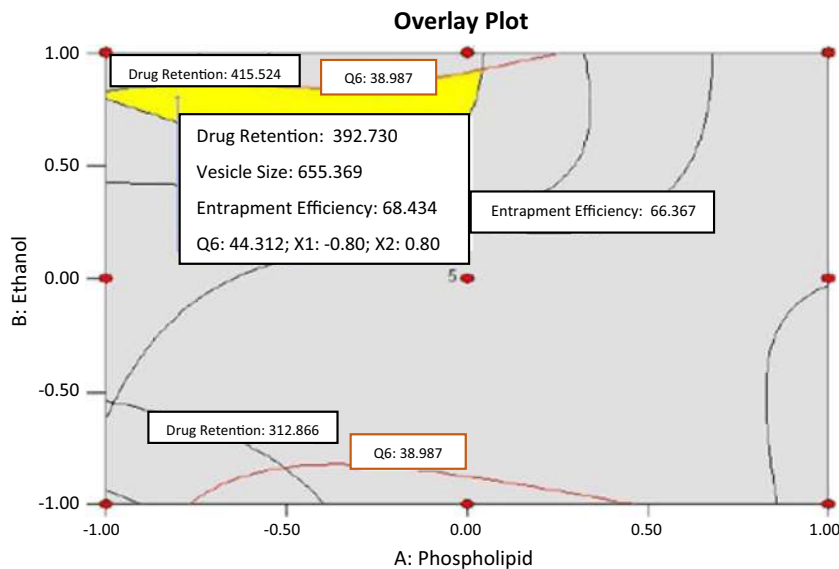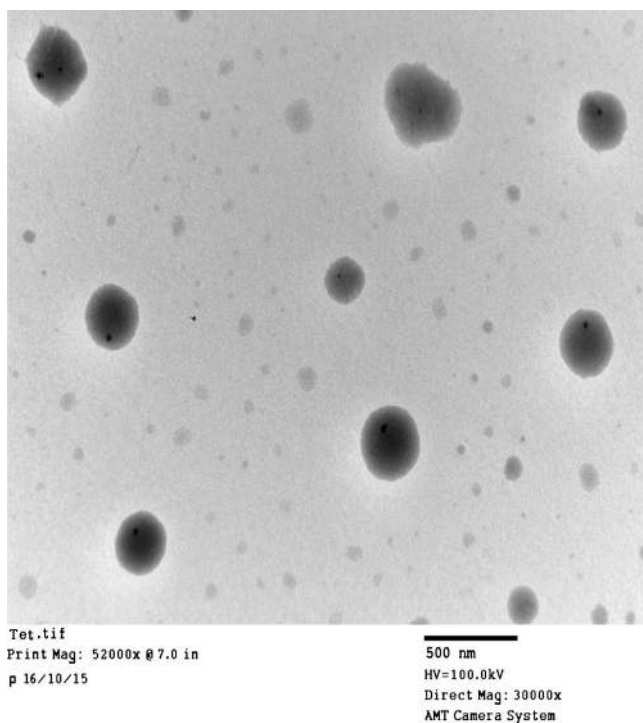$$Y1 = +274.12 - 104.43 \times A + 16.59 \times B \tag{2}$$

Fig. 5. Overlay plot showing the location of optimized transethosomal formulation

Table VIII. Validation of Experimental Results with Predicted Values and Percentage Error

Formulation code	Predicted value	Experimental value	Percentage error
Percentage drug entrapment			
OP	68.44	71.20	4.03
V1	66.66	64.20	−3.69
V2	71.07	70.36	−0.99
V3	56.15	58.06	3.40
V4	69.13	67.50	−2.35
V5	60.76	59.40	−2.2
Vesicle size (nm)			
OP	655.3	646.00	1.42
V1	662.49	621.70	−6.10
V2	674.2	675.80	0.20
V3	777.23	819.10	5.38
V4	660.4	667.70	1.11
V5	770.62	819.10	6.29
Q6 amount of drug permeated at 6 h ($\mu g\ cm^{-2}\ h^{-1}$)			
OP	44.30	47.20	6.54
V1	51.65	46.60	−9.77
V2	47.69	43.78	−8.20
V3	34.87	38.30	9.84
V4	52.48	56.90	8.42
V5	47.86	50.10	4.68
Skin retention ($\mu g\ cm^{-2}$)			
OP	392.71	357.00	−9.09
V1	312.28	283.00	−9.37
V2	475.79	496.40	4.33
V3	352.17	345.9.0	−1.78
V4	314.59	297.86	−5.32
V5	233.21	219.65	−5.82

OP optimized transethosome

Tet.tif
Print Mag: 52000x @ 7.0 in
p 16/10/15

500 nm
HV=100.0kV
Direct Mag: 30000x
AMT Camera System

Fig. 6. TEM image of optimized transethosomal formulation (OP) at ×30,000

$$Y2 = +\,42.07 + 18.88 \times A + 2.43 \times B + 3.26 + 24.58 \times A^2 - 4.69 \times B^2 \tag{3}$$

$$Y3 = +\,64 + 0.68 \times A + 5.57 \times B - 3.49 \times AB - 3.51 \times A^2 + 0.71 \times B^2 \tag{4}$$

$$Y4 = +\,693.99 + 1.01 \times A - 2.25 \times B + 14.12 \times AB \tag{5}$$

A positive sign represents a synergistic effect, while a negative sign indicates an antagonistic effect (36). In case of Y1, a negative coefficient of *A* represents the decrease in drug retention in the skin with an increase in the percentage of SPL 70, while a positive coefficient of *B* represents an increase in drug retention in the skin with the increase in the percentage of ethanol. Similarly, in case of Y2 and Y3, positive coefficients of *A* and B represent an increase in drug permeation and entrapment efficiency with an increase in the percentage of SPL 70 and ethanol. In case of response Y4, vesicle size increases with the increase in the percentage of SPL 70 and decreases with the increase in the percentage of ethanol.

A perturbation graph was plotted to find those factors that most affect the response. A steep slope or curvature in a factor shows that the response is sensitive to that factor. A relatively flat line shows insensitivity to change in that particular factor. In the case of Y1 and Y3, factors *A* and *B* show a steep slope. In case of Y2, factor *A* shows a steep slope and factor *B* shows a slight bend. In case of Y4, only a slight bend was observed for both factors. Figure 3 represents the perturbation plot for responses Y1, Y2, Y3, and Y4, respectively (36).

The effect of the two factors soya PC and ethanol on response factors, entrapment efficiency, vesicle size, drug permeation, and drug retention in the skin, respectively, is shown in Fig. 4. Further, it was observed from Table III that with an increase in the percentage of ethanol, an increase in drug retention in the skin (Y1) was observed. Ethanol fluidizes the skin lipid layer as well as the lipids present in the vesicles; thereby, it increases the fluidity as well as the flexibility of formulation. This ultimately causes deeper penetration of the drug in the skin tissues and thereby increases drug retention in the skin (37,38). Moreover, the presence of the edge activator (Span 80) has also provided a synergistic effect in drug retention in the skin. In case of skin permeation (Y2), a significant increase was observed with the increase in the percentage of SPL 70 and ethanol. Ethanol is a well-known permeation enhancer, and with the combined effect of SPL 70 and Span 80, its permeation efficiency has further been improved.

The vesicular size (Y4) of the developed transethosomes decreased with the increase in ethanol concentration and increased with the increase in phospholipid concentration. At the same time, the content of ethanol and phospholipid had a significant positive effect on entrapment efficiency (Y3) of transethosomal vesicles.

Search for Optimized Transethosomal Formulation and Validation Studies

Optimization of the developed transethosomes was performed to find the levels of factors *A* and *B*, with values for drug

Table IX. Vesicle Size of Optimized Transethosomes, Transfersomes, Ethosomes, and Liposomes Before and After Extrusion

Formulation	Before extrusion (nm) ($n=3$)	After extrusion (nm) ($n=3$)	Deformability index	Deformability/elasticity
Optimized transethosomes (OP)	646.0 ± 0.01	634.4 ± 0.02	0.982 ± 0.01***	12.73 ± 0.01***
Transfersomes (T)	647.4 ± 0.02	430.8 ± 0.01	0.665 ± 0.01***	8.60 ± 0.01***
Ethosomes (E)	615.4 ± 0.02	540.5 ± 0.01	0.878 ± 0.02***	10.81 ± 0.01***
Liposomes (L)	887.2 ± 0.02	239.8 ± 0.02	0.270 ± 0.02***	4.79 ± 0.02***

Statistical analysis: ANOVA/Bonferroni: $P < 0.001$ (***)

retention in the skin (Y1) in the range of 60 to 402.67 $\mu g/cm^2$, drug permeation (Y2) at 6 h in the range of 29.0 to 86.20 $\mu g\ cm^{-2}\ h^{-1}$, entrapment efficiency (Y3) in the range of 56.13 to 70.80%, and vesicle size d (90) (Y4) in the range of 638 to 710.68 nm, respectively. Under this model, the predicted responses Y1, Y2, Y3, and Y4 in the required range at A and B had values of 392.73 $\mu g\ cm^{-2}$ (drug retention in the skin), 44.312 $\mu g\ cm^{-2}\ h^{-1}$ (drug permeation at 6 h), 68.434% (entrapment efficiency), and 655.73 nm (vesicle size), respectively. By employing the graphical optimization method, the best fit optimum formulation was selected which contained −0.8 (2.2%) SPL 70 level and +0.8 (36%) ethanol level. Figure 5 depicts the optimized formulation in the design space overlay plot in the demarcated optimal region. Optimized transethosomal formulation showed an entrapment efficiency of 71.2%. The vesicle size of the optimized elastic vesicle formulation was 646 nm with a PDI of 0.341.

Comparison of the experimental and predicted responses along with the percentage error is listed in Table VIII. The percentage error ranged between −9.77% and 9.84%, *i.e.*, well within ±10%. These data showed that most of the predicted values were close to the experimental values. These indicated the excellent prognostic ability of the experimental design employed for the optimization of transethosomal formulation of piroxicam.

Evaluation of the Optimized Batch

Transmission Electron Microscopy

The TEM image of optimized transethosomes, *i.e.*, elastic vesicular formulation (OP), is shown in Fig. 6. The results obtained from drug-loaded optimized transethosomal formulation (OP) showed the morphology of the vesicles. Vesicles were observed at ×30,000. The vesicles observed were spherical in shape. The TEM image also revealed the bilayer structure of transethosomes (8,9).

Degree of Deformability

The elasticity of vesicles is one of the important parameters as it differentiates them from conventional vesicles (28,39). Deformability index and elasticity were chosen to access the flexibility of vesicles. Table IX shows the comparison of vesicle size of optimized transethosomes (OP), transfersomes (T), ethosomes (E), and liposomes (L) before and after extrusion. The deformability index in Table IX of optimized formulation was near 1 which showed that vesicles regained their size after extrusion while liposomes have a deformability index of 0.270, which showed that they are inelastic. The liposomes got ruptured during passage through the polycarbonate membrane. The deformability index of ethosomes was more than that of liposomes as these are also a type of flexible vesicles but it was lesser than that of optimized transethosomes. ANOVA/Bonferroni test was used to compare transethosomes, liposomes, transfersomes, and ethosomes. The deformability index was found significantly higher ($P < 0.0001$) for transethosomes as compared to liposomes, transfersomes, and ethosomes (5,9). Conventional liposomes showed the least elasticity, while the optimized transethosomes showed maximum elasticity as these were passed through a pore size that was 12.73 times smaller than that of their size. Thus, the obtained results clearly demonstrated the higher deformability of transethosomes containing both Span 80 as edge activator and ethanol.

Entrapment Efficiency, Drug Retention in the Skin, and Drug Permeation Study

Studies on entrapment efficiency, drug retention in the skin, and drug permeation were carried out for the optimized batch of transethosomes, liposomes, transfersomes, and ethosomes. The results are shown in Table X. ANOVA/Bonferroni test was applied to compare the results of entrapment efficiency (EE), drug retention in the skin, and drug permeation study, respectively, for all the aforementioned

Table X. Entrapment Efficiency (EE), Skin Retention, and Drug Permeation Study of Optimized Transethosomes, Transfersomes, Ethosomes, and Liposomes

Formulation	Entrapment efficiency (%) ($n=3$)	Skin retention ($\mu g\ cm^{-2}$) ($n=3$)	Drug permeation at 6 h ($\mu g\ cm^{-2}\ h^{-1}$) ($n=3$)
Optimized transethosomes (OP)	71.2 ± 0.02***	357 ± 0.02***	47.2 ± 0.02***
Transfersomes	68.8 ± 0.02***	128.2 ± 0.01***	35 ± 0.02***
Ethosomes	65 ± 0.02***	150 ± 0.02***	41 ± 0.02***
Liposomes	62 ± 0.01***	70 ± 0.01***	16.4 ± 0.02***

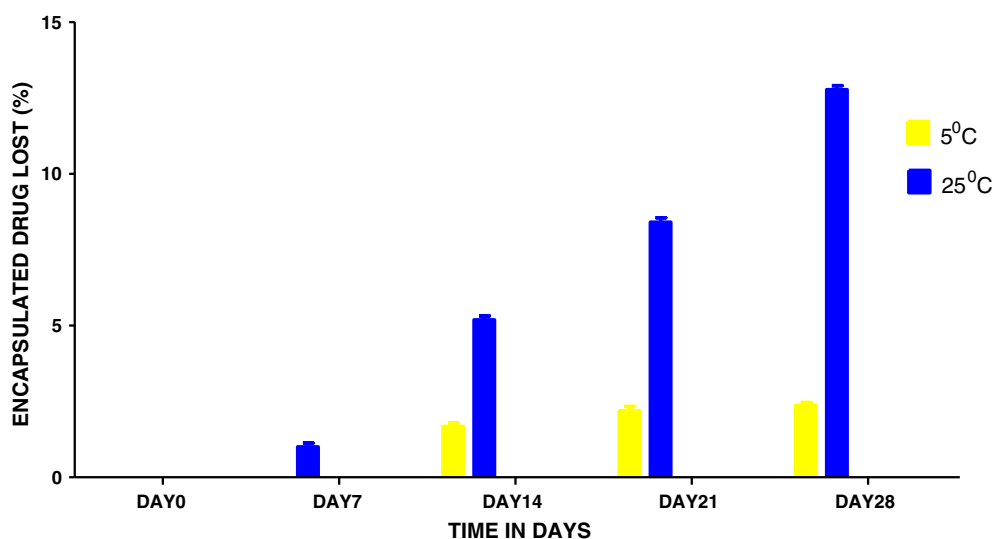Statistical analysis: ANOVA/Bonferroni: $P < 0.001$ (***)

Fig. 7. Bar diagram depicting the stability study of transethosomes at different temperatures

formulations. It was observed from the results that all the three parameters were found to be significantly higher ($P < 0.0001$) for transethosomes as compared to other vesicular formulations. The least response for all the three responses was found for liposomes. Hence, the results revealed the superiority of the developed elastic vesicles over other conventional vesicular delivery systems.

The increasing order for entrapment efficiency was as follows: optimized transethosomes > transfersomes > ethosomes > liposomes.
The increasing order for drug retention in the skin and drug permeation at 6 h was as follows: optimized transethosomes > transfersomes > ethosomes > liposomes.

Stability Studies

Figure 7 showed the stability data of optimized transethosomes. A negligible loss (2.37%) of entrapped drug was observed at $5 \pm 3°C$ after 28 days of storage. However, at $25 \pm 2°C$, comparatively more loss of the drug (12.78%) was observed. Thus, the results showed the instability of vesicles at room temperature. This could be due to the loss of rigidity of the vesicles and/or inability of the vesicles to retain the drug. The increase in fluidity (loss of rigidity) at a higher temperature may be due to phase transition which is generally responsible for drug loss. Hence, it is recommended that the developed transethosomes should be kept at refrigerated

conditions ($5 \pm 3°C$) to avoid drug loss and any other instability problems (5,22).

Characterization of the Vesicular Gel

Texture of Gel Formulation

OTG, MG, TG, EG, and conventional LG were characterized for texture analysis. The parameters studied were firmness, consistency, stickiness, and work of adhesion. The results are shown in Table XI. The firmness of OTG, EG, TG, LG, and MG was found to be 0.046, 0.065, 0.094, and 0.202 kg, respectively. Consistency of OTG, EG, TG, LG, and MG was found to be 0.031, 0.046, 0.080, 0.196, and 0.049 kg s, respectively.

In order to calculate maximum negative force, the stickiness of the gel was considered. The stickiness of OTG, EG, TG, LG, and MG was −0.035, −0.046, −0.067, −0.138, and −0.034 kg, respectively. A negligible difference in stickiness was observed between OTG and MG. However, differences were high in case of EG, TG, and LG as compared to OTG. Work of adhesion was calculated from the area of the negative region of the peak. OTG, EG, TG, LG, and MG had work of adhesion −0.016, −0.023, −0.032, −0.074, and −0.020 kg s, respectively. Thus, the above results revealed that there was negligible difference in the various rheological and texture characteristics of OTG and MG.

Table XI. Texture Parameters of Various Gel Formulations

Formulation code	Firmness (kg) ($n = 3$)	Consistency (kg s) ($n = 3$)	Stickiness (kg) ($n = 3$)	Work of adhesion (kg s) ($n = 3$)
OTG	0.046 ± 0.001	0.031 ± 0.002	-0.035 ± 0.001	-0.016 ± 0.001
EG	0.065 ± 0.001	0.046 ± 0.001	-0.046 ± 0.002	-0.023 ± 0.0012
TG	0.094 ± 0.0023	0.080 ± 0.003	-0.067 ± 0.003	-0.032 ± 0.002
LG	0.202 ± 0.0031	0.196 ± 0.011	-0.138 ± 0.002	-0.074 ± 0.002
MG	0.051 ± 0.001	0.049 ± 0.002	-0.034 ± 0.002	-0.020 ± 0.002

OTG optimized transethosomal gel, *TG* transfersomal gel, *EG* ethosomal gel, *LG* conventional liposomal gel, *MG* marketed gel formulation
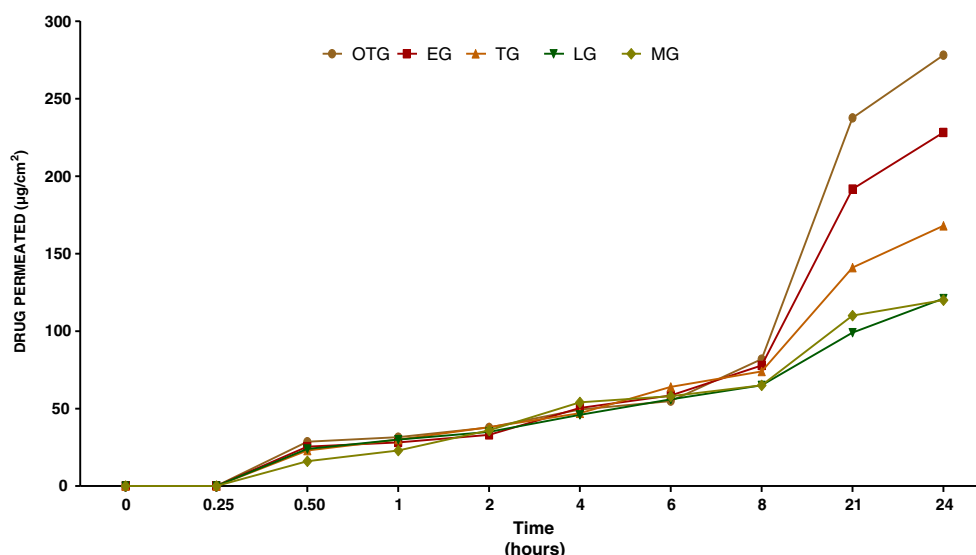
Fig. 8. Plot between permeation rate ($\mu g\ cm^{-2}\ h^{-1}$) of piroxicam permeated from various formulations *versus* time

Ex Vivo Drug Permeation Studies of Gel Formulation

The results of the drug permeation study of OTG, TG, EG, LG, and MG are shown in Fig. 8. From the data, it has been revealed that optimized transethosomal formulation showed the highest drug permeation through porcine skin as compared to other gel formulations. It was observed that permeation of transfersomal gel was less than that of ethosomal gel formulation. There was no significant difference found between the liposomal formulation and the marketed formulation. The highest permeation was observed with OTG formulation. This may be due to the combined effect of fluidization due to ethanol and the destabilizing effect of Span 80. Prolonged drug release of OTG may be attributed to the slow release of drugs from the vesicles. Permeation of all the formulations was found to be several times more than that of piroxicam gel formulations which is reported in the literature (40,41).

Analysis of the Release Mechanism by the Kinetic Model

The release kinetics of OTG, EG, TG, LG, and MG was studied for different kinetic equations (zero order, first order, Hixson Crowell, and Higuchi model). The best fit with a higher correlation coefficient (r^2) was chosen as the kinetic

model and given in Table XII. The results revealed that zero-order kinetics was the best fit model for OTG, EG, TG, LG, and MG formulation. Zero-order release provides a constant release of the drug and is suitable for topical drug delivery systems.

Drug Retention in Skin Studies

Figure 9 depicts the results of drug retention in the skin. In this study, comparatively higher retention of the drug was observed within the skin layers in the case of OTG as compared to other gel formulations. This is one of the reasons for the depot forming effect of the vesicular system. The observation of high drug retention with OTG formulation may be accounted for the characteristic flexibility of the vesicular membranes which helps in moving the drug across the tough horny layer of the skin while keeping it trapped in the dermal layers (33,35).

CONCLUSIONS

In the present study, transethosomes containing lipid, edge activator, and ethanol were formulated and evaluated for their drug retention in the skin, drug permeation at 6 h, entrapment efficiency, and vesicle size. CCD was used to

Table XII. Various Kinetic Models of Optimized Transethosomal (OTG), Transfersomal (TG), Ethosomal (EG), Conventional Liposomal (LG), and Marketed Gel (MG) Formulations

Formulation	Zero order		First order		Higuchi model		Hixson Crowell model	
	K	r^2	K	r^2	K	r^2	K	r^2
OTG	11.23	0.985	0.007	0.982	27.35	0.724	0.671	0.884
EG	8.770	0.990	0.005	0.989	22.81	0.777	0.636	0.893
TG	6.125	0.973	0.002	0.969	17.39	0.872	0.580	0.872
LG	4.049	0.919	0.002	0.908	12.61	0.937	0.510	0.823
MG	4.346	0.917	0.002	0.913	13.45	0.941	0.538	0.863

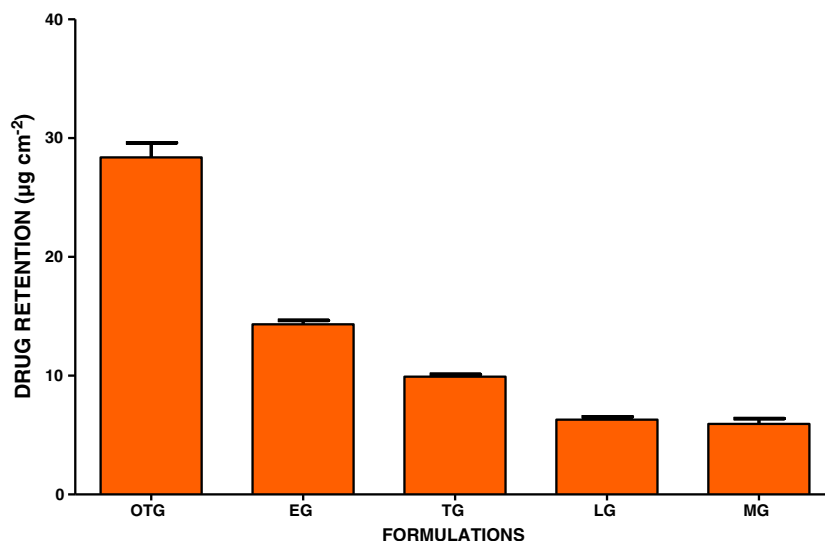K is the slope except in first order where $K = \text{slope} \times 2.303$

Fig. 9. Bar diagram showing drug retention (μg cm^{-2}) of piroxicam of various formulations

optimize the ratio of SPL 70 and ethanol. The developed transethosomal formulation was found to be more effective over the conventional vesicular delivery systems (*i.e.*, liposomes, ethosomes, and transfersomes) in terms of drug retention in the skin, degree of deformability, entrapment efficiency, and drug permeation at 6 h, respectively. The results of the stability studies revealed that the developed formulation was stable at refrigerated conditions ($5 \pm 3°$C) for 28 days. The optimized formulation was converted into gel and was evaluated for texture analysis, drug permeation, and drug retention in the skin. It was observed that the texture of developed OTG was found similar to MG formulation. The highest drug permeation, prolonged drug release, and highest drug retention in the skin were observed with OTG. Hence, it can be concluded that the developed transethosome was found superior over other vesicular delivery systems and has been successfully applied as a carrier to topical delivery in the form of gel.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest The authors declare that they have no competing interests.

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