Proniosomal gel containing oxymetazoline hydrochloride for Rosacea Treatment: Development, optimization, In-vitro and In-vivo characterization

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Abstract

The present proniosomal gel investigation was aimed to minimize the adverse effect associated with present topical proniosomal formulation and made to enhance its bioavailability and sustain release by noval pronisomal class of drug for treatment of rosacea. Oxymetazoline hydrochloride is a direct-acting alpha sympathomimetic and adrenergic agonist used to treat rosacea, a facial redness or ocular inflammation involving both the vesicular and tissue stroma. The pathology of rosacea was unknown, and was expected to hot beverages, alcohol consumption, high environmental temperature, sun exposures, menopause exacerbate the rosacea symptoms such as eythema, rash, and telangiectasia. Direct vasoconstriction of both the small arteries and veins has been established. Proniosome gel are prepared by coacervation phase separation method, using different concentration of non ionic surfactant(span and brij)and evaluated various parameter like surface characterization, entrapment efficiency, drug content, viscosity, in vitro % drug release, invitro antifungal activity, invivo study of different optimized formulation.In this investigation, we used a variety of surfactants, including span 40, 60, 80, and brij 35 and Brij 72, in various gel formulations and analysed. As a result, the optimized formulation of proniosome oxymetazoloine hydrochloride (SK723) transparent gel is a vesicle size312.5±0.3 and % entrapment efficiency 81.22±1.62 and % amount of drug release 85.37±0.45, highest drug content, compared to other formulation, indicate a stable gel. The pH and viscosity of prepared all proniosomal formulation were found in the range(6.3-7.1) .In vitro diffusion study was carried by the using treated cellophane membrane, % drug release study showed (56.12-79.42%)for all formulation. In the (SK723) formulation show a highest drug release (85.37%) compared to other formulation over a 24 hours period of time. the antifungal activity study was concluded that proniosomal gel formulation with drug (SK723) shows better zone of inhibition then the other and marketed formulation. The optimized formulation also show a good extrudability, and 92.45% drug content also show a effective controlled release property 85.37±0.45.The proniosome Brij 72 gel formulation exhibits improved drug absorption and release in vitro. The best results are obtained with a formula containing a balanced ratio of brij 72 and cholesterol (1:1). The zero and first order Higuchi equations are used to explain the release mechanism. As a result, the proniosome gel has better trapping efficiency and uses alcohol to act as a penetration enhancer.In vitro study,& in vivo study,they increase oxymetazoline's ability to penetrate the skin. Thus the present work proved that the oxyetazoline hydrochloride loaded proniosomal gel effective in treatment of rosacea.

Keywords: Proniosomal gel, transdermal drug delivery, Oxymetazoline, In-vitro drug release, stability Studies.

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INTRODUCTION

Conventional chemotherapy to treat intracellular infections is no longer effective because the drug's penetration into cells is limited. This can be remedied by using a vesicle delivery system. Encapsulation of the drug in a vesicular structure can be predicted to prolong the shelf life of the drug in the systemic circulation and thereby improve penetration into target tissues and reduce toxicity. Vesicular delivery also improves drug bioavailability, especially in cases of poor drug solubility. They can combine both hydrophilic and lipophilic drugs. This system also solves the problem of drug insoluble, unstable and rapidly degraded.[1]

Rosacea is a chronic skin condition that presents with a broad diversity of cutaneous manifestations Facial erythema, the most common primary characteristicof all subtypes of rosacea, is a mandatory diagnostic feature. Persistent centrofacial erythemais the predominant hallmark of patients with rosacea, especially in the erythema totelangiectatic rosacea (ETR) and the papulopustular rosacea (PPR). Oxymetazoline is a adrenergic alpha agonist, direct acting sympathomimetic used as a vasoconstrictor to relieve nasal congestion. The sympathomimetic action of oxymetazoline constricts the smaller arterioles of the nasal passage, producing a prolonged gentle and decongesting effect oxymetazoline relief of conjunctival hyperemia by causing vasoconstriction of superficial conjunctival blood vessels.

This new topical use of oxymetazoline hydrochloride in the form of 1% cream for the treatment of facial erythema of rosacea has been approved by USFDA on 19 jan 2017[4].Looking forward to exploit this new use of oxymetazoline hydrochloride in dermatology like to develop proniosomal drug delivery system for the treatment for Rosacea, a chronic disorder of the central face. Oxymetazoline is a 1A -adrenergic receptor agonist. It works as a vasoconstrictor. Intranasal use results in constriction of arterioles in the nasal mucosa to provide relief from congestion. Oxymetazoline has also been used in the eye to decrease redness. When applied to the skin, Oxymetazoline constricts the superficial vasculature. Inflammatory factors are the most likely cause of erythematotelangiectatic Rosacea rather than a treatable, underlying bacterial infection. Contributing factors are likely interplay between increased immune and angiogenesis responses.

proniosomes is a dry product that can be hydrated immediately prior to use, which will avoid many of the problems associated with water dispersion of niosomes and physical stability problems. (aggregate, merge, leak) can be minimized. These dry surfactant coated carrier formulations can be dosed as needed and rehydrated by rapid shaking in hot water. They are water-soluble carrier particles coated with surfactants and can be hydrated to form a niosomal dispersion immediately prior to use by rapid stirring in a hot water medium. The reported methods for pronisome preparation are the spraying of surfactants onto water-soluble carrier particles and the suspension method. This free-flowing, dry granular product, upon addition of water, disperses or dissolves to form a multistellar niosome suspension suitable for oral administration or by other systems such as drug targeting, controlled release control and improve drug penetration. But there are still some limitations when decoding and this can be avoided if the modulation is dry.[2] [9] [3].

A non-ionic surfactant of various structural types has been shown to be a useful substitute for phospholipids in the production of vesicle systems. Non-ionic surfactants form multiple aggregates in the form of micelles with large vesicles, which can be used for drug delivery. Steroids are important components of cell membranes and their presence in membranes leads to notable changes in bilayer fluidity and permeability. Cholesterol can be incorporated into a bilayer with a significantly higher molar ratio; however, by itself it does not form a double layer. Therefore, it can be used to manipulate the properties of membranes. It is amphoteric in nature, so it arranges such that the OH group faces the aqueous phase while the fatty chain aligns parallel to the hydrocarbon chain of the surfactant. Cholesterol acts as a fluid buffer because below the transition temperature it makes the membrane less orderly while above it the membrane becomes more orderly[8], [7].

MATERIALS:

Oxymetazoline hydrochloride drug obtained from gift sample of Lobachemie, Mumbai. Span 40, 60, 80 and Brij 35& Brij 72, were purchased from Central Drug House. Cholesterol and dialysis tubing were purchased from Hi-Media Laboratories (Mumbai, India). All other chemicals and reagents used were of analytical grade and were used without further purification.

Identification of Drug by Ultraviolet Spectroscopy

Identification of Oxymetazoline Hydrochloride was estimated by UV spectrophotometer. Absorption maxima (λ_{max}) of Oxymetazoline Hydrochloride was determined by placing 10 µg/ml solution of drug in phosphate buffer solution (pH 7.4) and spectra was recorded by using UV Spectrophotometer. The λ_{max} of Oxymetazoline Hydrochloride was found to be 380 nm which was similar with the standard value of pure Oxymetazoline Hydrochloride as reported in IP monograph hence, confirmed that the obtained sample was Oxymetazoline Hydrochloride.

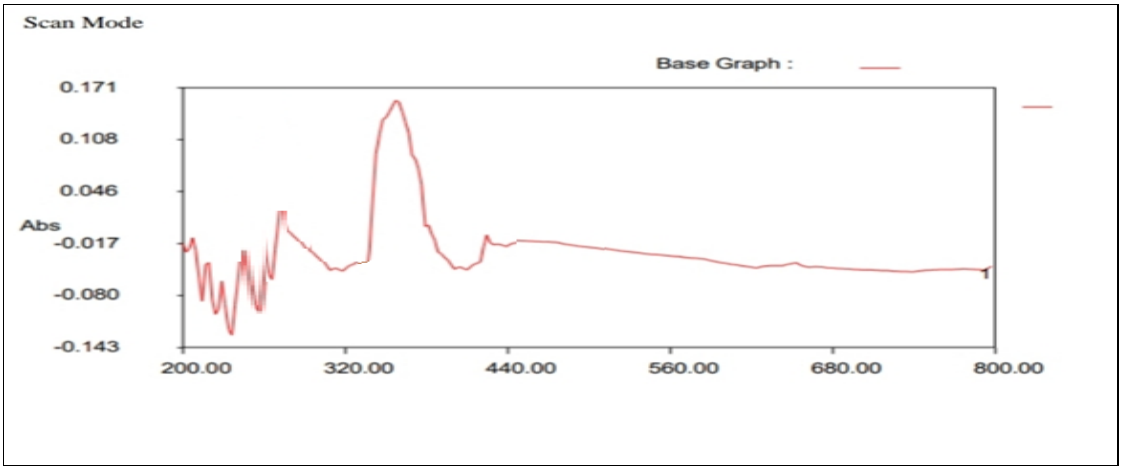

Fig.1 Spectrum of Oxymetazoline hydrochloride

Identification of Drug

The drug was identified by FT-IR spectroscopy. Fourier transform infrared spectroscopy (FT-IR) was performed by using potassium bromide disc method. Over the range of 4,000.00 to 650.00 cm^1 the samples were scanned in an inert atmosphere. Infrared spectroscopy was done for cholesterol, surfactant, drug and physical mixture of drug with surfactant and drug with cholesterol was used in the ratio of 1:1. The cholesterol, pure drug and physical mixture of cholesterol with drug and surfactant with drug were mixed separately with IR grade KBr in the ratio of 1:100. The discs were prepared by applying 15000 lb pressure in a hydraulic press. The IR spectrum of pure Oxymetazoline Hydrochloride shows a peak at 3310-3500 cm-1 which is attributed to N-H and 1330-1540 which is attributed to NO_2 stretching and peaks at 2100-2260 cm-1 is due to C=C aromatic stretching, peak at 1078.81 cm-1 is due to C=C bending and 3000-3300 cm-1 confirms the C-H bending (Fig 2). Hence, it confirms the structure of Oxymetazoline Hydrochloride.

IR Spectra of Oxymetazoline hydrochloride

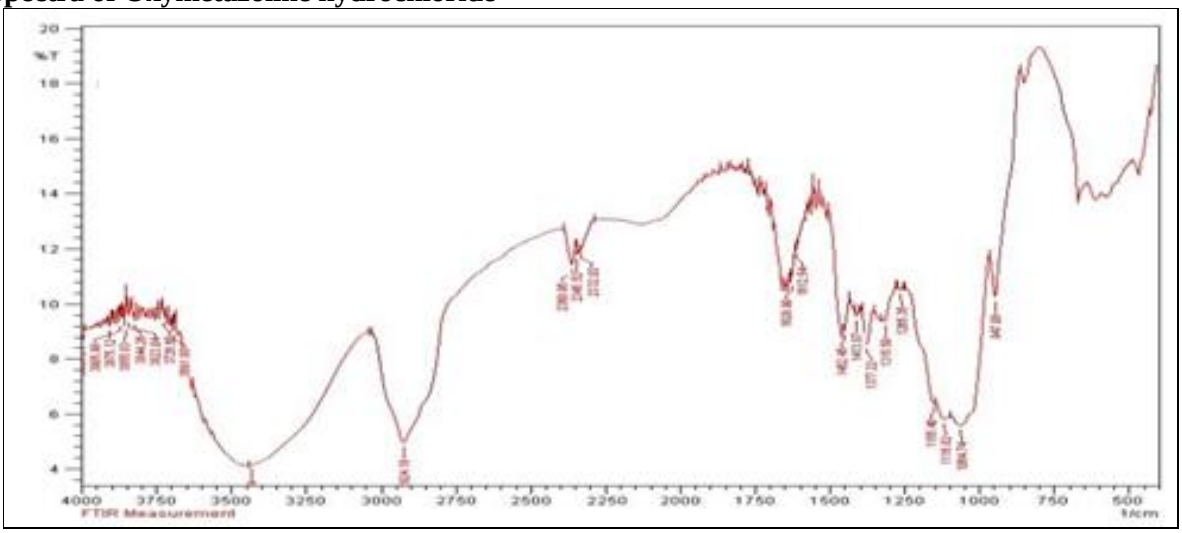

Fig2. IR Spectra of Oxymetazoline hydrochloride

FTIR spectra of brij 72

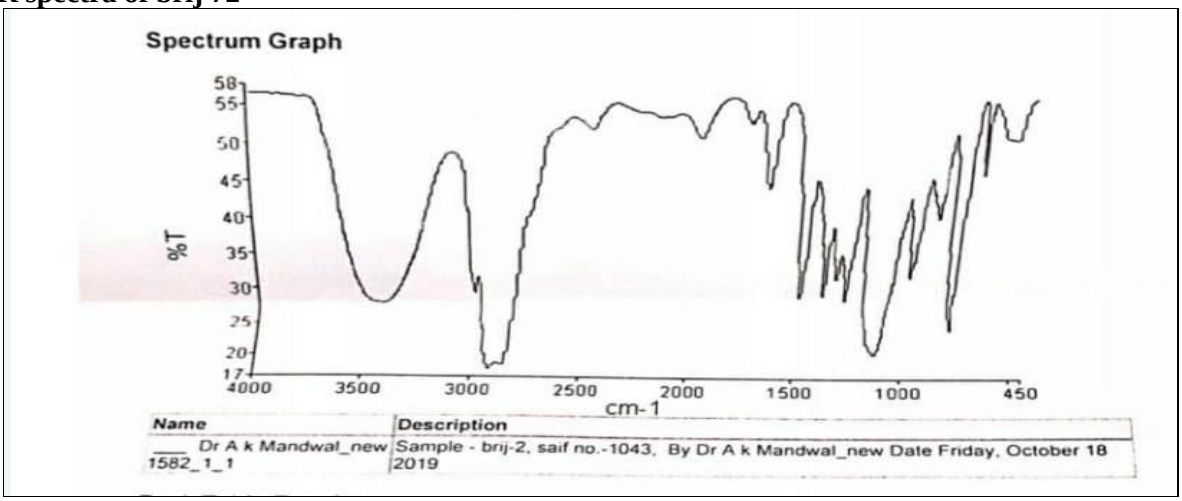

Fig 3. FTIR spectra of brij 72

FTIR spectra of Oxymetazoline Hydrochloride, Brij72 & cholesterol

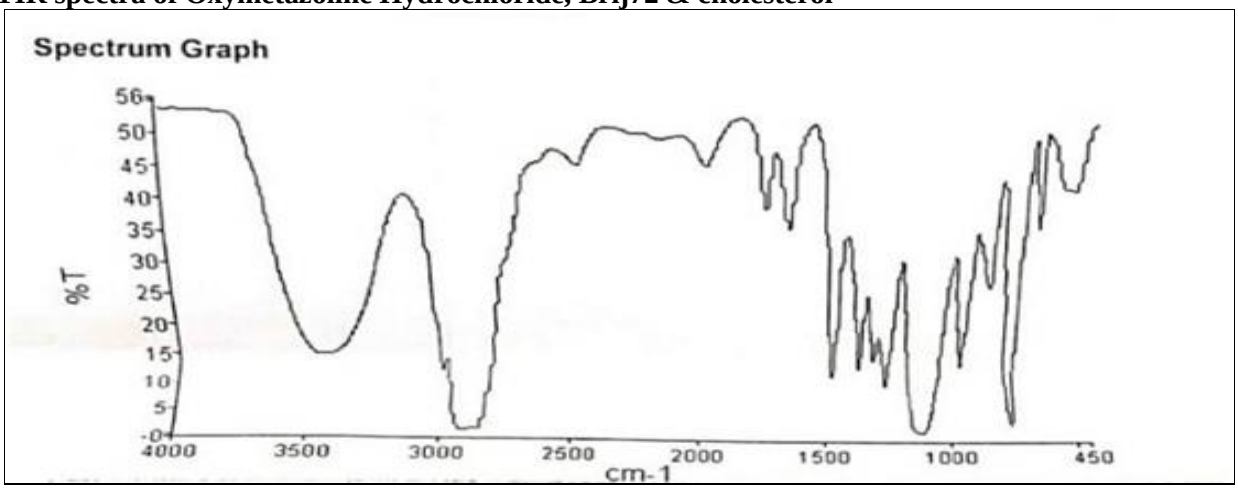

Fig4. FTIR spectra of Oxymetazoline Hydrochloride, Brij72 & cholesterol

Development of proniosome gel

Preparation of proniosome gel was adopted by the method given by Proniosome gel preparation involves mixing of surfactant, cholesterol, and the drug with a suitable alcohol. After mixing all the ingredients, it is covered with a lidto prevent the loss of solvent and warm on a water bath at 60° - 70°C until the surfactant dissolves completely [5].To it is added an aqueous phase, which may be purified water, dilute glycerol solution or an isotonic buffer solution like, phosphate buffer or saline solution. It is warmed again form a clear solution, which on storage for overnight under dark converts to proniosomal gel (Table 1). The ratio of surfactant, alcohol and the aqueous phase plays an important role in gel formation [15].

Table 1- Composition of optimized proniosomal gel formulations

S.No.	Drug (mg)	Span40 (mg)	Span60 (mg)	Span80 (mg)	Brij35 (mg)	Brij72 (mg)	CHL (mg)	Ethanol(ml)	Water (ml)
SK403	10	50	-	-	-	-	50	0.5	0.18
SK603	10	-	50	-	-	-	50	0.5	0.18
SK803	10	-	-	50	-	-	50	0.5	0.18
SK353	10	-	-	-	50	-	50	0.5	0.18
SK723	10	-	-	-	-	50	50	0.5	0.18

Evaluation of the prepared formulations

Organoleptic properties:

Proniosomal gels were characterized for appearance, color, and homogeneity by visual inspection. The physical appearance results are shown in Table 2. It was found that the formulae 1, 2 white creamy gel; formula 3 was viscous gel,

formula 4 was pale yellow gel; and the formulae 5, white viscous gel.

Table 2- Observation of proniosomal gel formulation

S.No.	Drug (mg)	Span 40(mg)	Span 60(mg)	Span 80(mg)	Brij 35(mg)	Brij 72(mg)	CHL (mg)	Ethanol (ml)	Water (ml)	Obsevation
SK403	10	50	-	-	-	-	50	0.5	0.18	white creamy gel
SK603	10	-	50	-	-	-	50	0.5	0.18	white creamy gel
SK803	10	-	-	50	-	-	50	0.5	0.18	viscous gel
SK353	10	-	-	-	50	-	50	0.5	0.18	pale yellow gel
SK723	10	-	-	-	-	50	50	0.5	0.18	white viscous gel

Optical microscopy

One drop of the formed gel was spread on a glass slide and examined for the vesicle structure using ordinary light microscope with varied magnification powers (×10 and ×40).[11] Photomicrographs were taken using a digital camera (Sony Cybershot DSCw55 7.2 megapixel, Tokyo, Japan).The optical photomicrographs of some formulations are shown in fig. 6 to 10.

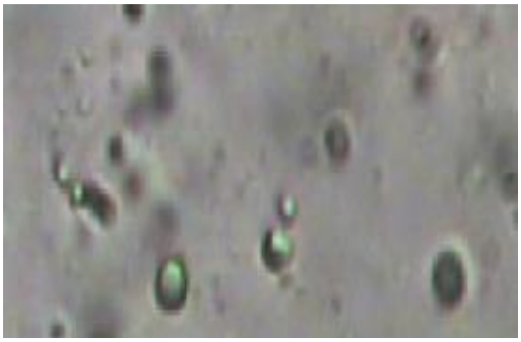

Fig.5 SK403Proniosomal gel derived noisome

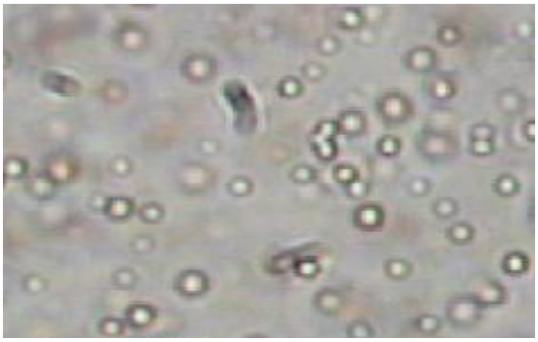

Fig.6 SK603 Proniosomal gel derived noisome

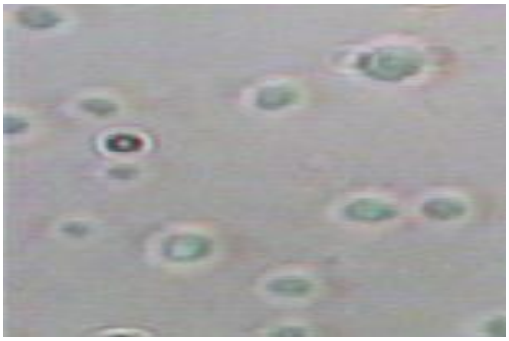

Fig.7 SK803 Proniosomal gel derived noisome

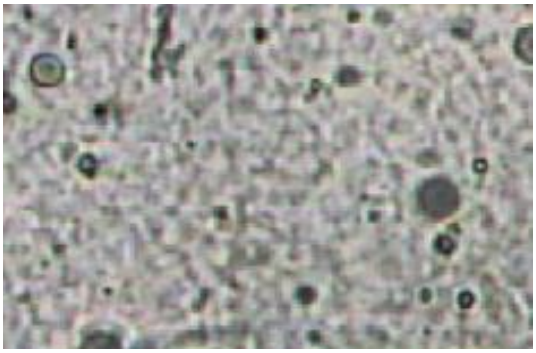

Fig.8 SK353 Proniosomal gel derived noisome

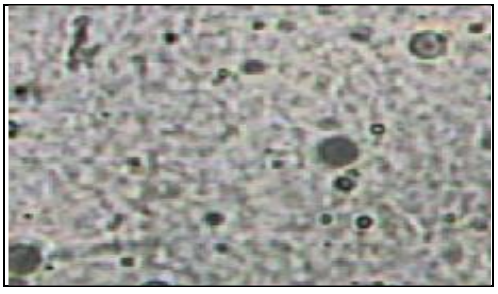

Fig. 9 SK723 Proniosomal gel derived niosome

pH measurement:

The pH of the gel was determined by digital pH meter(Model 420, ORION, USA). A sample of 0.1 g of gel was dissolved in 10 ml of distilled water and the electrode was then dipped into gel formulation and constant reading was noted.[12]

The readings were taken for an average of three times. Its lies from 6.7 to 7.1.

Solubility Determination Oxymetazoline Hydrochloride was freely soluble in water and methanol, soluble in dimethyl sulphoxide and PBS (pH 7.4), slightly soluble in ethanol and insoluble in acetone. (Table No. 3)

Table 3. Solubility in different solvent

S. No.	Solvent	Solubility
1.	Water	Freely soluble 1-10 parts
2.	Methanol	Soluble 10-30 partS
3.	Ethanol	Slightly soluble 100-1000 parts
4.	Acetone	Insoluble 10,000 and above
5.	Phosphate buffer (pH7.4)	Soluble 10-30 parts

Partition Coefficient: The partition coefficient of Oxymetazoline Hydrochloride was found to be 2.049 ± 0.0008. The value shows that the drug is lipophilic which is necessary to formulate a gel. This also shows that the drug is biphasic in nature which ensures easy permeation through [7]skin.(Table no. 4)

$$P_{o/w} = \frac{\text{Concentration of drug in n - Octanol}}{\text{Concentration of drug in water}}$$

Table 4. Partition coefficient of Oxymetazoline Hydrochloride in different solvent system

S. No.	Solvent system	Partition coefficient
1.	n-octanol/water	3.92
2.	n-octanol/phosphate buffer pH 7.4	1.21

Melting Point Determination(Capillary Method):

The melting point was determined by introducing a minute amount of drug into a small thin-walled capillary melting point tube sealed at one end by inserting the tip into a Bunsen flame. The drug was transferred from the open end to the bottom of the capillary tube, the bottom was tapped gently. A packed capillary was attached to a normal mercury thermometer then capillary was inserted into melting point apparatus and the temperature was observed at which melting begins and is complete.[9]

Melting point was found to be 162 ± 0.816$^{\circ C}$ (specification 162- 165$^{\circ C}$).

Particle size analysis of Oxymetazoline hydrochloride proniosomes:

The particle size (PS) and Polydispersity Index (PDI) of proniosomes were measured using a Zeta sizer 3000 PCS (Malvern Instr., England) equipped with a 5 mW helium–neon laser with a wavelength output of 633 nm. Measurements were made at 25°C, angle 90, and runtime at least 180 s.[14] The proniosomal gels were appropriately diluted with distilled water before measurements. Polydispersity Index was determined as a measure of homogeneity. Small values of Polydispersity Index (0.3 indicate high heterogeneity. [15]

Particle sizedistribution report:

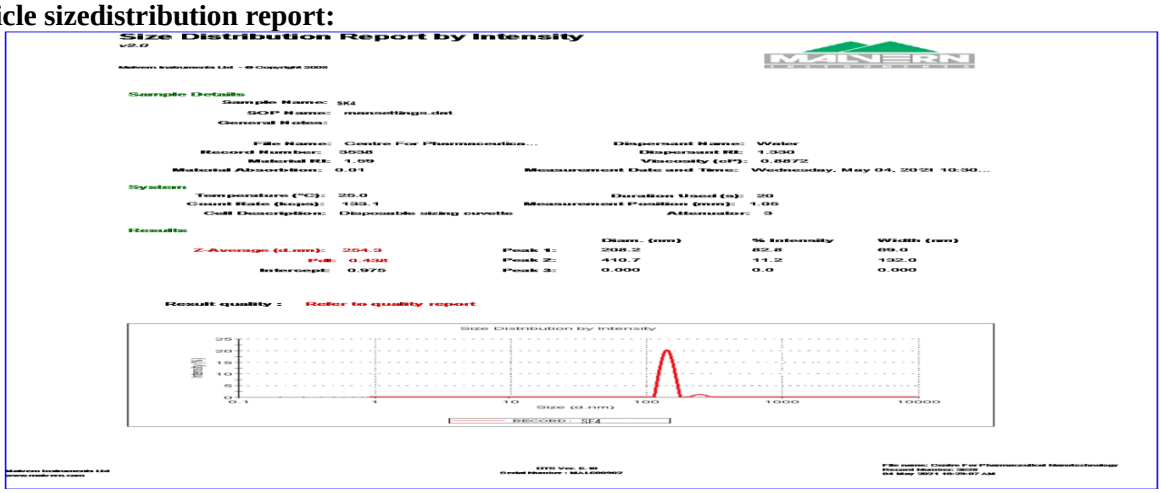

Fig. 10 Vesicle size (nm) vs. intensity (%) of formulation SK723

Vesicle Size of Different optimized Proniosomal gel Formulation

Table 5. Vesicle size values for different optimized gel formulations

S. No.	Formulation code	Vesicle size (nm) (Mean±SD)
1	SK403	375.2±0.42
2	SK603	330.6±0.22
3	SK803	305.4±0.31
4	SK353	265.4±0.36
5	SK723	315.6±0.23

n = 3 ($p < 0.05$)

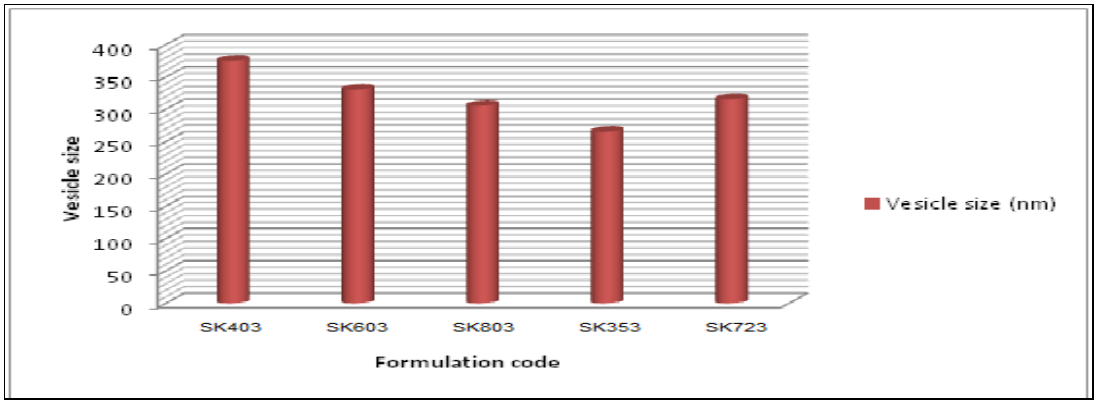

Fig.11: Vesicle size values for different optimized gel formulations

Zeta potential report:

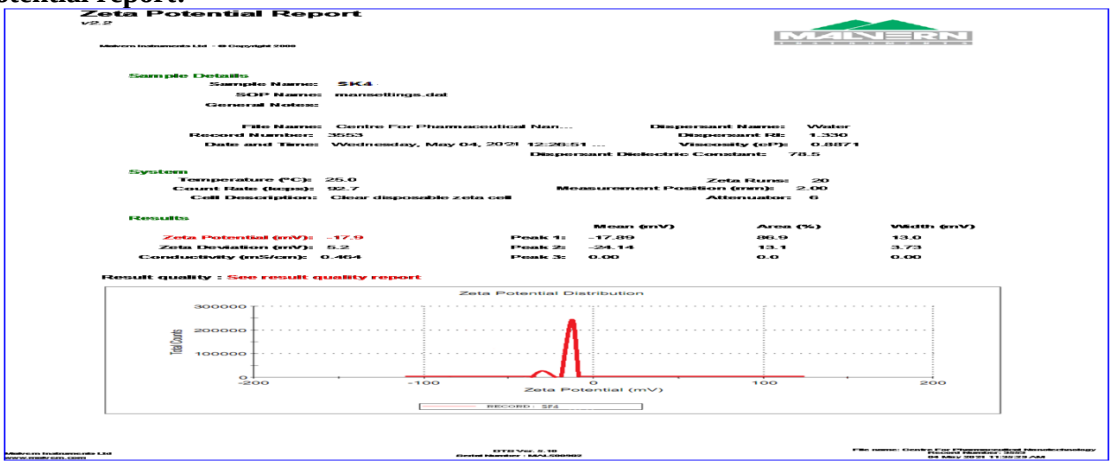

Fig.12 .Zeta potential report of formulation SK723

Zeta potential analysis

Charge on drug-loaded vesicles surface was determined using zeta potential (ZP) analyzer (A Brookhaven Instrument Corp). Analysis time was kept for 60 s, and average zeta potential and charge on the proniosome preparation after hydration with phosphate buffer saline pH 7.4 were determined at 25°C and three runs were carried out[16].(Fig. 13).

High-resolution transmission electron microscopy

The selected and prepared Oxymetazoline hydrochloridegel was characterized for its shape by transmission electron microscopy (JEOL Model - JEM 2100–200KV, Tokyo, Japan), using a 300 mesh carbon-coated copper grid and phosphotungstic acid (1%; w/v) as a negative stain. After being stained, the samples were allowed to dry at room temperature for 10 min for investigation. [17], (Fig. 14, 15 & 16)

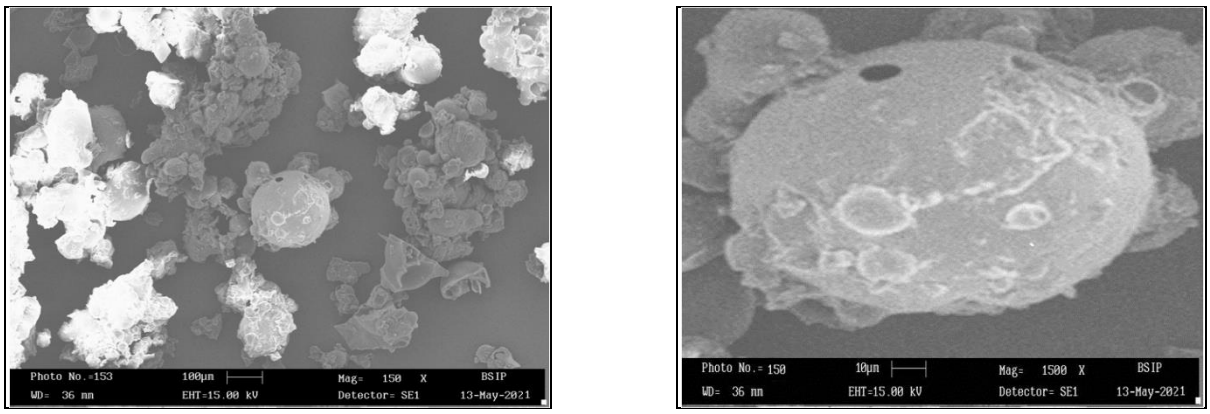

Fig.13, 14 Scanning electron micrograph of SK723 Proniosomal gel of optimized formulation.

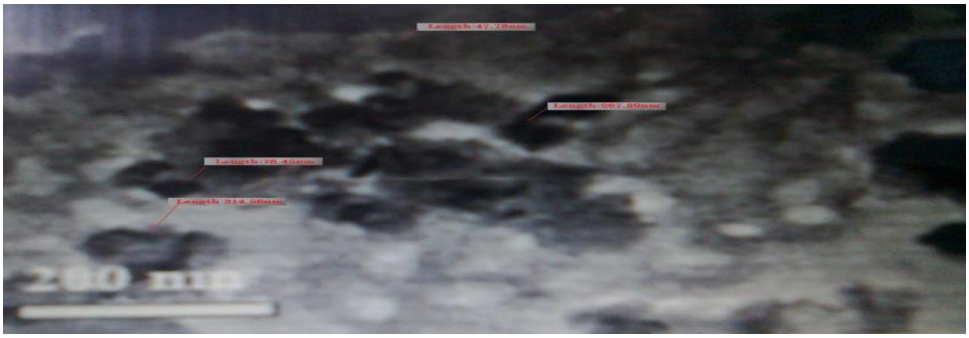

Fig.15 Transmission electron micrograph of SK723 Proniosomal gel of optimized formulation**.**

Determination of drug entrapment efficiency

A sample of 0.2 g of proniosomal gel was taken in a glass tube, and 10 ml of phosphate buffer (pH 7.4) was added. This aqueous suspension was sonicated in a sonicator bath (Rolex, India), followed by centrifugation at 9.000 rpm at 20°C for 30 min (Ultra centrifuge 5417R, Eppendorf, Hamburg, Germany). The supernatant was collected and assayed by using ultraviolet (UV) method (Shimadzu UV spectrophotometer (2401/PC), Japan) for unentrapped Oxymetazoline hydrochloride content at 260 nm.[11] The percentage of drug encapsulation (entrapment efficiency percentage [EE%]) was calculated by the following equation: EE% = (Total amount of drug − Unentrapped drug/Total amount of drug) ×100.

Table 6. Entrapment efficiency of optimized proniosomal gel formulation

Formulation code	% Entrapment Efficiency (Mean±SD)
SK403	51.78±32
SK603	55.60±54
SK803	64.60±65
SK353	69.90±40
SK723	81.22±25

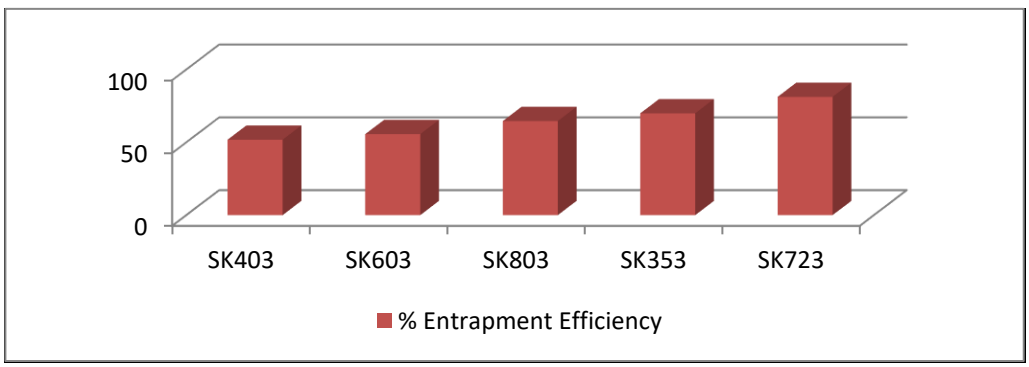

Fig. 16 Entrapment efficiency of optimized proniosomal gel formulation

Physical Appearance and Homogeneity-All the proniosomal gel formulations have colorless to pale yellow appearance.

Table 7. Physical Appearance and Homogeneity

S.No	Formulation Code	Physical Appearance
1.	SK 401	Colorless
2.	SK 602	Colorless
3.	SK 803	Colorless
4.	SK 353	Colorless
5.	SK 723	Colorless

Homogeneity- All the proniosomal gel formulations showed good homogeneity with absence of lumps

Table 8. Homogeneity-

S.No	Formulation Code	Homogeneity
1.	SK 401	Good
2.	SK 602	Fair
3.	SK 803	Good
4.	SK 353	Good
5.	SK723	Good

Table 9. Evaluation parameters of Oxymetazoline gel

S.No	Formulation Code	pH	Spreadability(g.cm/sec)	Gellingstrength (sec)
1.	SK401	6.8	21.99±0.21	65±1
2.	SK602	6.7	18.75±0.02	82±3
3.	SK803	6.7	26.30±0.32	68±4
4.	SK353	7.1	22.39±0.12	79±3
5.	SK723	6.9	28.86±0.34	65±3

n=3, (Mean±SD)

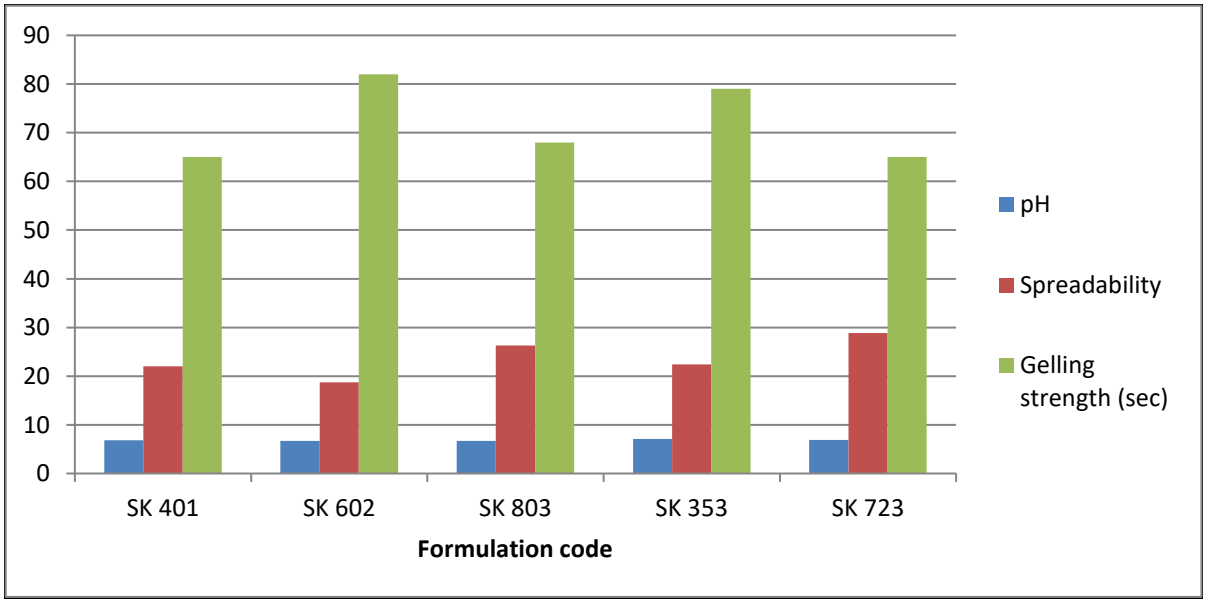

Fig. 17 Evaluation parameters of Oxymetazoline gel

Drug Content: Drug content was found in the range of 88 to 95%. The Formulation (SK723) showed maximum drug 95.25. Drug content of all the formulations were within the acceptable range which shows the proper mixing of the drug with the excipients.

Table 10 show Extruability and %drug content of following optimized formulation

S.No	Formulation Code	Extrudability	% Drug Content
1.	SK403	Good	88.56±0.12
2.	SK603	Good	95.25±0.35
3.	SK803	Good	90.78±0.28
4.	SK353	Excellent	89.46±0.26
5.	SK723	Good	92.45±0.41

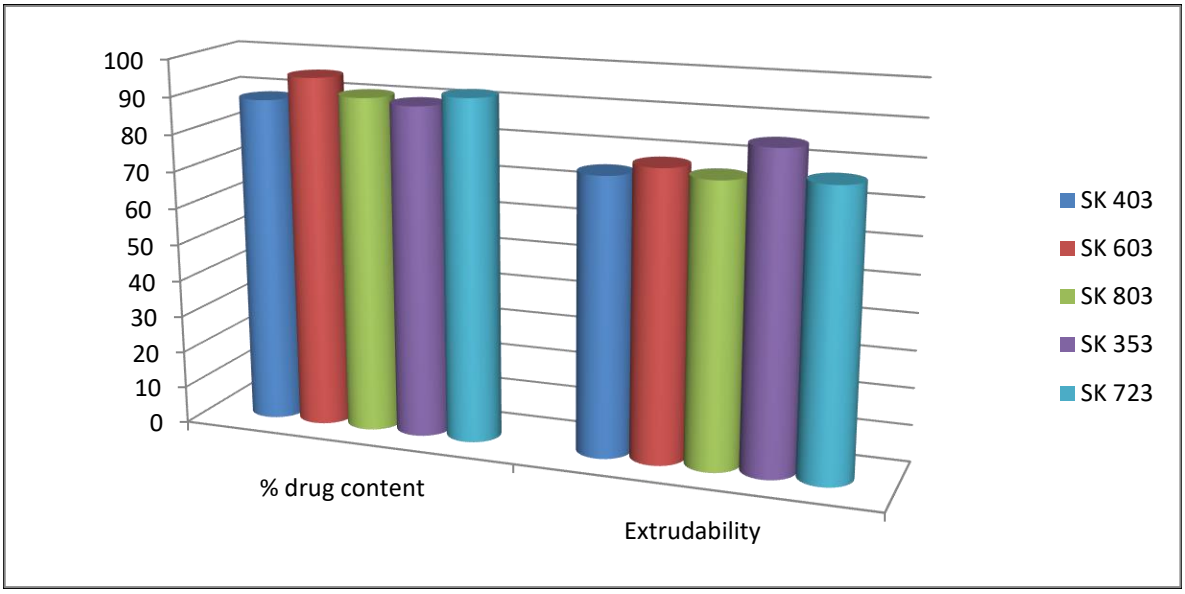

Fig.18 show Extruability and %drug content of following optimized formulation

In vitro release study

The *in vitro* drug release studies of proniosomal gel were carried out by means of treated cellophane membrane. *In vitro* release studies on proniosomal gel were performed using locally manufactured Franz-diffusion cell. The capacity of receptor compartment was 15 ml. The area of donor compartment exposed to receptor compartment was 1.389cm^2. The dialysis cellophane membrane (MMCO14KDC) was mounted between the donor and receptor compartment. A weighed amount of proniosomal gel was placed on one side of the membrane. The receptor medium was phosphate saline buffer pH 7.4. The receptor compartment was surrounded by a water jacket to maintain the temperature at 37±1°C. Heat was provided using a thermostatic hot plate with a magnetic stirrer. The receptor fluid was stirred by a Teflon-coated magnetic bead fitted to a magnetic stirrer (Bio-Craft Scientific Systems Pvt. Ltd., Agra). At each sampling interval, samples were withdrawn and were replaced by equal volumes of fresh receptor fluid on each occasion. Samples withdrawn were analyzed spectrophotometrically (Shimadzu-1700) at 380 nm. [21]

Amount of drug release profile from optimized proniosomal gel formulation in phosphate buffer saline pH 7.4

Table 11. Amount of drug release profile from optimized Proniosomal Gel formulation in phosphate buffer saline pH 7.4

Time (hrs)	SK403 (µg/cm^2)	SK603 (µg/cm^2)	SK803 (µg/cm^2)	SK353 (µg/cm^2)	SK723 (µg/cm^2)
0.5	13.63±1.16	14.87±0.47	16.07±0.50	21.30±1.20	23.06±0.67
1.0	21.97±1.08	23.77±0.57	26.35±0.59	34.20±1.10	36.86±0.56
2.0	32.05±0.90	37.41±0.67	37.65±0.69	47.26±1.03	50.86±0.12
3.0	46.76±0.98	53.20±0.61	56.50±0.84	73.99±0.95	76.10±0.45
4.0	71.79±0.83	82.8±0.70	82.84±0.89	109.3±0.88	113.16±0.45
5.0	105.49±0.79	112.9±0.78	124.34±0.99	161.9±0.45	171.90±0.98
6.0	188.56±0.72	203.2±0.89	224.23±0.98	242.6±0.87	256.35±0.23
8.0	339.05±0.62	365.9±0.86	399.24±1.06	436.7±0.87	465.38±0.56

10.0	603.8±0.54	658.6±0.56	717.89±1.89	794.2±0.56	837.3±0.45
12.0	1091.1±0.87	1180.3±0.87	1291.0±1.76	1379.8±0.56	1509.0±0.34
24.0	2173.8±0.23	2360.7±0.45	2562.8±1.65	2816.0±0.34	3025.5±0.45

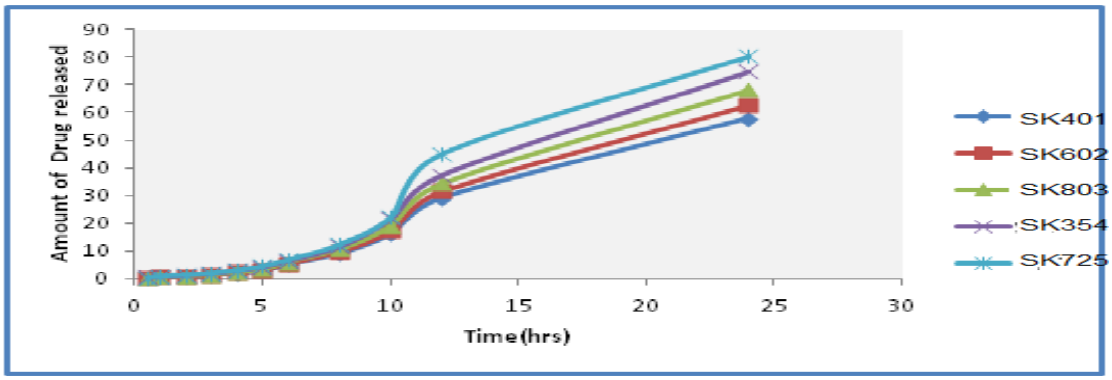

Fig. 19 Amount of % Drug release from different proniosomal gel formulation through rabbit skin in phosphate buffer pH **7.**

Table 12. Amount of drug release profile from different Proniosomal Gel formulation through rabbit skin in phosphate buffer pH 7.4

Time(hrs)	SK403	SK603	SK803	SK353	SK723
0.5	0.272±0.45	0.415±0.45	0.32±0.98	0.541±0.87	0.421±0.88
1.0	0.589±0.98	0.632±0.60	0.70±0.76	0.909±0.45	0.982±0.54
2.0	0.852±0.89	0.99±0.32	1.00±0.65	1.314±0.22	1.35±0.65
3.0	1.243±0.56	1.41±0.67	1.50±0.45	1.966±0.12	2.02±0.53
4.0	1.908±0.67	2.20±0.43	2.20±0.56	2.906±0.88	3.00±0.63
5.0	2.804±0.34	3.08±0.12	3.30±0.34	4.304±0.65	4.51±0.86
6.0	5.012±0.76	5.42±0.43	5.96±0.76	6.45±0.43	6.81±0.68
8.0	9.012±0.23	9.73±0.89	10.61±0.66	11.61±0.65	12.37±0.45
10.0	16.05±0.87	17.17±0.76	19.00±0.34	21.61±0.87	22.25±0.88
12.0	29.01±0.56	31.51±0.65	34.32±0.56	37.50±0.43	45.14±0.45
24.0	57.78±0.45	62.75±0.87	68.12±0.86	74.85±0.56	85.37±0.67

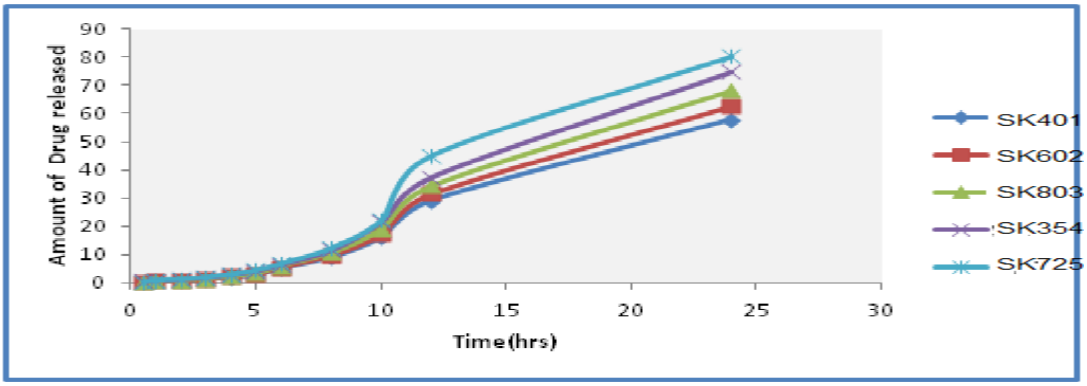

Fig. 20 Graph showing amount of % drug release from different Proniosomal Gel formulation through rabbitt skin in phosphate buffer pH 7.4

Data treatment for release in pbs ph 7.4

Zero order kinetic treatment for release data of oxymetazoline hydrochloride for encapsulated proniosomal gel

Table 13 Zero order kinetic treatment for release data

Formulation code	Equation of line	Correlation coefficient(r^2)
SK403	Y = 96.30X-234.84	0.951
SK603	Y = 104.48X-254.07	0.952
SK803	Y = 113.63X-276.36	0.952
SK353	Y = 123.96X-294.69	0.954
SK723	Y = 133.43X-318.9	0.951

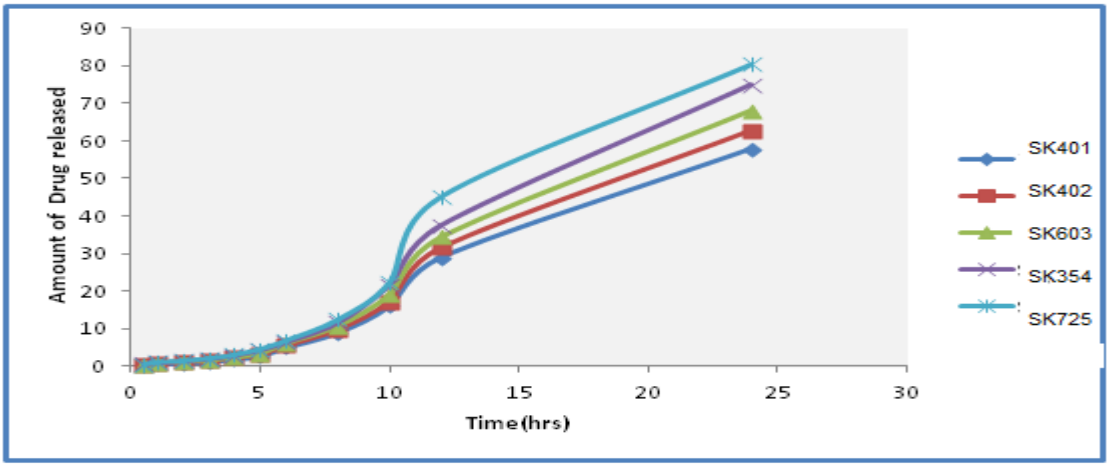

Fig. 21 Zero order kinetic treatment for release data

First order kinetic treatment for release data of oxymetazoline hydrochloride for encapsulated proniosomal gel

Table14 First order kinetic treatment for release data

Formulation code	Equation of line	Correlation coefficient(r^2)
SK403	Y = -0.0161X+ 2.045	0.931
SK603	Y = -0.0183X+ 2.0544	0.925
SK803	Y = -0.0211X+ 2.0642	0.922
SK353	Y = -0.0352X+ 2.078	0.914
SK723	Y = -0.0299X+ 2.0945	0.908

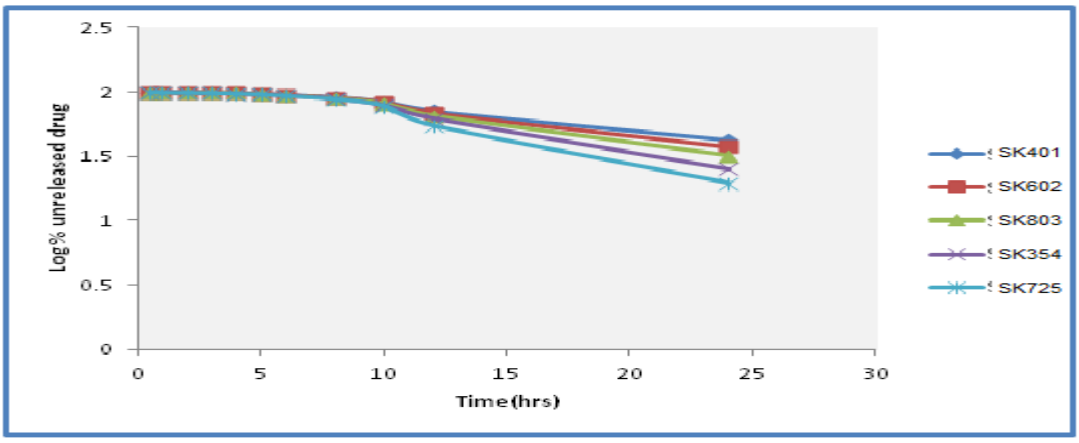

Fig.22 First order kinetic treatment for release data

Higuchi square root treatment for release data of oxymetazoline hydrochloride for encapsulated proniosomal gel

Table 15. First order kinetic treatment for release data

Formulation code	Equation of line	Correlation coefficient(r^2)
SK403	Y = 11.085X- 13.54	0.738
SK603	Y = 12.012X- 14.633	0.737
SK803	Y =13.082X-15.971	0.738
SK353	Y = 14.354X- 17.309	0.742
SK723	Y = 15.706X-18.951	0.738

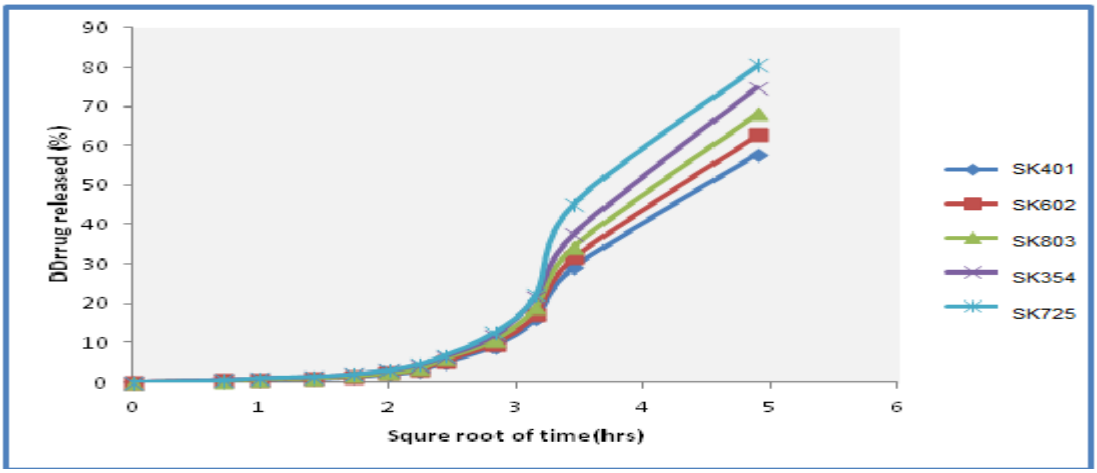

Fig. 23 Graph showing Higuchi square root treatment for release data

Physical stability studies

Stability study was carried out to investigate the degradation of drug from proniosomal powder formulation during storage. The stability study of all prepared formulation were performed by storing 4ºc, 25ºc and 45ºc for a period of 45 days. Throughout the study, proniosomal formulation was stored in aluminium foil sealed glass vials. The formulation was analyzed for the drug content spectrophotometrically. In the stability study one of the best formulation of each proniosomalgel (SK723) was selected for the stability studies on the basis of entrapment efficiency and in vitro release profile

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Stability studies data

Table 16 Drug leakage of proniosomal gel formulation SK723 at various temperature

Time(days)	4ºC		25ºC		45ºC	
	% drug retained	% drug lost	% drug retained	% drug lost	% drug retained	% drug lost
0	100	-	100	-	100	-
15	87.32±0.12	1.08±0.21	85.24±0.67	2.76±0.12	74.46±0.34	23.54±0.32
30	96.17±0.78	2.53±0.32	91.02±0.94	8.06±0.18	57.76±0.45	42.33±0.19
45	95.22±1.54	2.98±0.22	81.46±1.54	16.54±0.31	34.21±1.67	63.79±0.29

**Mean±SD, n=3. SD: Standard deviation

Stability study profile of SK723 at 25ºC±2ºC

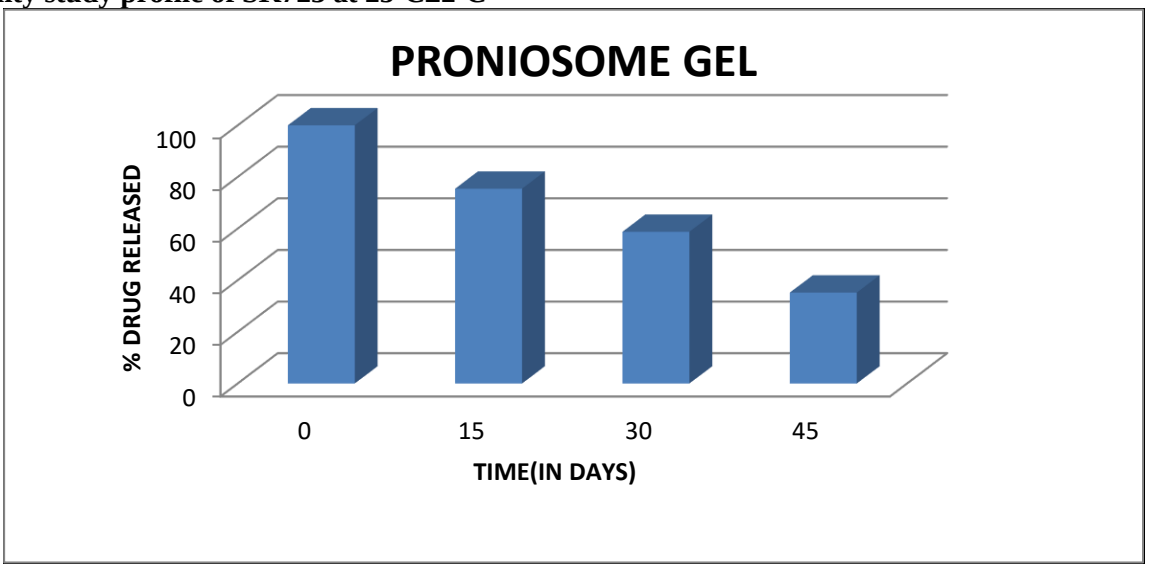

Fig.24 Graph showing stability study profile of SK723 at 25ºC±2ºC

Antifungal activity of formulation against *candida* strain

The microbiological assay is based upon a comparison of inhibition of growth of micro-organisms by measured concentrations of test compounds with that produced by known concentration of a standard antibiotic. Two methods generally employed a returbidometric(tube-dilution) method and cylinder plate (cup-plate) method.

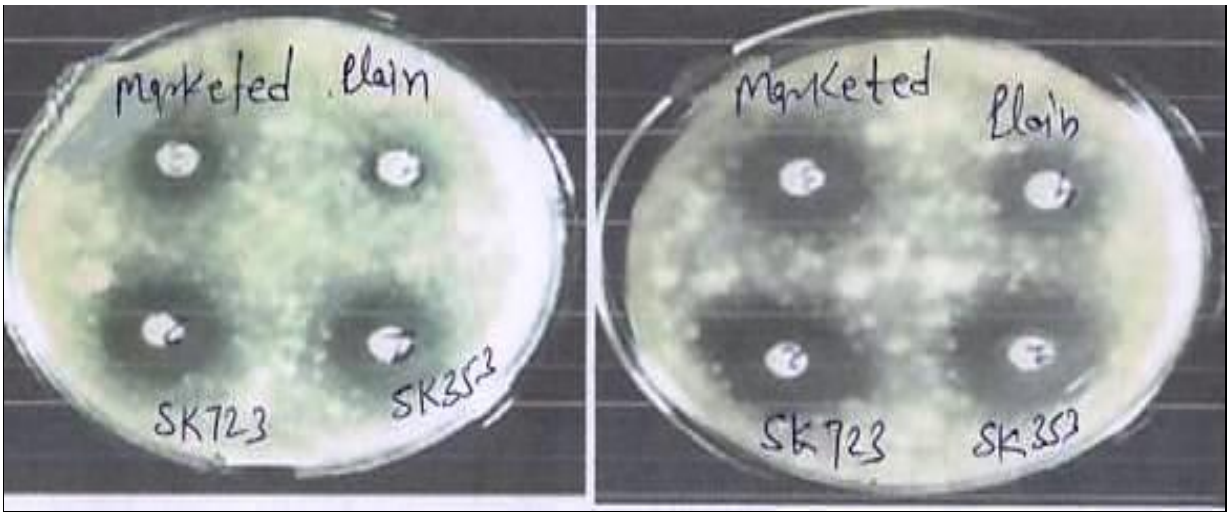

Fig. 25 & 26 *In vitro* antifungal activities

Antidermatophytic activity of formulation against (*trichophytonrubrum)*

The *invitro* antidermatophytic activity of optimized provesicularge laws performed using agar disc diffusion technique. Standard tests train of *Trichophyton rubrum*(MTCC 8477) was obtained from the Microbial Type Culture Collection Centre, Institute of Microbial Technology, CSIR, and Chandigarh. Further, microbial growth inhibitory properties of test formulations were determined by disc diffusion method. Sterile Sabouraud's Dextrose Agar medium was prepared using culture medium (Dextrose 40 gm, Peptone 10 gm, Agar 2 gm and Distilled water 1 L) at Temp 25□C and ph - 5.5. The plates were inoculated with 100µl standardized culture of test organism *Trichophytonrubrum* (aerobic, incubation time – 48 hrs) and exposed to air drying at room temperature under asceptic conditions for15min.The in-vtro antifungal study of optimized formulation was studied by cup-plate method. Following results of antifungal activity are shown in table. The zone of inhibition is more observed in oxymetazoline hydrochloride loaded proniosomal gel formulation (SK723) compared to other formulation and plain drug was taken as a standard. The marketed formulation (Brimonidine 1% cream) showed less zone of inhibition compared to optimized proniosomal gel formulation (SK723). From the following study it eas concluded that the prepared proniosomes gel gormulation exhibited promising antifungal activity.

Table 17 .*In-vitro* antifungl ativity

S.no	Formulation	Zone of Inhibition
1.	Plain drug	19±0.05
2.	Marketed product (brimonidine 1%)	12
3.	SK401	-
4.	SK403	3
5.	SK603	9
6.	SK723	16
7.	SK353	9.5
8.	SK803	4

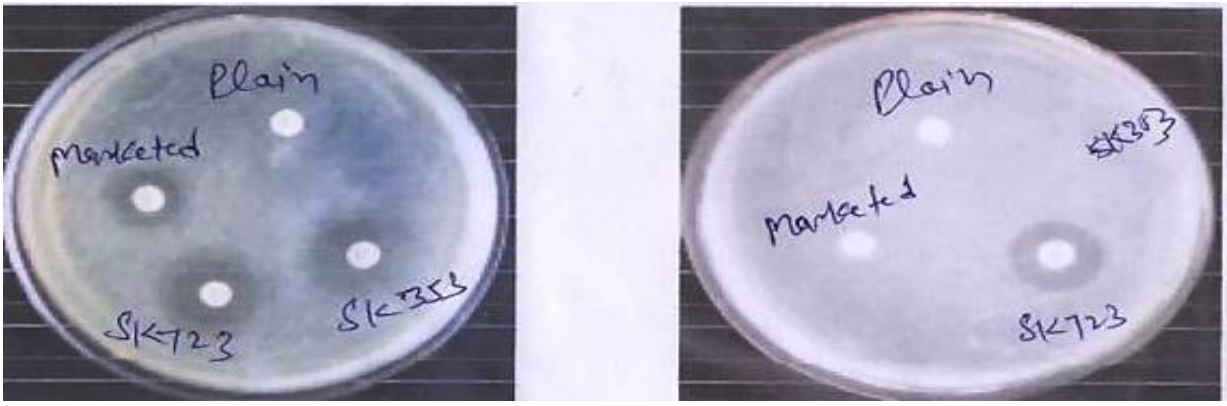

Fig. 27& 28 *In vitro* antidermatophytic activity

The formulations (proniosomal preparation SK723; plain drug marketed gel) were initially dissolved in DMSO and prepared at concentrations of 1000, 750,500,250µg/ml .Then antifungal disks (6mmindiameter) impregnated with test formulation (prepared in DMSO) at conc. mentioned were placed on surface ofinoculatedmediaplates.Theagarplateswereincubatedat25^{0C}for3days.Clearzonesofinhibitiononagarsurfacearoundthediscwasmeasuredinmm,includingthediameterofthedisc[38,39].

In-vivo study

The in-vivo studies are important to evaluate the physical availability of drug and from developed delivery system. These studies are generally performed either by observing the pharmacological response or by constructing a drug plasma concentration versus time profile and determining different pharmacokinetic parameter using a suitable animal model. Nine male white rabbits (2.50-2.75Kg), obtained from the Animal house, Institute of pharmacy, Dr. A. P.J. Abdul Kalam university Indore M.P. were used in this study. Animals were not studied until after two-week environmental adjustment period. Rabbits were kept individually in metal cages, fitted with wire floors to reduce coprography.

Plasma drug concentration study

Table 18. Plasma concentration of drug and formulation

S.No.	Time(hrs)	Plasma concentration of oxymetazoline hydrochloride (µg/ml)		
		Plain drug	MarketedGel	Proniosomegel (Sk723)
1	0	0	0	0
2	1	12.48±1.34	19.23±3.15	25.12±3.61
3	2	35.35±3.06	39.86±4.36	58.33±3.81
4	4	49.21±2.71	92.16±2.14	168.23±1.21
5	6	43.27±3.21	165.23±3.56	199.87±2.11
6	8	30.32±4.12	123.95±1.48	170.23±3.12
7	12	15.64±7.32	90.23±3.52	150.45±4.67
8	16	11.01±2.17	49.42±2.09	122.12±3.32
9	24	7.23±1.07	36.21±5.40	90.47±4.57

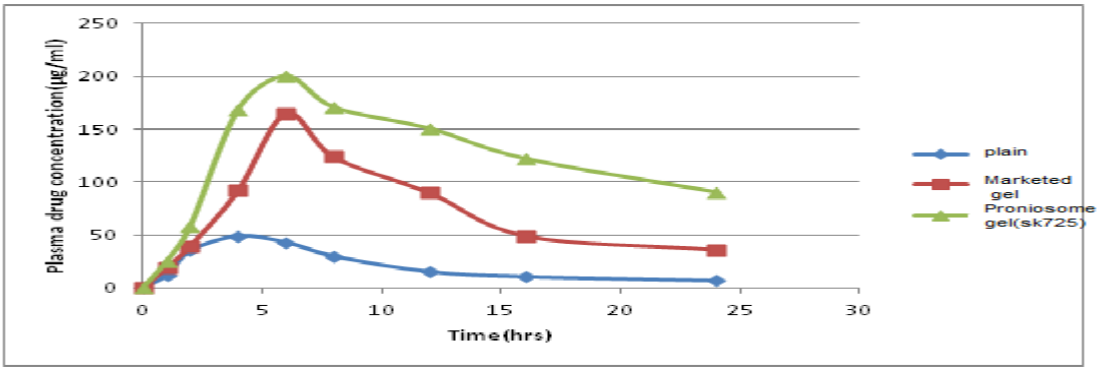

Fig. 29 Plot between mean plasma concentrations (µg/ml) of oxymetazoline hydrochloride and time for gel, powder and plain formulations

Pharmacokinetic parameter of oxymetazoline hydrochloride

Table 19. Results of pharmacokinetic assay of drug and proniosome formulation of oxymetazoline hydrochloride

S.No.	Formulation code	C_{max}(µg/ml)	AUC(µg.hr/ml)
1	Plain drug solution	48.21±2.71	479.67±1.45
2	Marketed gel	146.23±3.56	1757.12±3.32
3	Proniosomal Gel formulation(SK723)	198.77±2.11	2946.90±2.76

Comparative study profile of Marketed product and optimized proniosome gel formulation

Table 20. Comparative study profile of Marketed product and optimized proniosome gel formulation

S.No.	Formulation code	Entrapemet Efficiency	In vitro % drug released	C_{max} (µg/ml)	AUC (µg.hr/ml)
1.	Marketed product	74.32%±1.51	70.32±0.89	145.23±3.56	1675.12±3.31
2.	ProniosomalGel formulation(SK72)	81.47%±1.55	85.37±0.67	198.77±2.11	2946.90±2.76

Skin Irritation Study The proniosomal gel formulation (100 mg) was evenly applied to an area of about 6 cm of skin of left lateral surface of the rabbit under a gauze patch. Right untreated shaven surface was considered as control area which was also covered with gauze patch.

Table 22. Skin Irritation Study

	Time (hrs)	No. of hours				
		1	2	3	4	5
Skin Irritation	SK 403	No Skin Reaction	No Skin Reaction	No Skin Reaction	No Skin Reaction	No Skin Reaction
	SK 603	No Skin Reaction	No Skin Reaction	No Skin Reaction	No Skin Reaction	No Skin Reaction
	SK 803	No Skin Reaction	No Skin Reaction	No Skin Reaction	No Skin Reaction	No Skin Reaction
	SK 353	No Skin Reaction	No Skin Reaction	No Skin Reaction	No Skin Reaction	No Skin Reaction
	SK 723	No Skin Reaction	No Skin Reaction	No Skin Reaction	No Skin Reaction	No Skin Reaction

RESULTS AND DISCUSSION

Proniosome gel containing Oxymetazoline hydrochloride were prepared by a coacervation phase separation method. The entrapment efficiency of proniosomal gel was found to be in the range of 40.0% - 82.56 %. The entrapment efficiency of proniosomal gel was attributed due to the amphiphillic nature of the drug. The entrapment efficiency was found maximum for SK723 formulation due to higher HLB value, of the formulation, which result in larger vesicle hence more entrapment of drug into the vesicle.

The effect of cholesterol on Oxymetazoline hydrochloride entrapment was varied according to the nonionic surfactant used, cholesterol was found to have little effect on the Oxymetazoline hydrochloride entrapment was obtained when 10% of cholesterol was incorporated into niosome, prepared from span 40 & span60, followed by decrease in encapsulation efficiency of the drug upon further increase in cholesterol content.

As the Brij72: CHL (SK723) formulation showed the highest entrapment. It was selected for further optimization the total concentration of surfactant mixture Brij 72: Cholesterol was kept constant, the ratio of Brij 72: cholesterol was varied from 10: 90 to 90: 10 to investigate the effect of this ratio on Oxymetazoline hydrochloride. Formulation Brij72 containing 50:50, Brij72/ CHL ratios showed the highest entrapment that is 81.3%. The increase in entrapment efficiency with increase in cholesterol content can be explained by the fact that cholesterol intercalated into the bilayer preventing the leakage of the drug through the bilayer. This could be due to the surfactant chemical structure, all span surfactant here same head group and different alkyl chain, increasing the higher alkyl chain length is leading to higher entrapment efficiency. The entrapment

efficiency order is Brij72>span 60 >brij 35>span 40> span80.The cholesterol content affects the membrane elasticity making the membrane rigid. A decline in the entrapment efficiency beyond a certain cholesterol level. Surfactant used to make non-ionic surfactant vesicle have low aqueous solubility, however freely soluble non-ionic surfactant such as Brij72can form micelles on hydration due to the presence of more polar head group in the chain, in the addition of cholesterol they abolish the more polar part present in surfactant mainly due to lipophillic in nature and help in formation of vesicle in the equimolar ratio of Brij72 and cholesterol show better result.

The proniosome formation takes place from Brij72with the presence of cholesterol, the length of alkyl chain show a crucial factor of permeability, Brij72have long lauryl(c12) chain, thus the long chain influence the HLB of the surfactant and also lead to the higher drug entrapment efficiency and also show better stability of the proniosome using Brij72. Cholesterol is one of the most important additives included in the formulation in order to prepare stable niosome. Cholesterol stabilize bilayer, prevents leakiness and retards permeation of solutes enclosed in the aqueous core of these vesicle. The release study was conducted for all the optimized formulation showing better entrapment efficiency, optimum vesicle size, Spherical surface morphology). Most of the formulation were found to have alinear release and the formulation (SK723) was found to provide approximately 85.37% release with in a period of 24hrs. The amount of drug release from different proniosomal gel formulation was found in order of SK723>SK603>SK353>SK803> SK403.

The formulation SK723 (50% Brij 72 and 50% cholesterol) showed highest amount of % drug released (85.37%). The enhanced % drug release obtained from the proniosomal gel system could be justified on the basis of dual function performed by ethanol present in the proniosomal formulations, length of side chain, i.e. fluidizing both the vesicular lipid bilayer and greater malleability to the vesicles and enhancing permeability of the skin. Overall, the data clearly indicate that the proniosomal gel formulation SK723 (50% w/w cholesterol and 50% w/w Brij-72) showed the highest entrapment efficiency (82.56 %), optimum size (315.6 nm), highest cumulative amount of % drug released. Thus justifying itself as an optimized formulation and used for further skin *in-vivo* studies.

*Invitro*antifungalsusceptibilitytestsareusedtodeter mineclinicaloutcomebaseduponanoptimizationofa ntifungaltherapy.Standard test strain of *Trichophyton rubrum*(MTCC8477) and *Candidaalbicans* (MTCC 227)were obtained from the Microbial Type Culture Collection Centre, Institute of Microbial Technology, CSIR, Chandigarh(Table 9).The lowest concentration at which the isolate is completely inhibited (as evidenced by the absence of visible fungal growth) is recorded as the minimal inhibitory concentration or MIC. The drug microbiological inhibition data obtained for test formulationsSK723,SK353, against *Trichophyton rubrum* was subjected to statistical Mann–Whitney-U test for pair wise comparison with control preparation (plain drug marketed gel) (Fig 28).For all comparisons $*P < 0.05$ was considered as significant. The data were reported as mean+SD (n=3) (Table10). Antifungal susceptibility test in gis evolving very rapidly since last decade and has now become are levant clinical and diagnostic tool [133]. Further anticandidal studies were performed for optimized formulations. The tests revealed that during study period of 3 days, the formulations SK603, SK353 and SK723showedgradual increase in zone of inhibition due to the controlled release of medicament. The gel containing vesicles showed initial burst of drug (due to availability of unentrapped drug) and after 2 hours showed sustained release (due to gradual release of medicament). The antifungal activity of vesicle gel prepared showed sustained release of medicament by regular intervals when compared to drug Brij 35 gel and marketed preparation against *Candida* This *in-vivo* study of different formulations was carried out by means of plasma drug concentration measurement of plain Oxymetazoline hydrchloride, proniosomal gel, marketed Preparation, were applied to an area of about 6 cm of skin of left lateral surface of the rabbit under a gauze patch. Right untreated shaven surface was considered as control area which was also covered with gauze patch an equivalent amount of 5.5 mg Oxymetazoline hydrchloride for 24 hrs. The proniosomal gel, marketed Preparation and plain drug suspension showed the maximum blood level concentration 199.87 ± 2.11, 165.23 ± 3.56 µg/ml, 49.21 ± 2.71 µg/ml respectively (Fig. 7.57 and Table 7.46). Proniosomal gel formulation showed nearly four times better blood level concentration than plain

Oxymetazoline hydrchloride, representing greater permeation. Plasma levels of Oxymetazoline hydrchloride after application of plain Oxymetazoline hydrchloride suspension were low (C_{max}=49.21 ± 2.71 µg/ml) while those after application of the proniosomal gel formulation gradually increased and reached a peak level of 199.87 ± 2.11µg/ml at 6 hr post administration and maintained for longer times. The area under the curve (AUC) of the proniosomal gel was found to be 3055.90 ± 2.76 µg.hr/ml (Table 7.47) which was much larger than 1768.12 ± 3.32 µg.hr/ml being AUC of marketed Preparation. Marketed Preparation suspension formulation also showed higher drug level (165.23 ± 3.56 µg/ml) in comparison to plain Oxymetazoline hydrchloride suspension, yet lower as compared to proniosomal gel formulation. It may also noted that the concentration of Oxymetazoline hydrchloride observed higher in blood after topical application of proniosomal gel than *in-vitro* drug release study of proniosomal liquid formulation. It is probably due to intrinsic action of gel containing proniosomes acting as a reservoir in the skin, might release drug very soon, penetrate deeper into the skin layers and come in contact with blood vessels in the dermis resulting in higher serum drug concentration and maintain drug level for prolong period of time. The slower release rate of drug from Oxymetazoline hydrchloride suspension is due to barrier properties through which drug has to pass for systemic `delivery.

Kinetic data treatment

To find out the kinetics and mechanism of drug released from all the formulations of Oxymetazoline hydrochloride encapsulated proniosomes, the data were treated according to zero order, first order and Higuchi's equation pattern. In the case of proniosomal gel the correlation coefficient of the formulation (SK723) was found 0.951 in zero order equation pattern. When the data were plotted according to first order equation, the correlation coefficient was found to be 0.908. Hence the formulation (SK723) follows zero order kinetics. These results pointed to sustained release delivery of drug. This slow release pattern of entrapped drug may indicate the high stability of the Proniosomal formulation.

Conclusion

In conclusion, based on the result presented in Table 20, Proniosomal gel formulation have demonstrated promising characteristics as drug carriers. The vesicle size of proniosomal gel derived niosome was larger compared to other forfulation derived from the Proniosomal sources. The cumulative release of drug from proniosomal gel SK723 reached be 85.37% at 24th hour., showing a constant and controlled profile between 10^{th} to the 24^{th} hour, this indicates that the Proniosomal gel exhibited a good controlled release profile.

In contrast, other proniosomal formulations showed controlled release from the 11th to the 24th hour, with a controlled release value of 71.42% at the 24th hour. Therefore, it can be concluded that the release profile of proniosomal gel SK723 was better than other proniosomal formulations. In terms of stability, proniosomal gel showed good stability at both room temperature and refrigeration temperature, exhibiting a transition phenomenon. Thus, it is recommended to store the proniosomal gel formulation at either refrigeration or room temperature to minimize drug loss.

The improved stability of proniosomal gel may be attributed to the presence of ethanol in the proniosomal gel system, which provides greater stability to the vesicles compared to other vesicular systems. Additionally, proniosomal gel formulation showed higher entrapment efficiency and utilized alcohol as a penetration enhancer. This resulted in an increase in percutaneous permeation of Oxymetazoline hydrochloride in in vitro experiments. The proniosomal gel ensured sustained release of the drug, leading to a prolongation of its therapeutic activity, possibly through the accumulation of Oxymetazoline hydrochloride in the skin.

These findings are highly encouraging and confirm that proniosomes are a promising carrier for topical administration, offering enhanced drug delivery through the skin. This opens up various opportunities for the development of suitable therapeutic strategies via the topical route. The formulation is easy to scale up as the procedure is simple and does not involve lengthy processes or the unnecessary use of pharmaceutically unacceptable additives. It also enables direct fabrication of transdermal patches without the need for dispersing the vehicle into a polymer matrix.

Based on stability studies, it was concluded that proniosomal gel possesses a longer shelf life and greater stability compared to conventional forms. These results highlight the potential of

proniosomal gel formulations as effective and stable drug delivery systems for topical applications. [6, 9, 13]

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Authors' contributions
All the authors have contributed equally.

Conflicts of interest
The authors have declared no conflicts of interest.

References
1. Shahiwala A;Misra A; Studies in topical application of niosomally entrapped nimesulide. J Pharm Pharm Sci. 2002; 5 :220-225.
2. Khandare JN., Madhavi G., Tamhankar BM. Niosomesnovel drug delivery system. East Pharm. 1994; 37: 61-64.
3. JY. Fang, Yu. S, Wu PC, YB. Huang, YH Tsai. In-vitro skin permeation of estradiol from various proniosomes formulation. Int J Pharm. 2001; 215:91-99.
4. C. Hu, DG Rhodes. Proniosomes. A Novel Drug Carrier Preparation. Int J Pharm. 1999; 185: 23-35.
5. Abubkar O Nur, Zhang JS. Recent progress in sustained/controlled oral delivery of captopril. An overview. Int J Pharm. 2000; 194:139-146.
6. Zhou XH, Li Wan PA. Stability and in-vitro absorption of captopril, enalapril and lisinopril across the rat intestine. Biochem Pharmacol. 1994; 47:1121- 1126.
7. Tripathi KD. Essentials of Medical Pharmacology. New Delhi, India, Jaypee Brothers, 2003; 449-454.
8. Jain NK, Vora B, Khopade AJ. Proniosome based transdermal delivery of levonorgestrel for effective contraception. J Control Rel. 1998; 54:149-165.
9. Nagarsenker MS, Londhe VY, Nadkarni GD. Preparation and evaluation of liposomal Formulation of tropicamide for occular delivery. Int J Pharm. 1999; 190: 63-71.
10. Bhatia A, Kumar R, Katare OP. Tamoxifen in topical liposomes: Develoment, characterization and invitro evaluation. J Pharm Pharm Sci. 2004; 7:525-259.
11. Joshi YM, Bachman WR, Jani NB. New pharmaceutical composition in the form of beadlets and method. European Patent, 1988.
12. Singh J, Robinson DH. Controlled release kinetics of captopril form tableted microcapsules. Drug Dev Ind Pharm.1988; 14: 545-560.
13. Leung SHS, Robinson JR. The contribution of anionic polymer structure feature to mucoadhesion. J Contrl Rel.1998; 5: 223-231.
14. Sheth PR, Tossounian J. The hydrodynamically balanced system (HBSTM): A novel drug delivery system for oral use. Dev Ind Pharm. 1984; 10:313-339.
15. Seta Y, Higuchi F, Kawahara Y, Nishimura K, Okada R. Design and preparation of captopril sustained release dosage form and their biopharmaceutical properties. Int J Pharm. 1988; 41: 245-254.
16. Volland C, Woleff M, Lissel T. The influence of terminal gamma sterilization on captopril containing poly CD, L-lactic co Glycolide microsphere. J Control Rel.1994; 31:298-305.
17. F Glazener, K Blake, and M Gradman. Bradycardia, hypotension, and near-syncope associated with afrin (oxymetazoline) nasal spray. N Engl J Med. 1983; 12:731-739.
18. Thrush DN. Cardiac arrest after oxymetazoline nasal spray. J Clin Anesth. 1995; 7: 512-514.
19. Yerikala Ramesh, Kavya Teja P, Karuna Santhi K, Saraswathi H, Neeharika P, Saideepthi B, Formulation and evaluation of Losartan loaded Proliposomal gels: International Journal of Pharmacometrics and Integrated Biosciences. 2017; 2:173-178.
20. S Gizurason. The relevance of nasal physiology to the design of drug absorption studies: Adv. Drug Deliv. Rev. 1993; 11: 329-347.
21. LL. Ning. Characteristics of ophthalmic in situ gel and issues in progress. Chin. J. New Drugs. 2007; 16 :988-990.
22. Saudagar. Formulation Development and Evaluation of Topical Gel of Lisinopril Dihydrate: Scholars Academic Journal of Pharmacy.2016 ; 5: 277-283.
23. Kanahaiya. Agrawal. In-Situ Gel Formation for topical Drug Delivery System: Asian Journal of Biomedical and Pharmaceutical Sciences. 2011; 1: 01-07.
24. Kavitha D. Formulation and evaluation of intra Topical gel of Levofloxacin: European journal of pharmaceutical and medical research. 2017; 4: 249-253.

25. PatilSonali K. Formulation and Evaluation of Topical Gel for Alzheimer Disease: International Research Journal of Pharmaceutical and Biosciences. 2015; 2:41-58.
26. Schreier H, Bouwstra J. Liposomes and niosomes as topical drug carriers: Dermal and transdermal drug delivery. J Control Release. 1994; 30: 1-15.
27. Touitou E, Dayan N, Bergelson L, Godin B, Eliaz M. Ethosomes-novel vesicular carriers for enhanced delivery: Characterization and skin penetration properties: J Control Release. 2000; 65: 403-18.
28. Patel KK, Kumar P, Thakkar HP. Formulation of niosomal gel for enhanced transdermal lopinavir delivery and its comparative evaluation with ethosomal gel: AAPS Pharm Sci Tech. 2012; 13:1502-10.
29. Ammar HO, Ghorab M, El-Nahhas SA, Higazy IM. Proniosomes as a carrier system for transdermal delivery of tenoxicam. Int J Pharm. 2011; 405:142-52.
30. Oommen E, Tiwari SB, Udupa N. Niosome entrapped B-cyclodextrin methotrexate complex as a drug delivery system. Indian J Pharm.1999;31:279-84.
31. Alsarra IA, Bosela AA, Ahmed SM, Mahrous GM. Proniosomes as a drug carrier for transdermal delivery of ketorolac: Eur J Pharm Biopharm. 2005; 59: 485-90.
32. Tank CJ, Borkhataria CH, Baria AH. Formulation and evaluation of aceclofenac loaded maltodextrin based proniosomes :Int J Chem Technol Res. 2009; 1:567-73.
33. Azarbayjani AF, Tan EH, Chan YW. *In vitro* drug release of niosomesprepared from various proniosomal haloperidol by Proniosomal formulations with non-ionic surfactants. Biol Pharm Bull.2009: 32:1453-8.
34. Rhodes DG. Proniosomes a novelcarrier preparation. Int J Pharm. 1999; 185:23-35.
35. Duttshukla N, Tiwari M. Proniosomal drug delivery systems-clinicalapplications. Int J Res Pharm Biomed Sci. 2011 ;29:880-7..
36. Baldwin HE. Diagnosis and treatment of rosacea: state of the art. J Drugs Dermatol, 2012; 11:725-730.
37. National Rosacea Society. Rosacea riddle now threatens more than 16 million Americans [press release]. Barrington, IL: National Rosacea Society; 2010.
38. Tan J, Blume-Peytavi U, Ortonne JP, et al. An observational cross-sectional survey of rosacea: clinical associations and progression between subtypes. Br J Dermatol.2013; 169:555-562.
39. Wilkin J, Dahl M, Detmar M, et al. Standard classification of rosacea: report of the National Rosacea Society Expert Committee on the Classification and Staging of Rosacea. J Am Acad Dermatol. 2002; 46:584-587.
40. Wilkin JK. Rosacea: pathop.hysiology and treatment. Arch Dermatol,1994;130:359-362.
41. Thimmaraju D. R, Kurnool A.N. et. al. Proniosomal gel formulation and characterization of an antifungal drug (Butenafine Hcl) loaded proniosomes for topical delivery.Global journal of medical research.2020; 20(5):35-46.
42. Goswami S, Nakhuru KS, Das J , Srivastava RB. Phytochemicals and antidermatophytic activity of methanolic extract of *Cestrum nocturnum* leaves. Ind Drugs. 2009; 46: 619-22.
43. Pakshir K, Bahaedinie L, Rezaei Z, Sodaifi M, Zomorodian K. *Invitro* activity of six antifungal drugs against clinically importantdermatophytes. Jundishapur J Microbiol. 2009; 2: 158-63.
44. Barros ME, Santoro DA, Hamdan JS. *In vitro* methods for antifungal susceptibility testing of *Trichophyton* sp. Mycol Res. 2006; 110: 1355- 60.
45. Yenisehirli G,Tuncoglu E,Yenisehirli A,Bulut Y.*In vitro* activities of antifungal drugs against dermatophytes isolated in Tokat, Turkey. Int J Dermatol 2013; 52:1557-60.
46. Barry AL, Pfaller MA, Rennie RP etal. Precision and accuracy of fluconazole susceptibility testing by broth microdilution, Etest, and disk diffusion methods. Antimicrob Agents Chemother. 2002; 46: 1781-84.
47. Panigrahi L, Ghosal SK, Pattnaik S, Maharana L, Barik BB. Effect of permeation enhancers on the release and permeation kinetics of lincomycin hydrochloride gel formulations through mouse skin. Ind J Pharm Sci. 2006; 68: 205-11.
48. Williams FM. *In vitro* studies-how good are they at replacing *in vivo* studies for measurement of skin absorption? Environ Toxicol Pharmacol. 2006; 21:199-03.
49. Csóka I, Csányi E, Zapantis G, Nagy E, Fehér-Kiss A, Horváth G, Blazsó G, Eros I.*In vitro* and *in vivo* percutaneous absorption of topical dosage forms: Case studies. Int J Pharm.2005; 291:11-19.