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Formulation and Characterization of Flexible Phosphatydilcholine Vesicles for Systemic Delivery of Piroxicam

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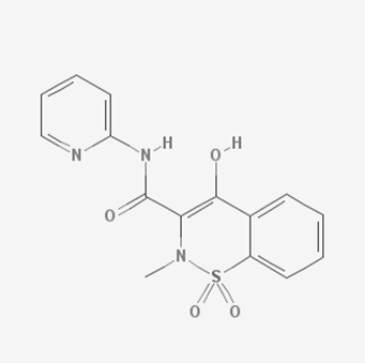
Abstract

The purpose of this study was to formulate and optimise the piroxicam loaded transfersomes for the bioavailability enhancement. Transfersomes had been prepared by rotary evaporation method and characterized for various parameters including determination of vesicle size, shape and size distribution, drug entrapment studies and *in vitro* skin permeation and histopathological studies. On the best drug entrapment efficiency and permeation studies through wistar rat skin was found for the formulation of TF PIROX4 and found to be 91.4 and 73.16. Histopathological examination and skin irritation studies had been done which showed 0.26 value for transfersomal gel, which means all the excipients used in this formulation were safe for transdermal drug delivery system. Further pharmacodynamic studies had been performed in wistar rats and compared with conventional oral capsule, conventional piroxicam gel and transfersomal gel. The result showed much higher therapeutic effect of piroxicamtransfersomal formulation as compared to the conventional formlations available in market and other Novel drug delivery systems designed for Piroxicam. Finally the stability studies for piroxicamtransfersomal gel had been carried out.

Key words: Transfersomes,Piroxicam,Phosphotedylcholine, Sodium cholate, Osteoarthritis, transdermal delivery.

INTRODUCTION:

About 74% of drugs are administered orally but they are not giving the desired effect always.To overcome the disadvantages of oral drug administration the transdermal drug administration has been used (1). poor permeability characteristics of the skin is one of the very important points mentioned in researches which lead to aim of increasing dermal and systemic drug delivery in a reproducible and reliable way(2). There are various advantages in dermal drug delivery, as instant, convenience, patient compliance and elimination of the first-pass effect(3) and it also provides the controlled delivery of the drug for extended period of time(4).The greatest problem for transdermal delivery is the barrier property of the stratum corneum (SC)(5,6). Piroxicam is one of the most potent non-steroidal anti-inflammatory drugs which used in treatment of the symptoms of rheumatoid and osteoarthritis, primary dysmenorrhoea, postoperative pain; and act as an analgesic, especially where there is an inflammatory component. It was first developed by Pfizer and Co. Piroxicam entered into medical practise in 1970's.It is an effective analgesic and anti-inflammatory agent in treatment of rheumatoid arthritis, osteoarthritis, ankylosing spondilitis and acute pain in musculoskeletal disorders and in acute gout(7). If piroxicam administer trough transdermal route it can overcome it`s adverse effects and more concentrations, this is the desirable factor for an anti-inflammatory agent(8,9,10). Osteoarthritis (OA) is one of the most common
and problematic chronic diseases which indicates it`s symptoms on elder stages. The concept of this disease is to involve the entire joint organ, including the subchondral bone, menisci, ligaments, periarticular muscle, capsule, and synovium(11).



MATERIALS AND METHODS

Materials

Piroxicam was received as a gift sample from ZydusCadila Pharmaceuticals (P) Ltd. Phosphatidylcholine was received from Lipoid, Germany. Monosodium iodoacetate and dextran were purchased from Sigma Aldrich Co. Carbapol 940, methanol and ethanol (Analytical grade) from SD. Fine Chem. Ltd., was purchased from S.D Fine chemicals, New Delhi. All other chemicals used were of analytical grade and were used as received. Double-distilled water was used for all experiments.

METHOD

Formulation of Transfersome

Preparation of vesicular formulation

Transfersomes were prepared by conventional rotary evaporation sonication method(12,13). The mixture of 15ml methanol- chloroform (1:1), phospholipids and surfactant were taken in a clean, dry round bottom flask and the drug was added to the above mixture. The organic solvent was removed by vacuum rotary evaporation above the lipid transition temperature. Last traces of solvent were

removed under vacuum overnight. The deposited lipid film was hydrated with drug solution in PBS (pH 7.4) by rotation at 100 rpm for 1 h at room temperature. This was given milky colloidal or suspension system. The LUV vesicular obtained is observed using microscope at 45x to have rough idea of formation of lipid bilayer The resulting vesicles were swollen at room temperature to get large multilamellar vesicles (LMLVs). To prepare smaller vesicles, LMLVs were probe sonicated at 4°C at 40W for specific range of time (10-30min). For optimization various amount of SPC and surfactant (sodium cholate) was taken and effect of sonication time was evaluated for optimum vesicle size, entrapment efficiency and amount of drug permeated from skin (flux). Table.1 shows composition of different developed formulations.

Preparation of transferosomal gel

On the basis of thermodynamic stability studies and physicochemical evaluation the optimized transfersome TF PIROX4 was selected to be formulated into gel. Piroxicamtransfersomal gel was prepared by dispersing the carbapol 940 in a sufficient quantity of distilled water. After complete dispersion, the 1% carbapol 940 solution was kept for 24h for complete swelling. Then other ingredients Triethanolamine was added to obtain a homogeneous dispersion of gel with desirable pH. The optimized transfersome was then mixed with the gel in 1:1 ratio with genle stirring.

Formulation of placebo transfersome

Formulation of placebo has been done by the same method without incorporating drug.

Evaluation of developed formulation

Determination of Vesicle size

Vesicle size of the transfersome was determined by photon correlation spectroscopy, which analyzes the fluctuations in light scattering due to Brownian motion of the particles, using a Zetasizer 1000 HS (Malvern Instruments, UK). Light scattering was monitored at 25ºC at a 90º angle. Vesicle size distributions are shown in Fig.1.(a)-Fig.1.(m). Vesicle sizes of different formulation are shown in Table.2.

Drug entrapment studies

Separation of unentrapped drug from the prepared transfersomes was carried out by centrifugation method(14). Transfersomal suspension centrifuged at 25000 RPM for 35 min and after that vesicles were disrupted using 0.1% Triton x 100 and then entrapped drug was analyzedspectrophotometrically (U.V/Visible spectrophotometer, Shimadzu) for the drug content estimation at λ_{max} of 254nm. The amount of entrapped drug expressed as % was calculated from the following equation:

$$\% \text{ Entrapment Efficiency} = \frac{\text{Entrapped drug (mg)}}{\text{Total drug (mg)}} \times 100$$

The result of % entrapment efficiency for various formulations is shown in Table.2.

Animal maintenance and care

The study was performed as the recommendations of the committee for the purpose of control and supervision of Experiments on Animals (CPCSEA) guidelines for Laboratory Animal Facility, The Gazette of India, 1998. Protocol for general procedures and use of animals for conducting this study has been reviewed and approved by the Institutional Animal Ethics committee, and source of animals was Central Animal House Facility, JamiaHamdard form no 858. Skin irritation test was performed using six female Wistar Albino rats, weighing 200-250gm. The animals were kept under standard laboratory conditions, at a temperature of 25±1°C and relative humidity of 55±5%. The animals were housed in polypropylene cages, six per cage, with free access to standard laboratory diet and water *ad libitum*.

***In vitro* skin permeation studies**

In vitro skin permeation studies were performed on a Franz diffusion cell. with an effective diffusion area of 4.5cm^2 and 10ml of receiver chamber capacity, using rat abdominal skin The full thickness of rat skin was excised from the abdominal region and hairs were removed with a hair removing cream. The subcutaneous tissue was removed surgically and the dermis side was wiped with isopropyl alcohol for removing adhering fat(15).The cleaned skin was washed with distilled water and stored at -21°C until further use. The skin was brought to room temperature and mounted between the donor and receiver compartments of the Franz diffusion cell where the stratum corneum side was facing the donor compartment and the dermal side was facing the receiver compartment. Initially, the donor compartment was empty and the receiver chamber was filled with ethanol and phosphate buffer (PB) pH 7.4 in the ratio (6:4%,*V/V*)

The receiver fluid was stirred with a magnetic rotor at a speed of 600 rpm and the assembled apparatus was connected to Marriote bottle from the inlet where the temperature of water was maintained at 37±1°C. The whole receiver fluid was replaced with fresh one after every 30 min to stabilize the skin. It was found that the receiver fluid showed a negligible absorbance after 2.5 h and beyond indicating complete stabilization of the skin. After complete stabilization of the skin, 1ml transfersome formulation (2mg/ ml Piroxicam) was placed into the donor compartment and sealed with paraffin film to provide occlusive conditions. Samples were withdrawn at regular intervals (1, 2, 3, 4, 5, 6, 7, 8, 10, 12 and 24 h), filtered through 0.45-mm membrane filter and analyzed for drug content by in house validated RP-HPLC method. The results are shown from Fig. 2- Fig.14 .

Data analysis

The cumulative amount of Piroxicam permeated through the skin (Q,µg/cm^2) was plotted as a function of time (h). The drug flux (permeation rate) at steady state (Js,µg/cm^2/h)was calculated from the slope of linear portion of the curve. The formula for calculating cumulative amount of drug permeated is shown below:

$$Cumulative amount of drug permeated = \frac{\text{Concentration } \left(\mu \frac{g}{ml}\right) \times \text{volume of diffusion cell} \times \text{Dilution factor}}{\text{Area (cm}^2\text{)}}$$

Flux = Slope of steady state portion of the plotbetween cumulative amount of drug permeated per cm^2 Vs time (µg/ml/cm^2/h)

$$Permeability coefficient\ (P_b) = \frac{\text{Flux}}{\text{Drug concentration in donor compartment } \left(\mu \frac{g}{ml}\right)}$$

Since the permeation study of drug loaded with 2 mg of drug as it is to be applied in the mounted skin in the donor

compartment therefore permeation study of transfersome was done with 2 mg of drug loaded transfersome.

Evaluation of the transfersomal gel(12)

Spreadability

Spreadability was measured on the basis of slip and drag character of gels. A modified apparatus consisting of two glass slides containing gel in between, with the lower slide fixed to a wooden plate and the upper one attached to a balance by a hook was used to determine spreadability, which was calculated using the following formula and the results are shown in Table.3.

$$S = M \times \frac{l}{t}$$

Where,

S= Spreadability in gcm/s

M= mass of gel placed between the two slides

l= length of slide

t= time taken by the upper slide to detach

Extrudability

It is a usual empirical test to measure the force required to extrude the material from tube. The method applied for determination of applied shear in the region of the rheogram corresponding to a shear rate exceeding the yield value and exhibiting consequent plug flow one such apparatus is described by *Wood et al*(16).

Procedure: In the present study, the method adopted for evaluating gel formulation for extrudability was based upon the quantity in percentage of gel and gel extruded from lacquered aluminium collapsible tube on application of weight in grams required to extrude at least 0.5cm ribbon of gel in 10 seconds. More quantity extruded better was extrudability. The measurement of extrudability of gel formulation was in triplicate and the average value was presented.

The extrudability was then calculated by using the following formula:

$$Eg = Wg/A$$

Where,

Eg is Extrudability,

Wg is applied weight to extrude gel from tube (in gm)

A is area (in cm^2).

Drug content determination

For determination of drug content, about 1gm of the gel was weighed in a 100ml volumetric flask and dissolved in methanol; it was diluted appropriately and analyzed at 254nm by UV spectrophotometry. Results are shown in Table.3.

Homogeneity

A small amount of transfersomal gel was pressed between the thumb and the index finger to check the consistency and homogeneity of transfersomal gel.

Viscosity

The viscosity of the formulations was determined using Brookfield DV III ultra V6.0 RV cone and plate rheometer (Brookfield Engineering laboratories, Inc., Middleboro, Ma) operated under the specifications as shown in Table.3.

Measurement of pH

The apparent pH of the gel was measured by pH meter (AccumentAB 15, Fisher scientific, USA) in triplicate at 25 ± 1^oC . Results are indicated in Table.3.

***In vitro* drug permeation of Piroxicam from transfersomal gel**

In vitro skin permeation studies were performed on a Franz diffusion cell with an effective diffusional area of 4.5cm^2 and 20ml of receiver chamber capacity, using rat abdominal skin. The full thickness of rat skin was excised from the abdominal region and hairs were removed with an hair removing cream.The subcutaneous tissue was removed surgically and the dermis side was wiped with isopropyl alcohol to remove adhering fat. The cleansed skin was washed with distilled water and stored at -21^oC until further use. The skin was brought to room temperature and mounted between the donor and receiver compartments of the Franz diffusion cell where the stratum corneum side was facing the donor compartment and the dermal side was facing the receiver compartment. Initially, the donor compartment was empty and the receiver chamber was filled with ethanol:phosphate buffer saline pH 7.4 (6:4v/v).The receiver fluid was stirred with a magnetic rotor at a speed of 600 rpm and the assembled apparatus was connected to Marriote bottle from the inlet where the temperature of water was maintained at 37±1^oC. The whole receiver fluid was replaced with fresh one after every 30min to stabilize the skin. It was found that the receiver fluid showed a negligible absorbance 2.5 h and beyond indicating complete stabilization of the skin, 1g Piroxicamtransferosomal gel was (2mg/g^{-1}) was placed into the donor compartment and sealed with paraffin film to provide occlusive conditions. Samples were withdrawn at regular intervals (1, 2, 3, 4, 5, 6, 7, 8, 10, 12 and 24 h), filtered through 0.45-mm membrane filter and analyzed for drug content by in house validated RP-HPLC method. The results are shown in Fig.16.

***In vitro* kinetic analysis of transfersomal gel**

From the skin permeation studies, percentage drug remaining was calculated using the cumulative percentage of drug permeated and the rate constant for zero order and first order for *in vitro* skin permeation studies were calculated after each time interval standard deviation (SD) and coefficient of variation (C_v) were also calcuted. The results are shown in Table 4-5.

Zero order rate constant (K_o)

$$K_{o=X/t=\frac{percentagedrugpermeated}{Time\,(h)}}$$

First order rate constant (K_1)

$$K_1 = \frac{2.303}{T}\log(\frac{C_o}{C})$$

Where,

Co=Initial drug concentration

C=Amount of drug remaining at time t.

C_v was calculated using the following formula:

$$C_v = \frac{SD}{Average} \times 100$$

Histopathological Examination(18)

Abdominal skins of wistar rats were treated with optimized Piroxicamtransfersome. After 24h, rats were sacrificed and the skin samples were taken from treated and untreated (control) area. Each specimen was stored in 10% formalin solution in methanolic PBS pH 7.4. The specimens were cut into section vertically. Each section was dehydrated

using ethanol, embedded in paraffin for fixing and stained with hematoxylin and eosin. These samples were then observed under optical microscope and compared with control sample. In each skin sample, three different sites (epidermis, dermis and subcutaneous fat layer) were scanned and evaluated for mechanism of skin permeation enhancement. The photomicrograph of control and treated skin sample is shown in Fig.19 and 20 respectively.

Skin Irritation Study

This test was carried out to confirm the skin compatibility of the formulation .A single dose of the formulation was applied to the left ear of the rat and the right was considered as a control. The development of erythema was monitored for seven days using the method as reported (19).

For carrying out DSC studies, transfersome and control (aqueous Piroxicam suspension) were applied separately to rat skin sections. After 24h of applicant, approximately 10mg of rat skin, treated with TF and control, was blotted on an inert paper and washed to remove the formulation sticking on the surface. Skin samples were freeze dried prior to the DSC measurement. DSC was recorded on Perkin Elmer. DSC instrument (Norwalk USA) with an intracooler (Model FC-60-PED, Perkin Elmer, USA) Samples were heated from 50 to 300^{o}C at a heating rate of 5^{o}C/min to obtain a satisfactory endotherm peak resolution with the minimum temperature lag effects.

DSC is a powerful technique that can detect the melting and transition of skin lipids upon interaction with certain vehicles and penetration enhances (20). The DSC thermogram of control treated and transfersome treated rat skin is shown in fig.21 and 22.

Dose calculation for the rats

Dose for the rats was calculated based on the body weight of the rats according to the surface area ratio(21). For 200gm rat, Piroxicam dose will be 0.36 mg i.e. 1.8 mg/kg weight.

Assessment of anti inflammatory activity

Inflammation will be induced in rats as per method reported by Tamura et al 2002.

Induction of acute paw edema in rats (22)

Young Wistar rats, weighing 200-250gm were randomly devided into 3 groups: control, treatment group with conventional gel. The animals were kept under standard laboratory conditions, at temperature of 25 ± 1^{o}C and relative humidity of 55 ± 5%. The animals were housed in polypropylene cages, six per cage, with free standard laboratory diet and water *ad libitum*. Dose for the rats was calculated based on the weight of the rats according to the surface area ratio. The abdominal region of the rats was shaved 12h before starting the experiments except in control group. Transfersomal gel was applied on the shaved abdominal region of all animals (except in control group) half an hour before subplanter injection of Piroxicam in right paw. Paw edema was induced by injecting 0.1ml of 1% v/v homogenous suspension of Dextran in distilled water. The volume of paw was measured at 1, 2, 3, 6, 12 and 24h after injection using pletesmometer. The amount of paw swelling was determined time to time and expressed as percent edema relative to the initial hind paw volume. Percent inhibition of edema produced by each formulation-treated group was calculated against the respective control group using the equation:

$$\%Inhibition = \frac{\%Edema(control) - \%Edema\ (formulation)}{\%Edema\ (control)} \times 100$$

Assessment of osteoarthritis in rats

Osteoarthritis will be induced in albino wistar rats as per method of Moore et al, 1998 (23). **Procurement, groupings and hosting of animals**

30 Wistar rats (15 male, 15 female rats) of weight 200-250g, supplied by the Central Animal Housing Facility of JamiaHamdard were used. The animals were kept under standard laboratory conditions of 12 hours light/dark cycle at 28±2^{o}C. Animals were provided with pellet diet and water *ad libitum*. They were mark with picric acid solution and were grouped into 5 groups such that each group had six animals. The study was approved by the Institutional Animal Ethics Committee (Form no.858).

Comparison between piroxicamtransfersomal gel and prepared conventional gel sample by using Dextran induced rat paw edema model.

Results of Anti-inflammatory activity were compaired using Dunnett test of one-way analysis of variance (ANOVA) and are shown in Table 6. shows the efficacy of piroxicamtransfersomal gel when compared with prepared conventional gel.

Induction and treatment of osteoarthritis

The Group I which served as control received no treatment during the whole study. Group II served as toxic control group, Group III (Piroxicam loaded transfersome), Group IV (marketed Piroxicam gel formulation), Group V (oral Piroxicam suspension) served as treatment groups. Injection of mono-iodoacetate (MIA), an inhibitor of glycolysis, into the femro-tibial joint of rats promotes loss of articular cartilage similar to that noted in human OA. Arthritis was induced by a single intraarticular injection of iodoacetate into the knee joint of rats anesthetized using light ether anaesthesia. A 10mg/ml concentration of monosodiumiodoacetate (MIA) was prepared using injectable saline as the vehicle. After appropriate anaesthesia each rat was positioned on their back and the left leg was flexed 90^{o} at the knee. The patellar ligament was palpated below the patella and the injection was made into this region. Each rat received 0.025ml intraarticular injection into the left knee using a glass gas tight syringe with a 27 gauge 0.5 inch needle except that in the control group. Care was taken not to advance the needle too far into the cruciate ligaments.

Albino Wistar rats were dosed with transfersomal gel formulation at 24h intervals (O.D) for the first 7 days after iodoacetae injection. Accordingly a 7-day dosing protocol was reported. Control, Toxic control and Treatment groups consisted of 6 rats each. Rats were sacrificed 7 days after iodoacetate injection and the left joint was immediately disarticulated and fixed in 10% buffered formulation for24-48h prior to capturing the image. The images of the slide prepared from control, toxic control and treatment group were taken and are shown in Fig. 22 to 27.

Stability Study

Transfersomal gel was stored at different temperature for 3 months. Then the consistency and concentration of Piroxicam were investigated. For the estimation of drug content UV spectroscopic method was used.

Stability studies as per ICH guidelines

Three packs of transfersomal gel were subjected to these studies. The samples were subjected to 40±2°C and 75±5% RH. At the end of 0,30,60,90 days, samples were withdrawn, diluted with methanol and analysed using UV method (Table 27). A graph was plotted between log percent drug remaining Vs time (Fig.28).The slope of the straight line from the graph was determined and degradation rate constant (K) was calculated by using the equation:

Slope = -K/2.303

Accelerated stability studies according to WHO for the determination of shelf life

Replicas of formulation (C3) were prepared and packed in vials lamitube. The lamitube were stored at 30±0.5°C, 40±0.5°C and 50±0.5°C for 90 days. Samples were withdrawn at intervals of 0,30,60,90 days, diluted with methanol and analysed using UV . The logarithmic percent of drug remaining in the formulation was plotted against time (in days) (Fig.29). Slopes of the straight lines for each temperature were obtained and degradation rate constants (K) were calculated using the following equation:

Slope = -K/2.303

Where, K is degradation rate constant.

The effect of temperature on degradation was studied by plotting log K vs 1/T (Arrhenius plot) (Fig.30).

Shelf life was calculated by substituting K_{25} in the equation.

$t_{0.9} = 0.1054/K_{25}$

Where, $t_{0.9}$ is the time required for 10% degradation of the drug and is referred to as shelf life.

RESULTS:

Various formulation of transfersomes have been developed using dry film hydration method and effect of SPC to surfactant ratio along with the effect of sonication time has been studied. It was obvious from the experiment that, increase in the amount of surfactant to SPC ratio resulted in increased entrapment efficiency of the formulation to a certain ratio. It was seen that the highest entrapment efficiency of 91.4 was obtained for the formulation 15:85 (4).

It was observed that with increase in sonication time, the vesicle size and entrapment efficiency was reduced. By observing the results the formulation (6), with 10 min sonication time has the largest vesicle size in between other formulas, which was 253.04.The smallest vesicle size is for formulation (2) with a sonication time of 30 min and the particle size observed is 74.35. Increase in the sonication time also reduces entrapment efficiency value slightly which can be explained by exaggerated treatment of vesicle for a longer period of time. It was found that the optimum sonication time for better entrapment is 20 minutes.

The optimum quantity of phosphalipid was found to be 85mg , most optimum quantity of sodium cholate was found to be 25mg and the best results obtained was with sonication time of 20min. Increase or decrease in the any of the above parameters will lead to reduction in the values of vesicle size and entrapment efficiency respectively. The best results were observed in the 4th formulation which was named as TF PIROX4, in which vesicle size was found to be 91.4nm and entrapment efficiency was found to be 100.18%.

On the basis of permeation studies, it was found that the formulation TF PIROX4 exhibited highest permeation profile. The cumulative amount of Piroxicam permeated from TF PIROX4 was 84.42% at the end of 24h and skin permeation rate (flux) of Piroxicam was 73.16µg/cm2/h while all the other transfersomes exhibited lesser percentage of drug permeation and flux.

As the lipid phase reach an optimum concentration i.e. 85mg the permeation increased significantly with formulations.

Thus, it was found that after 85mg lipid and 25mg surfactant mixture the drug permeation continued to decrease. Hence, transfersome TF PIROX4 was selected as the optimized formulation and was subjected to further studies.

The vesicle size distribution of transfersome (TF PIROX4) was similar to colloidal dispersions, with an average vesicle size of 100.18 as shown in Fig.1(d). A value of polydispersity index (PDI), which is a measure of uniformity of vesicle size within the formulation was estimated. The transfersome formulation exhibited a narrow size distribution and was found to be 0.0204. The Morphology and structure of the transfersome (TF PIROX4) vesicle was found to be spherical as shown in Fig.15. The result of transmission electron microscopy (TEM) measurements was in close agreement with the vesicle size measured by dynamic light scattering (DLS).

The spreadability of the transfersomal gel was found to be 7.2 g cm/s.

The values of spreadability 7.2g cm/s indicate that the gel was easily spreadable by small amount of shear and possessed acceptable bioadhesion. The extrudability of the transfersomal gel was found to be 9.86±0.020. Drug content was found to be 2.35 µg/ml. Viscosity of the transfersomal gel was found to be 511±0.16 cps. pH of the formulation was found to be 6.5±0.162. The value for flux for optimized transfersomal gel was found to be 73.13µg/cm²/h and permeability coefficient was found to be 3.66.

From the in vitro skin permeation studies study it was found that transfersome formulation TF PIROX4 exhibited satisfactory results as the cumulative amount of drug permeated from other formulations was comparatively lesser than TF PIROX4. To compare the skin permeation of Piroxicam marketed gel formulaion (0.05%w/w) with transfersome TF PIROX4, in vitro skin permeation was done. It was seen that compared to marketed gel formulation there was more than14 times increase in flux from transfersomal gel.

Table.1. Composition of different formulations

S.NO.	Formulation code	SPC (mg)	Surfactant (mg)	Drug (mg)	Sonication Time (min)
1	TF PIROX1	75	25	20	20
2	TF PIROX2	85	25	20	30
3	TF PIROX3	75	15	20	30
4	TF PIROX4	85	15	20	20
5	TF PIROX5	95	25	20	20
6	TF PIROX6	85	5	20	10
7	TF PIROX7	95	5	20	20
8	TF PIROX8	95	15	20	30
9	TF PIROX9	85	5	20	30
10	TF PIROX10	95	15	20	10
11	TF PIROX11	75	5	20	20
12	TF PIROX12	75	15	20	10
13	TF PIROX13	85	25	20	10

Table.2. Vesicle size and entrapment efficiency of developed formulations.

S.NO.	Name of Formulation	Vesicle size (nm)	Entrapment Efficiency(%)
1	TF PIROX1	76.47	74.3
2	TF PIROX2	94.72	73.8
3	TF PIROX3	74.35	70.2
4	TF PIROX4	100.18	91.4
5	TF PIROX5	109.75	62.1
6	TF PIROX6	253.04	70.2
7	TF PIROX7	106.82	65.8
8	TF PIROX8	75.84	65.6
9	TF PIROX9	81.79	63.9
10	TF PIROX10	234.63	61.1
11	TF PIROX11	76.38	59.8
12	TF PIROX12	154.67	73.9
13	TF PIROX13	175.81	72.5

TF is abbreviation for transfersome.
PIROX is abbreviation for piroxicam.

Table .3.Evaluation of transfersomal gel

Piroxicam Transfersome	Mean Spreadability (gcm/s)	Drug content (µg)	Mean Viscosity (cps)	Mean pH	Mean Extrudability
	7.2±0.413	2.35	511±0.16	6.5±0.162	9.86±0.020

Table.4. Kinetic profile of *in vitro* skin permeation data of Piroxicam transfersomal gel

S.NO.	Time (h)	Cumulative % drug permeated	% of drug remaining	Zero order rate constant	First order rate constant
1	1	3.71	96.29	3.71	0.03781
2	2	6.60	93.4	3.30	0.03415
3	3	8.69	91.31	2.896	0.03031
4	4	12.79	87.21	3.197	0.03422
5	5	15.35	84.65	3.07	0.03334
6	6	19.42	80.58	2.34	0.03599
7	7	23.78	76.22	3.397	0.038799
8	8	25.68	74.32	3.21	0.03711
9	10	34.74	65.26	3.47	0.04269
10	12	50.61	49.39	4.22	0.058795
11	24	84.42	15.58	3.52	0.07748
Average				3.302727	0.041881
SD				0.4751	0.01401

Table.5. Kinetic profile of *in vitro* skin permeation data of Piroxicam conventional gel

S.NO.	Time (h)	Cumulative % drug permeated	% of drug remaining	Zero order rate constant (K_o)	First order rate constant(K_1)
1	1	1.0	99	1	0.010052
2	2	4.12	95.88	2.06	0.0210401
3	3	5.20	94.8	1.7333	0.017803
4	4	6.14	93.86	1.535	0.015844
5	5	6.43	93.57	1.286	0.0142802
6	6	6.89	93.11	1.1483	0.011900
7	7	7.11	92.89	1.0157	0.0105382
8	8	7.57	92.43	0.9463	0.0101395
9	10	7.79	92.21	0.779	0.008111
10	12	8.15	91.85	0.6791	0.00708572
11	24	8.96	91.04	0.3733	0.0039120
			Average	1.141454	0.0118823
			SD	0.4877	0.004993

Table.22. Grading scale for skin irritation

0	No apparent cutaneous involvement
1	Faint but definite erythema
2	Moderate erythema
3	Severe erythema

Table.23. Skin irritation score

SI.NO.	Rat number	Score after (days)							Mean Score
		1	2	3	4	5	6	7	
1	1	0	0	1	0	1	0	1	0.43
2	2	0	1	0	0	0	0	1	0.29
3	3	0	0	0	1	0	0	0	0.14
4	4	0	1	0	0	0	1	0	0.29
5	5	0	0	0	0	1	0	0	0.14
6	6	1	0	0	0	0	1	0	0.29

Table.6. Anti-inflammatory effect of Piroxicam transfersomal gel in Dextran induced rat paw edema model.

Group	Formulation	NO.	Av. Group Wt (g)	Time (h)	Mean % Edema ± S.D.	% Inhibition
I	Control (Piroxicam only)	6	225g	1	51.2±1.2	
				2	62.5±1.7	
				3	71.6±1.1	
				6	33.2±1.8	
				12	26.3±1.4	
				24	12.7±1.6	
II	Transfersomal gel	6	250g	1	39.6	22.7
				2	45.2	27.7
				3	50.4	29.6
				6	20.8	37.3
				12	15.7	40.3
				24	5.1	59.8
III	Prepared Conventional gel	6	230	1	49.8	2.7
				2	59.2	5.2
				3	66.9	6.6
				6	31.1	6.3
				12	23.7	9.9
				24	9.8	22.8

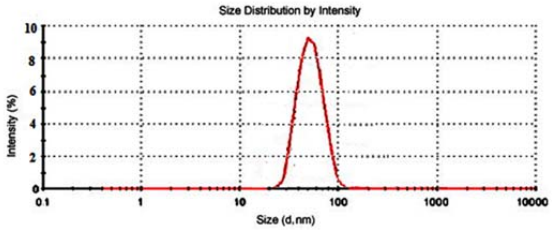


Fig.1. (a). Vesicle size and it`s distribution of formulation TF PIROX1 = 76.47 nm.

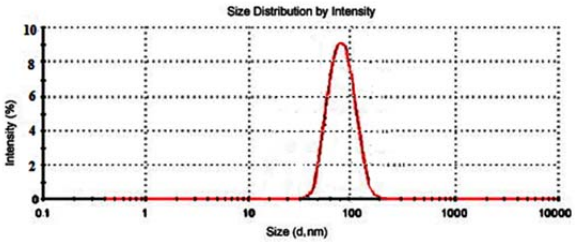


Fig.1.(b). Vesicle size and it`s distribution of formulation TF PIROX2 = 94.72 nm.

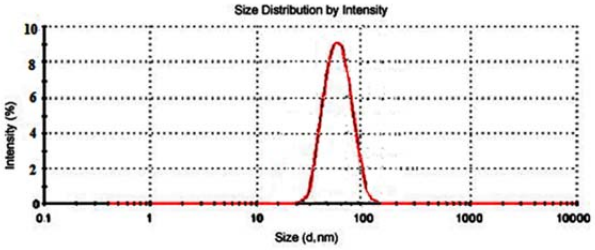


Fig.1.(c) Vesicle size and it`s distribution of formulation TF PIROX3 = 74.35 nm.

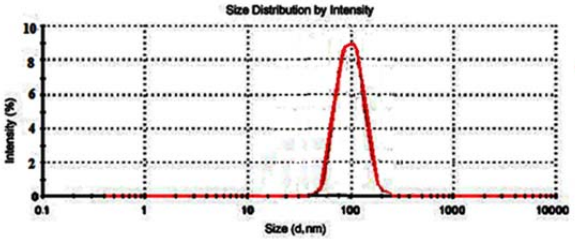


Fig.1.(d). Vesicle size and it`s distribution of formulation TF PIROX4 = 100.18 nm.

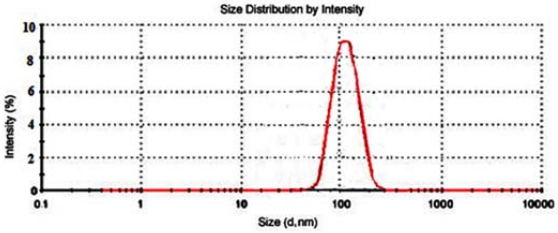


Fig.1.(e) Vesicle size and it`s distribution of formulation TF PIROX5 = 109.75 nm.

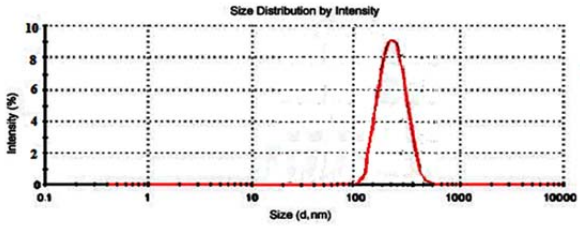


Fig.1.(f). Vesicle size and it`s distribution of formulation TF PIROX6 = 253.04 nm.

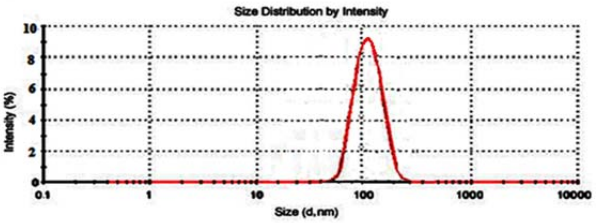


Fig.1.(g) Vesicle size and it`s distribution of formulation TF PIROX 7 = 106.82 nm.

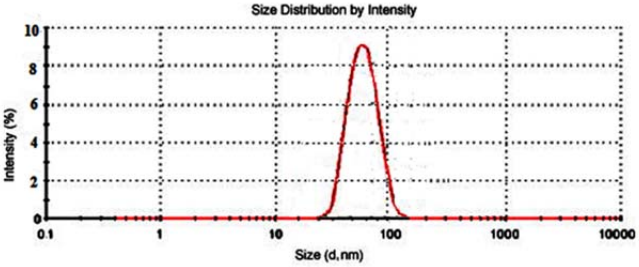


Fig.1.(h). Vesicle size and it`s distribution of formulation TF PIROX8 = 75.84 nm.

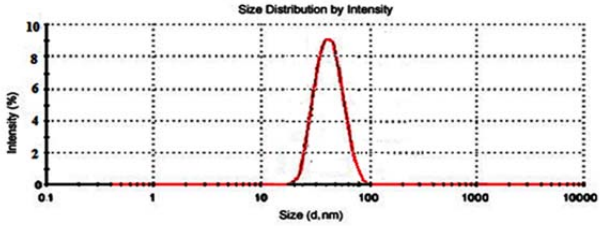


Fig.1.(i) Vesicle size and it`s distribution of formulation TF PIROX9 = 81.79 nm.

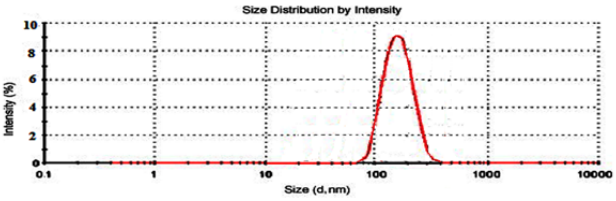


Fig.1.(j)Vesicle size and it`s distribution of formulation TF PIROX10 = 234.63 nm.

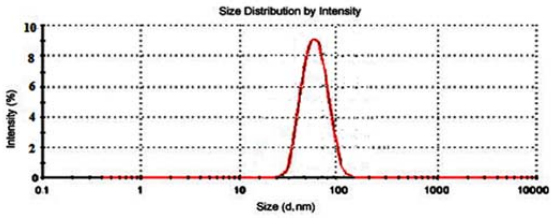


Fig.1.(k). Vesicle size and it`s distribution of formulation TF PIROX11 = 76.38 nm.

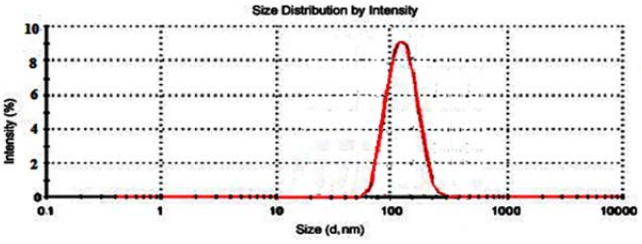


Fig.1.(l) Vesicle size and it`s distribution of formulation TF PIROX12 = 154.67 nm.

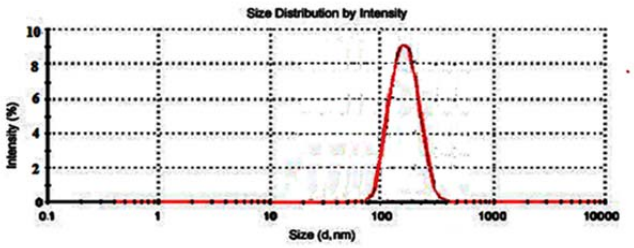


Fig.1.(m) Vesicle size and it`s distribution of formulation TF PIROX13 = 175.81 nm.

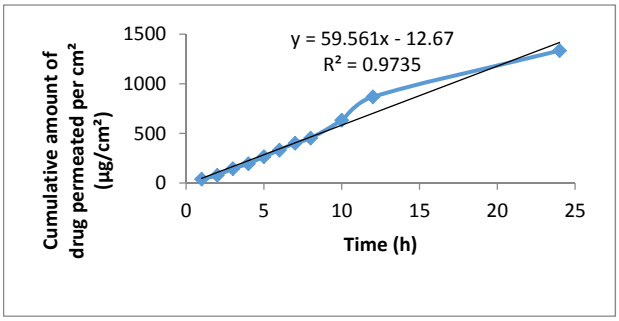


Fig.2. *In vitro* skin permeation of Piroxicam from transfersome TF PIROX1

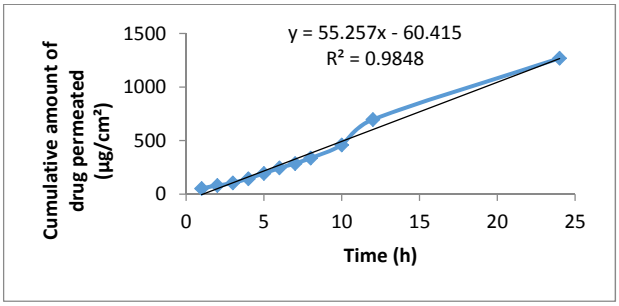


Fig.3. *In vitro* skin permeation of Piroxicam from transfersome TF PIROX2

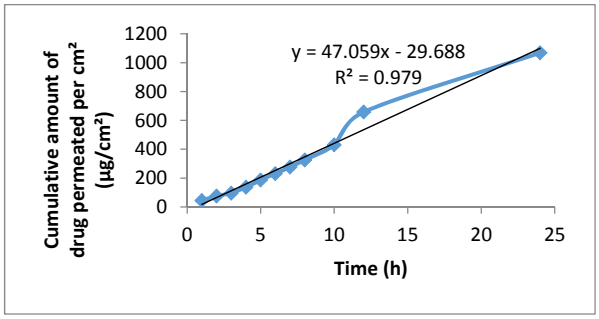


Fig.4. *In vitro* permeation of Piroxicam from transfersome TF PIROX3

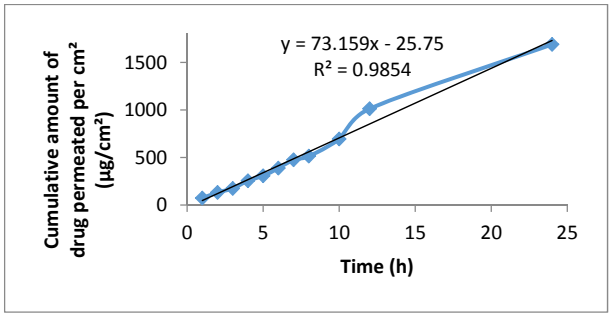


Fig.5. *In vitro* skin permeation of Piroxicam from transfersome (TF PIROX4)

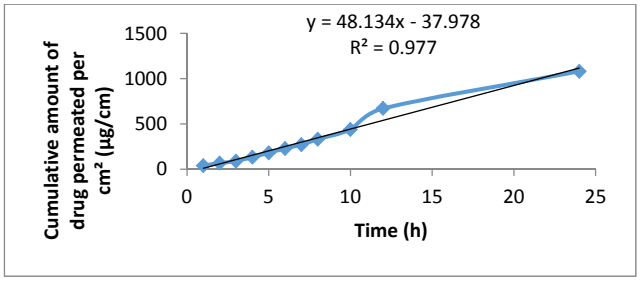


Fig.6. *In vitro* skin permeation of Piroxicam from transfersome TF PIROX5

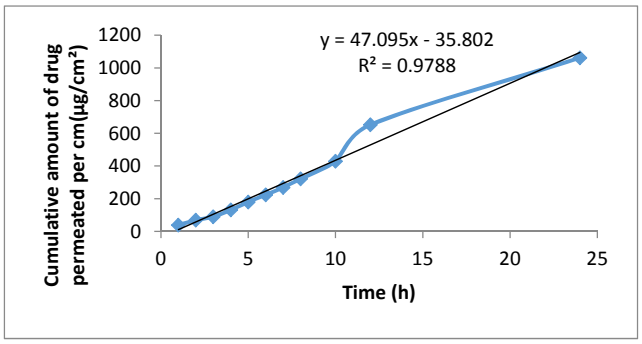


Fig.7.*In vitro* skin permeation of Piroxicam from transfersome TF PIROX6

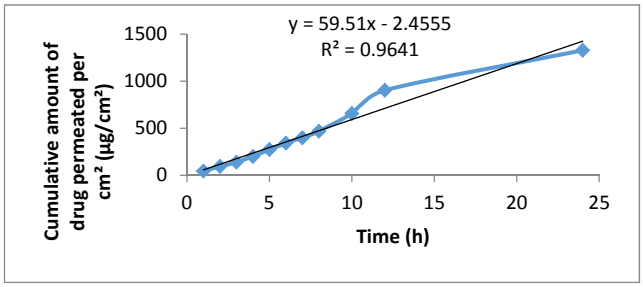


Fig.8. *In vitro* skin permeation of Piroxicam from transfersome TF PIROX7

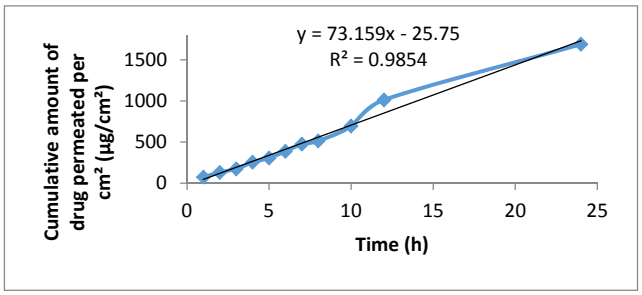


Fig.9. *In vitro* permeation of Piroxicam from transfersome TF PIROX8

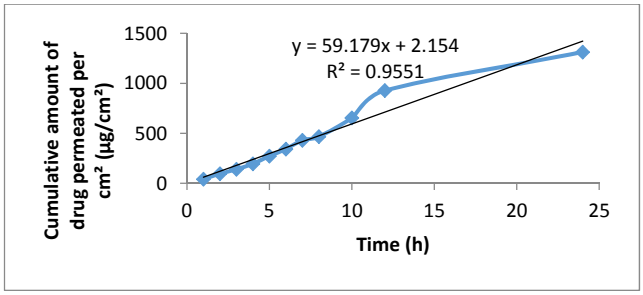


Fig.10. *In vitro* permeation of Piroxicam from transfersome TF PIROX9

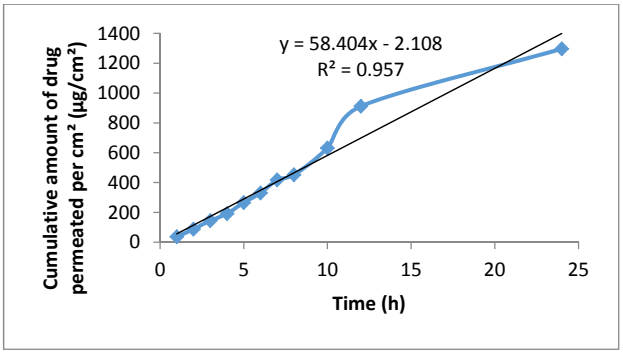


Fig.11. *In vitro* permeation of Piroxicam from transfersome TF PIROX10

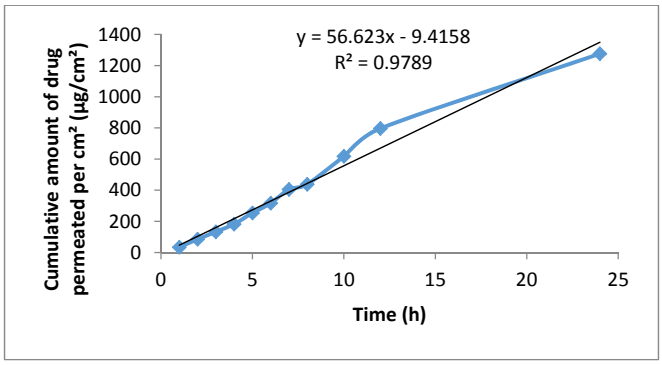


Fig.12. *In vitro* skin permeation of Piroxicam from transfersome TF PIROX11

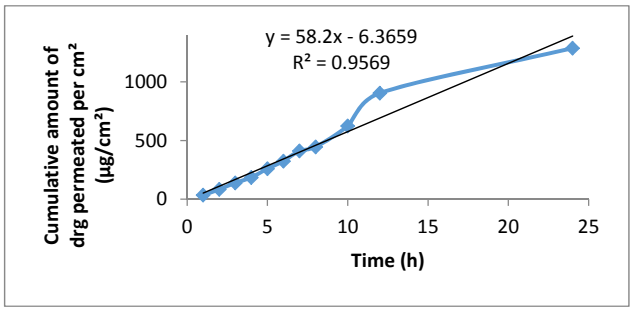


Fig.13. *In vitro* permeation of Piroxicam from transfersome TF PIROX12

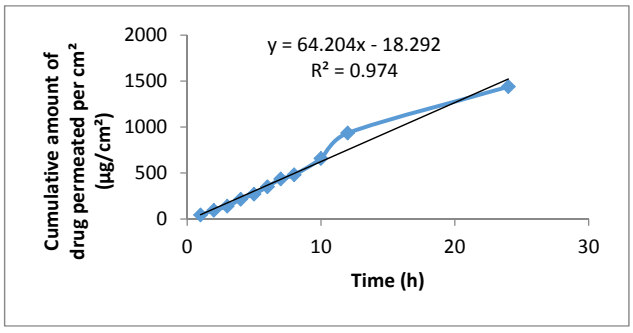
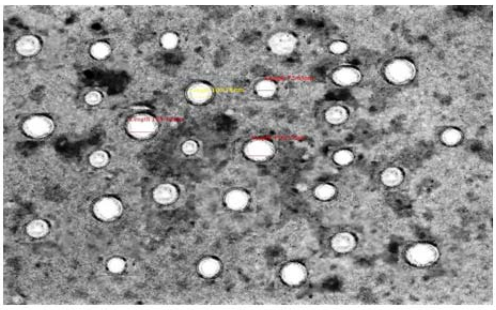


Fig.14. *In vitro* skin permeation of Piroxicam from transfersome TF PIROX13



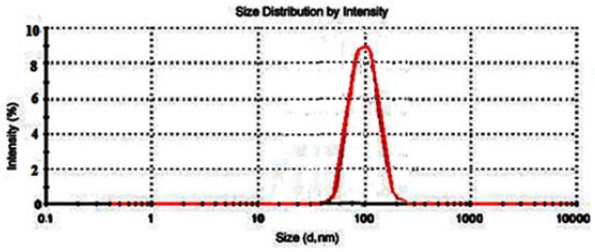


Fig.15. TEM image of optimized formulation and Vesicle size and it`s distribution of formulation TF PIROX4 = 100.18 nm.

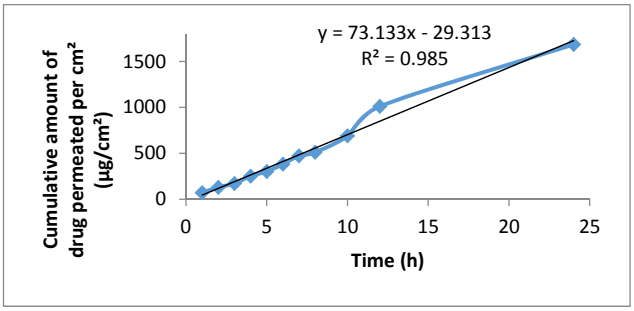


Fig.16. *In vitro* drug permeation of Piroxicam from transfersomal gel.

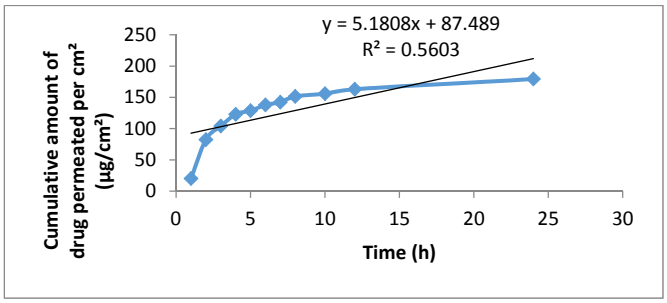


Fig.17. *Ex vivo* permeation of Piroxicam from marketed formulation gel 0.5% w/w.

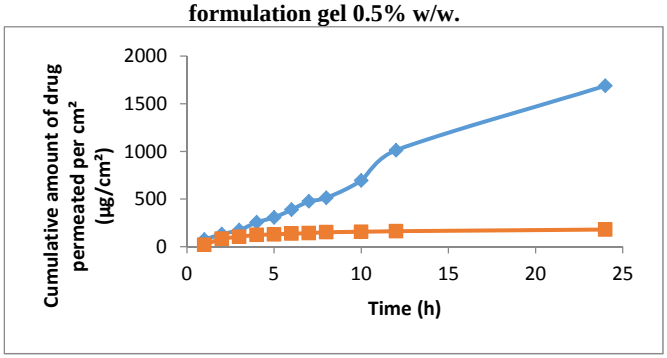


Fig.18. Comparative permeation profile of transfersomal gel and Marketed gel formulation(0.5% w/w)

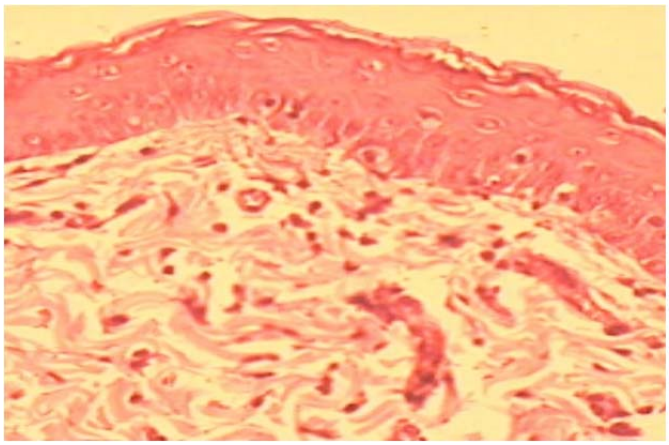


Fig.19. Photomicrograph of control skin

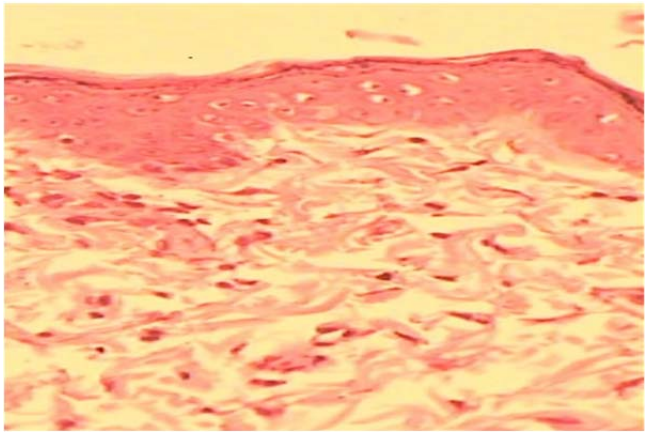


Fig.20. Photomicrograph of treated skin

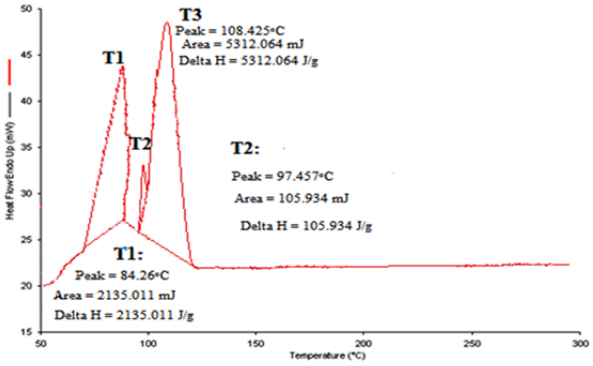


Fig.21. DSC thermogram of control treated rat skin

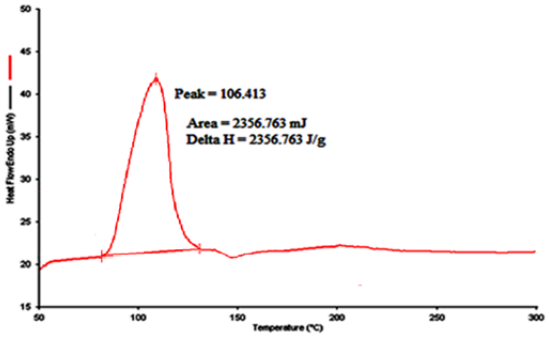


Fig.22. DSC thermogram of transfersomal gel treated skin

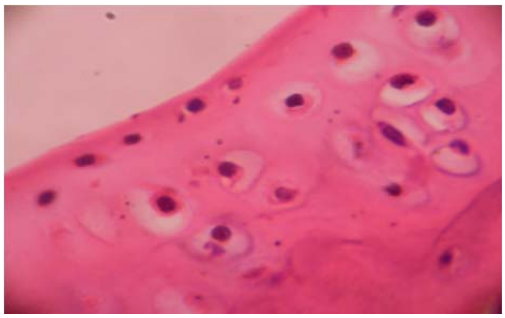


Fig.23. Control

Fig.24. Toxic control

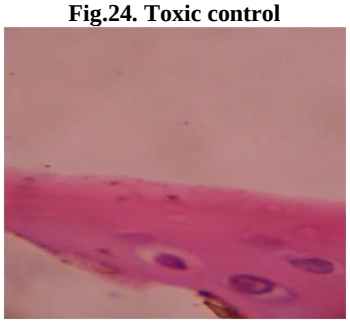


Fig.25. Treatment (oral piroxicam suspension)

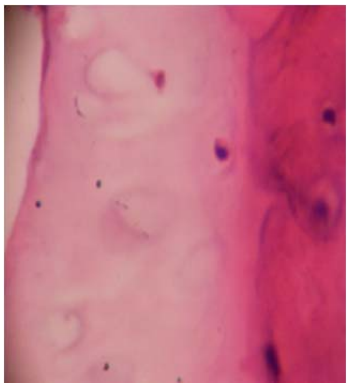


Fig.26.Treatment (marketed piroxicam gel formulation)

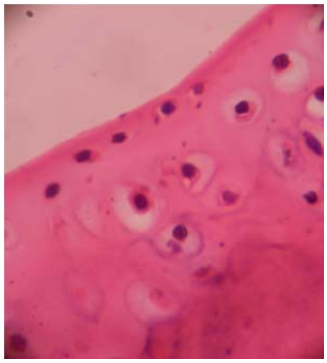


Fig.27. Treatment (Piroxicam transfersomal gel)

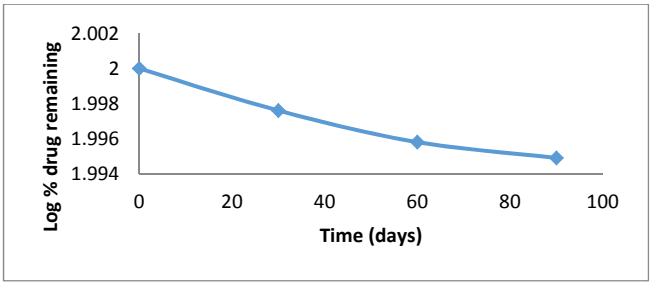


Fig.28. Degradation kinetics of Piroxicam in transfersomal gel(40±2°C &75±5% RH)

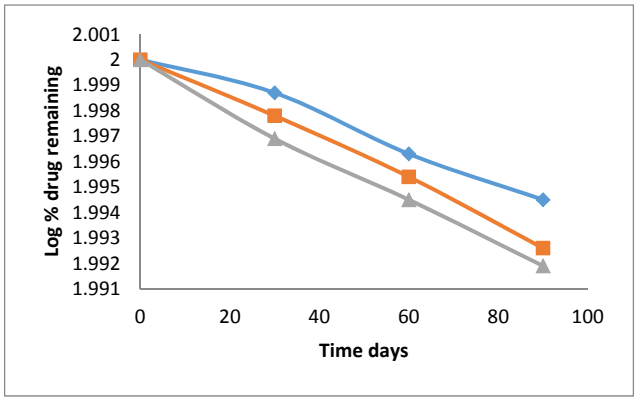


Fig.29. Log percentage remaining vs time plot for transfersomal gel

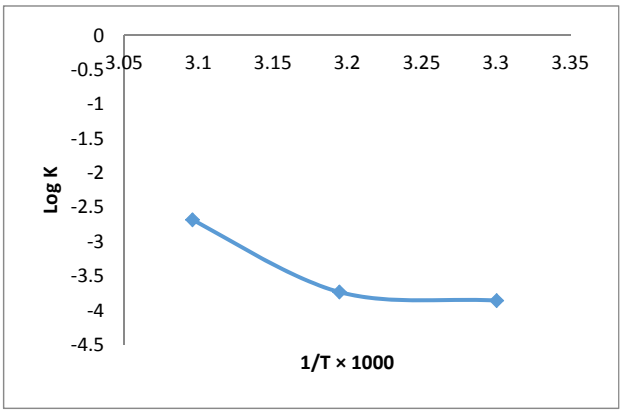


Fig.30. Arrhenius plot for the calculation of shelf life

The data of the skin permeation studies of the transfersomal gel was evaluated in order to ascertain permeation kinetics. From the permeation studies the rate constant for zero and first order rate kinetics were calculated for each time interval and their coefficient of variation (C_v) and standard deviation (SD) were calculated. It was seen in the case of transfersomal gel that small value of $C_v$was observed for zero order kinetics and more of C_v was observed for first order kinetics. Therefore, it was found that permeation from transfersome followed zero order permeation kinetics.

In vitro permeation data of conventional gel was also evaluated for permeation kinetics. The studies conducted on conventional gel revealed that less C_v was observed for first order rate kinetics while more $C_v$was found for zero order kinetics.

Thus it was concluded that the permeation of the drug from transfersomal gel through the skin follows zero order kinetics.

The optical photomicrographs of control (untreated skin) showed normal skin with well defined epidermis and dermal layers. Keratin layer was well formed and lied just adjacent to the top most layer of the epidermis. Dermis was devoid of any inflammatory cells. Skin appendages were within normal limits (Fig.19).When the skin was treated with transfersomal gel formulation for 24 h, significant changes were observed in the skin morphology. Low power photomicrograph of skin sample showed epidermis with a prominent keratin layer, a normal dermis and subcutaneous tissues. Dermis does not show any edema or inflammatory cell infiltration. The disruption of lipid bilayers was clearly evident as distinct voids and empty spaces were visible in the epidermal region.

There were no apparent sign of skin irritation (erythma and edema etc.) observed on visual examination of skin specimens treated with transfersomal formulation.

Van Abbe *et al* (20) mentioned that a value of skin irritancy score between 0 and 9 indicates that the applied formulation is non irritant and safe for human skin. The mean value of skin irritancy score for formulation transfersomal gel was found to be 0.26. This value indicates that all excipients used in formulation were safe for transdermal drug delivery.

DSC thermogram of untreated rat epidermis revealed three endotherms (Fig.21). The three endotherms were recorded at 84,26°C (T1), 97.457°C (t2), 108.425°C (T3) respectively. T3 produced a sharp and prominent peak at 108.425°C which is attributed to SC proteins and the second endotherm (having the lowest enthalpy) was attributed to sebaceous section and to minor structural rearrangement of lipid bilayer. The first endotherm (T1) appeared due to the melting of SC lipids (24).

It was observed that both T1 and T2 endotherm were shifted to lower melting points in thermograms of SC treated with transfersomal gel formulation.

According to the previous studies, formulation TF PIROX4, which was showing the best results among all other Transfersomal formulations of piroxicam was selected for Transfersomal gel formulation for the anti inflammatory effects. The percent inhibition value after 24h administration was found to be high for Transfersomal gel i.e. 59.8% as compared to prepared conventional gel sample (22.8%).The difference was extremely significant at 1% level of significance ($p<0.01$) when compared with Transfersomal gel.

It was observed that Piroxicam when applied transdermally in the form of transfersomal gel reduced the cartilage necrobiosis of the tibial plateau and decreased proteoglycan destruction due to its matrix metalloproteinase inhibition ref. The transfersomal formulation of Piroxicam showed much higher achievement in treatment of osteoarthritis as compared to its conventional formulations which are already available in the market. In the control image Fig.23. the cartilage remains intact featuring prominent cell density and proteoglycan. In the toxic control image Fig.24, it was seen that there was an extension of the area of central chondrocyte necrobiosis with cartilage thinning in the surrounding area, marked loss of proteoglycans and a decreased cell density. This is due to the increase in activity

of matrix metalloproteinase and a decreased cell density. This is due to the increase in activity of matrix metalloproteinase enzymes which cause the loss of proteoglycans and thinning of cartilage was observed. The treatment images 25, 26 and 27 showed slight loss of proteoglycan with a decreased cartilage thinning and slight decrease in cell density.

The value of K at 25°C (K_{25}) was extrapolated from the Arrhenius plot (Fig.28) and shelf life was calculated by substituting k_{25} in the equation for calculating shelf life. Shelf life was found to be 1.286 years. The result showed that formed transfersomal gel is stable. Thus the prepared transfersomal gel, if stored properly, can be effective for a period of 1.286 years from the date of its manufacturing.

DISCUSSION

The observation that shows increase in the amount of surfactant to SPC ratio resulted in increased entrapment efficiency of the formulation to a certain ratio can be probably explained by enhancing emulsification effect of sodium cholate (surfactant) over the phospholipid to a certain degree.

According to the results appeared the entrapment efficiency and vesicle size of different formulations were dependant on the ratio of lipid:surfactant and sonication time.

The content of lipid and surfactant mixture in transfersome affected the skin permeation rate of Piroxicam significantly.

As reduction in the drug permeation, which could be because of the fact that, at higher content of lipid and surfactant mixture the affinity of Piroxicam to the transfersome is increased while its affinity to stratum corneum is decreased.

The spreadability plays a significant role in patient compliance and helps in uniform application of gel to the skin. A good gel takes less time to spread and will have high spreadability.

. In order to ascertain that whether the zero order kinetics achieved in the case of transfersomal gel above was because of the formulation or due to the nature of the skin.

This shows that the permeation of drug from the conventional gel through the skin followed first order kinetics.

Observations of histopathological studies support the in vitro skin permeation data of Piroxicam.

The skin irritation test was performed to confirm the safety of the transfersomal gel formulation.

DSC of rat skin indicated that the components (lipid and surfactant) of the transfersome enhanced skin permeation of piroxicam through disruption of lipid bilayers. Change in peak area and transition temperature (peak shift) indicated increase in fluidity of skin bilayer which could be the mechanism of permeation enhancement.

The enhanced anti-inflammatory effects of ultraflexibleTransfersomal gel formulation could be due to enhanced permeation of Piroxicam through the skin.

It is reported that iodoacetate injection into the knee of rats results in histopathological features of OA such as a loss of proteoglycan, cartilage degeneration, osteophyte formation, a dose dependent reduction in spontaneous locomotion(25) and altered gait (26). In this study we have demonstrated the important role of Piroxicam in the chondroprotection against iodoacetate induced knee osteoarthritis in albino wistar rat model.

The results of pharmacodynamic studies can be due to the antiinflammatory effect of Piroxicam which result from the reversible inhibition of cyclooxygenase, causing the peripheral inhibition of prostaglandin synthesis. The prostaglandins are produced by an enzyme called Cox-1. Piroxicam blocks the Cox-1 enzyme, resulting into the disruption of production of prostaglandins. Piroxicam also inhibits the migration of leukocytes into sites of inflammation and prevents the formation of thromboxane A2, an aggregating agent, by the platelets.

CONCLUSION:

The present study is my M.Pharm thesis carried out on July 2011 till August 2012 which developed and evaluated the transdermal transfersomal gel of piroxicam, since the oral administration piroxicam causes severe hypoglycaemic reaction. The transfersomal formulation of Piroxicam showed the much higher achievement in treatment of osteoarthritis as compare to its conventional formulations which are already available in the market. Further research is recommended for validating the efficacy and safety of the above formulation by long term pharmacokinetic and pharmacodynamic studies in healthy volunteers and patients.

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