MAJOR RESEARCH PROJECT

Formulation and evaluation of niosomal in-situ gel for ocular drug delivery system using newer bioadhesive polymer

F No. 43-503/2014(SR)

Final Report Submitted

To

University grants commission

Bahadur Shah Zafar Marg, New Delhi 110002

Submitted by

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DECLARATION AND CERTIFICATE

I hereby declare and certify that, the Major Research Project entitled **"**Formulation and evaluation of niosomal in-situ gel for ocular drug delivery system using newer bioadhesive polymer**"** F No. 43-503/2014(SR) is a bonafide record of research work carried out by me during the year 2015-2018. Further certify that the work presented in the report is original and carried out according to the plan in the proposal and guidelines of the University Grants Commission.

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CERTIFICATE

This is to certify that the work incorporated in the report entitled "**Formulation and evaluation of niosomal *in-situ* gel for ocular drug delivery system using newer bioadhesive polymer**" F No. 43-503/2014(SR) is an original contribution submitted to the UNIVERSITY GRANTS COMMISSION, Bahadur Shah Zafar Marg, New Delhi by **Dr. Pravin D. Chaudhari** based on the research work carried out by him during the year 2015-2018.

Such material has not been submitted to any other university / institute for any financial support. The literature related to the problem investigated has been appreciated cited and duly acknowledged wherever facilities and suggestions have been availed of.

Date: **Dr. Pravin D. Chaudhari**

Place: **Principal Investigator**

Forwarded through

Principal

CONTENTS

Annual Report of Major Research Project

First Year

The Chemicals were procured from different pharmaceutical companies. Prednisolone sodium phosphate (API) was obtained from Sai Lifesciences Private Limited, Hinjewadi, Pune.

The synthesis of polymers were carried out one by one with different concentrations of crosslinking agent (Divinyl glycol, divinyl benzene, 2,5-dimethyl-1,5-hexadiene). Synthesis was carried out at different temperatures with different curing times in order to obtain maximum yield. Completion of reaction was checked by TLC.

The polymers were evaluated for different parameters like density, polymer hydration, and effect of pH, ions and time on polymer swelling property. The samples of polymer were sent to university of Pune for DSC and XRD evaluation purpose. FTIR spectroscopy was performed to determine the probable structure and presence of functional group responsible for mucoadhesion. Bioadhesion test was performed on goat conjunctival membrane using modified tensiometer which was made out of weighing balance.

The irritation potential and tolerability was determined ex vivo by HET CAM test. In vivo Draize skin and eye irritation test was performed to determine irritation potential in rabbits.

Second Year

Preformulation study was carried out for API as well as excipients in order to confirm their purity and identity. UV analysis was done in order to find out the λmax of drug in water, phosphate buffer pH 7.4 and simulated tear fluid. Compatibility studies were carried out by FTIR and DSC.

Preliminary batches of noisome were prepared for selection method of preparation (thin layer formation), selection of surfactant (span 60), and ratio of cholesterol and span 60 (1:1). These batches were evaluated for drug content, entrapment efficiency and particle size. The batches prepared for selection of ratio cholesterol and span 60 were also evaluated for in vitro diffusion study and then optimized batch was obtained.

The optimized batch was further evaluated for polydispersity index, zeta potential, Transmission electron microscopy, digital motic microscopy. Stability studies were performed according to the guidelines of ICH.

Preliminary batches of blank in situ gel were prepared by cold method where poloxamer 407 and poloxamer 188 were dissolved in water in varying concentrations and the ratio was fixed by measuring the gelation temperature. A series of batches were short listed which showed gelation temperature at 37°C.

Third Year

The shortlisted blank in situ gel batches were further incorporated with bioadhesive polymer (100 mg) and niosomal pellets. The niosomal pellets were prepared by freeze drying process. Benzalkonium chloride was added as preservative and the pH was adjusted by addition of 0.1M NaOH if required.

This final formulation was evaluated for other gelling properties like gelling strength, gel melting temperature, gelling time, pH, mucoadhesive strength, spreadability. The rheogram of gel was studied by measuring viscosity at different temperature conditions and different shear rates.

The in vitro and ex vivo drug diffusion study was carried out by using Franz diffusion cell containing cellophane membrane and goat conjunctival membrane respectively. In vivo pharmacodynamics study included Draize skin and eye irritation test in order to find any irritation caused by this formulation in rabbits. In vivo pharmacokinetic study was carried out in rabbits where aqueous humor concentration was measured where paracentesis technique was used to withdraw aqueous humor samples.

Sterility studies was carried out to determine contamination if any in the formulation. Stability study of the whole formulation was carried out according to ICH guidelines.

INTRODUCTION

Origin of the research problem:

Development of any ophthalmic formulation for the treatment of eye disorders or diseases is a very difficult and most challenging to the pharmacists. In the development of ophthalmic formulation main constraint is the anatomy, physiology and biochemistry of the eye because it renders the eye acquisitively impervious to foreign substances. Although continues efforts are being developed to the improvement and optimization of ophthalmic formulation, successful treatment of various eye pathologies depends on intrinsic activity of the drug, the ability of the drug to cross numerous biological barriers to reach a biosphere and maintenance of the drug in the biosphere for an extended period. The ophthalmic formulations are not developed at faster rate as compared to other drug delivery routes such as oral, transdermal, transmucosal, etc. The challenge to the formulator is to circumvent the protective barriers of the eye without causing permanent tissue damage. Delivery of the drug to the eye is complicated by the efficient removal mechanisms in the precorneal area, which serves to maintain a good refractive surface. In an idealized case, the drug delivery system is based on the pharmacodynamic and pharmacokinetic parameter of drug. The absorption of drug into the eye requires a prolonged precorneal residence time and good corneal permeation however for most drugs corneal permeation is low.

The vast majority of existing ophthalmic delivery systems such as solutions, suspensions, ointments, etc. are still fairly primitive and inefficient. Inspite of some limitations eye drops (solution or suspension) are most prescribed dosage form. About 90% of available ophthalmic formulations in United State are eye drops and similar percentages are still presumably valid for the current global market. Eye drops are relatively easy to prepare and sterilize. Repeated application can cause biochemical or mechanical injury as well as sensitivity reactions resulting in blepharitis or conjunctivitis. The local and systemic toxicity can be significantly minimized by intervention with newer ophthalmic delivery systems. Frequent local instillation of antiglaucomaagents, antibiotics, antiviral and sulfonamide provide an unusually high drug and preservative concentration at the epithelial surface.

To overcome the disadvantages regarding conventional dosage forms vascular systems were used as -

Niosomes

Niosomes are non-ionic surfactant vesicles obtained on hydration of synthetic nonionic surfactants, with or without incorporation of cholesterol or other lipids. This is systems

similar to liposomes, can be used as carriers for both hydrophilic and lipophilic drugs. It is promising vehicle for drug delivery and being non-ionic, less toxic and improves the therapeutic index of drug by restricting its action to target cells and by preventing drug degradation from different enzymes. Niosomes are microscopic lamellar structures obtained on admixture of non-ionic surfactant of the alkyl or dialkylpolyglycerol ether class and cholesterol with subsequent hydration in aqueous media.

Structure of niosome:

Niosomes may act as a depot, releasing the drug in a controlled manner. The therapeutic performance of the drug molecules can also be improved by delayed clearance from the circulation, protecting the drug from biological environment and restricting effects to target cells. It can also be used as vehicle for poorly absorbable drugs to design the novel drug delivery system. It enhances the bioavailability of drug by crossing the anatomical ocular barriers. Niosomes provides better drug concentration at the site of action.

Types of niosomes

i. Small Unilamellar Vesicles (Size = 0.025-0.05μm)

ii. Multilamellar Vesicles (Size = > 0.05 μm)

iii. Large Unilamellar Vesicles (Size = > 0.10 μm)

Components of Niosomes:

1. Drug
2. Surfactant
3. Cholesterol
4. Charge
5. Solvent (ether or ethanol)
6. Phosphate buffer

Polymeric system development for increased bio-adhesion and residence time:

A series of cross-linked, swellable polymers was synthesized from monomer i.e acrylic acid, others with various cross-linking agents to produce a range of polymers differing in charge densities and hydrophobicity. The densities, rate, and extent of hydration of the polymers were determined. An increase in the number of hydrophobic groups in the polymer structure reduced hydration whereas the density of the polymer was unaffected. A sensitive in vitro method for measuring adhesion of polymer to tissue from the rabbit eye was developed. Polymers of acrylic acid loosely cross-linked **(0.3%,** w/w) with three different agents, Poly (ethylene Glycol) PEG 400, showed the higher degree of bioadhesion. while poly (acrylic acid-poloxamer 407) showed reduced bioadhesion. The small percent of cross-linking agent,

irrespective of physicochemical properties, did not contribute substantially to bioadhesion, whereas the starting monomer had a large effect. The effect of pH on the bioadhesion of poly(acry1ic acid- PEG 400) was studied at constant temperature, ionic strength, and osmolality. The polymer showed maximum adhesion at pH 5 and 6 and a minimum at pH 7. Ocular bioadhesion studies of cross-linked polymers in rats were studied. Other polymer derivatives are synthesized and studied for hydration and bioadhesion properties.

Pathology of Eye

The eye consists of a number of individual anatomic and functional elements. In keeping with this, pathology of the eye covers the conjunctiva, cornea, uvea, lens, retina, vitreous and optic nerve. All these elements may responds to systemic diseases. A major proportion of blinding diseases are due to primary ophthalmic disorders such as glaucoma, cataract and macular degeneration.

One of the most common infections is bacterial conjunctivitis. It is associated with swelling of the eyelid and yellowish discharge. Sometimes it causes the eye to itch and affected the eyelids, particularly upon waking. The conjunctiva appears red and sometimes thickened. Often both eyes are involved.

In a few cases, the inflammation does not respond well to the initial treatment with eye drops. In those rare cases a second visit to the office should be made and other measures undertaken. In severe infection, oral antibiotics are necessary. Covering the eye is not a good idea because a cover provides protection for the germs causing the infection. If left untreated, conjunctivitis can create serious complications, such as infections in the cornea, lids and tear ducts.

In situ gelling system:

In-situ gel is drug delivery system that are in sol form before administration in the body, but ones administered undergo gelation, to form sol to**gel** (from the lat.*gelu*—freezing, cold, ice or *gelatus*—frozen, immobile) is a solid, like material that can have properties ranging from soft and weak to hard and tough. Gels are defined as a substantially dilute cross-linked system, which exhibits no flow when in the steady-state. By weight, gels are mostly liquid, yet they behave like solids due to a three-dimensional cross-linked network within the liquid. It is the crosslinks within the fluid that give a gel its structure (hardness) and contribute to stickiness. In this way gels are a dispersion of molecules of a liquid within a solid in which the solid is the continuous phase and the liquid is the discontinuous phase.

Interest in New Ophthalmic Drug Delivery System

To overcome the disadvantages of eye drops various ophthalmic drug delivery systems such as hydrogels, micro and nanoparticles, liposomes, collagen shields, etc. have been investigated. Drug delivery as it pertains to the eye is a generic term which is defined broadly as representing an approach to controlling and ultimately optimizing delivery of the drug to its target tissue in the eye. An optimum ophthalmic drug delivery system would be one which can be delivered in eye-drop form with no creation of blurred vision or irritancy and which would need no more than one to two instillations each day. The benefits to the patient are simplicity a diminished frequency of administration, lower toxicity and side effects.

METHODOLOGY

The study was divided into two parts namely:

Section I: Synthesis and evaluation of water insoluble but swellable bioadhesive polymer

Section II: Formulation and evaluation of niosomal in situ gel using newly synthesized polymer for ocular drug delivery.

Section I

Synthesis and evaluation of water insoluble but swellable bioadhesive polymer

Synthesis procedure

In 100 g acrylic acid was dissolved a mixture of 1 g of benzoyl peroxide (initiator). The cross-linking agent (divinyl benzene/ divinyl glycol/ 2,5-dimethyl-1,5-hexadiene) was added in three different concentrations (0.3/0.6/1 g) as shown in table no. 1. with stirring in to a solution containing 800 g of magnesium sulfate (MgS04. 7Hz0) in 100 mL of distilled water and refluxed at temperature of 95^0C. Polymerization was achieved within 30 min of reflux. After polymerization, the mixture was maintained at the same temperature with stirring for two hours of curing time. At the end of the reaction, the mixture was diluted with 150 mL of hot water and then repeatedly washed with equal portions of water. The washed cross-linked polymer was dried in a hot air oven at 90°C for a specified time before being ground to the required size (30-40 mesh).

Table No. 1 Synthesis scheme for polyacrylic acid along with the concentrations

Monomer (100 g)	**Cross-Linking agent**	**Quantity (g)**	**Polymer code**	**Initiator**
Acrylic acid	Divinyl glycol	0.3	DG1	
	Divinylbenzene		DB1	

	2,5,-Dirnethyl-l,5hexadiene		HD1	
Acrylic acid	Divinyl glycol	0.6	DG2	
	Divinylbenzene		DB2	
	2,5,-Dirnethyl-l,5hexadiene		HD2	
Acrylic acid	Divinyl glycol	1	DG3	Benzoyl peroxide
	Divinylbenzene		DB3	
	2,5,-Dirnethyl-l,5hexadiene		HD3	

Physicochemical Properties of Polymers

1. **Yield and Density**

The density of each polymer was determined in a 2 ml specific gravity bottle at 25°C. Benzene of known density (0.874 g/ml) was used as the medium because no swelling of the polymer in benzene was observed.

2. **Polymer hydration**

Each polymer was weighed 0.2g and allowed to hydrate in 10 mL of distilled water at 25°C in a 25-mL graduated cylinder. The volume of the hydrated polymer was measured at 5-min intervals until there was the rise in hydrated volume was constant.

A) Effect of pH on swelling of polymer

In 100 ml test solution, 50 mg polymer was introduced and allowed to hydrate at 37°C for 24 h with occasional stirring to remove trapped air bubbles. The pH of the solution was constantly checked and adjusted with saturated sodium hydroxide solution, if required, to maintain pH. After **24** h, the fully hydrated polymer was transferred to a 10-mL graduated cylinder and allowed to settle. After several hours, the increase in hydration volume was measured. The test solutions were HCL of pH 1.2 and 2, 0.1 M monobasic potassium phosphate of pH 3,4,5,6,7.

B) Effect of time on swelling ratio

Polymer (0.1 g) was placed in 10 ml distilled water. At different time intervals like 5, 10, 15, 20, 25,30, 35,40,50,60,90,120,150,180,210,240 min, weight of swollen polymer was noted. The swelling ratio was calculated as

Swelling ratio = weight of swollen polymer- initial weight of polymer/ initial weight of polymer

C) Effect of ions on swelling ratio

Polymer (0.1 g) was dissolved in 0.1 M different solutions for 2 hours. The solutions were prepared in a manner where polymer was exposed to different monovalent and divalent ionic solutions. The hydrated polymer was weighed after 2 hours and the swelling ratio was determined by formula as mentioned in procedure.2.B.

3. FTIR spectrometry

Infrared spectrums of polymers were determined on Fourier Transform Infrared Spectrophotometer (FTIR 4100, Jasco) using KBr dispersion method. The base line correction was done using dried potassium bromide. The samples to be analyzed and KBr were previously dried in oven for 30 min and mixed thoroughly with potassium bromide in 1:300 (sample: KBr) ratio in a glass mortar. These samples were then placed in a sample holder and scans were obtained at a resolution of 2 cm-1 from 4000 to 400 cm-1.

4. Ex vivo Mucoadhesive strength determination

The mucoadhesive force which is defined as detachment stress of the polymer was determined by using a modification of weighing balance. Fresh goat lower eyelids of both eyes were bought from slaughter house and placed in an aerated saline solution until used. One of the eyelids was secured over a weighted glass vial using a rubber band so that the conjunctiva faced outwards. The vial was then placed in a jacketed beaker thermostated at 37°C containing 500 ml pH 7.4 isotonic Sorensen buffer, and positioned under the scale. Another eyelid was placed on rubber stopper of another vial which was hanged on the balance. One vial was connected to the balance and the other fixed with polymer gel and the height was adjusted so that the polymer is placed between mucosal sides of both vials. Water from the burette was allowed to fall drop by drop till the detachment of vials. Mucoadhesive force was determined from minimal weights of water that detached the vial.

Mucoadhesive strength (dynes/cm^2) = mg/A

Where,

m = Weight required for detachment in g

g = acceleration due to gravity (980 cm/s^2)

A = area of mucosa exposed (cm^2)

5. Differential Scanning Calorimetry

DSC measurements were performed on a differential scanning calorimeter (DSC Mettler STAR SW 9.20, Switzerland). Nitrogen gas was purged at a flow rate of 50ml/min in order to maintain inert atmosphere. In a sealed aluminium pan, all accurately weighed samples were placed and the heating of samples was carried out at the rate of 10^0C/min under nitrogen gas flow (20 ml/min) for 40-340^0. An empty aluminum pan was used as reference.

6. X Ray Diffraction analysis

X-ray diffraction patterns of the polymer samples were recorded using Philips PW3710 Analytical XRD B. V. X-ray diffractometer using Cu K 2α rays with a voltage of 40 kV and a current of 25 mA. Samples were scanned for 2θ from 5 to 500. Diffraction patterns of polymers were obtained using an X-ray diffractometer (mod. D8 Discover, Bruker, USA).

7. Conjunctival (HET-CAM) test

Fertilized hen's eggs were freshly bought from poultry farm and were kept in incubation chamber at temperature 37.5± 0.5 ◦C and 66 ± 5% relative humidity (RH) for a period of three days. On third day, eggshells were broken and inner content was exposed on petri plate. The main criterion for this test was only viable embryo with intact CAM and yolk sacs were used and further incubated. The conjunctival irritation potential of the polymers was investigated. On tenth day, 0.1 g (solid) of each polymer was placed on the membrane. The test was carried out in triplicates. Following were the chemicals used as standards in order to compare the degree of irritation. Sodium hydroxide (0.5M) was used as positive control strong irritant, acetone as moderate irritant, propylene glycol as slight irritant and normal saline solution as negative control. The blood vessels and capillaries were inspected for irritation effects. The irritation effects considered in order to classify the polymers on degree of irritation after instillation for 5 min were hyperaemia, haemorrhage and clotting. Based on how much time is required for each effect to occur by each polymer, a time-dependent numerical score was assigned (Table no. 2). A single numerical value was calculated by addition of numerical values obtained for each irritant response and which ultimately determined degree of irritation potential (Table no. 2). The classification system used to determine irritation potential was similar to that used in Draize test.

Table no. 2 Irritation scores and interpretations used in HET-CAM test.

	Score			Cumulative score	Irritation assessment
Effect/time (min)	0.5	2	5	0-0.9	None
Hyperemia	5	3	1	1.0-4.9	Slight
Haemorrhage	7	5	3	5.0-8.9	Moderate
Clotting/coagulation	9	7	5	9.0-21.0	Severe

8. Primary skin irritation test

Two healthy albino rabbits were used for the experiment. Animal husbandry was conducted in accordance with the "Guide for the Care and use of Laboratory Animals," NIH publication No.85-23.

Methods: The backs of the animals were cleaned free of fur with a razor atleast 4 hours before application of the sample. One ml sample of the least irritant polymer obtained from ex vivo test was then applied to the particular site to an area of skin approximately 1" × 1" (2.54 × 2.54 cm) square. The sample applied site was covered with a nonreactive tape. Animals were returned to their cages. After a 24 hours exposure, the tape was removed and the test sites were wiped with tap water to remove the test sample. At 24 and 72 hours after test sample application, the test sites were examined for dermal reactions in accordance with the FHSA- recommended Draize scoring criteria (Appendix 1).The Primary Irritation Index (P.I.I.) of the test sample was calculated following test completion.

9. In vivo Draize eye irritation test

The Draize test was performed on white albino rabbits. In this test 100 µg test sample was placed into the lower cul-de-sac of rabbit's right eye (1.5-2 kg, 13 week of age). Left eye was treated as a control. Rabbits' eyes were observed periodically for redness, swelling and watering of the eye at 1 hr., 4 hr. and every 24 hr. for 7 days. Three rabbits were used for test substance. These parameters were calculated from weighted scores for each part of the rabbit eye such as (cornea, iris and conjunctiva) and also from the sum of these scores. The maximal average Draize total scores (MAS) are classified into non-irritants (0 MAS<0.5), slight irritants (0.5 MAS<15), mild irritants (15 MAS<25), moderate irritants (25 MAS<50) and severe irritants (50 MAS) (26). Approval of the institutional animal ethics committee (Approval

No.MCP/IAEC/01/2016) was obtained prior to the commencing of the study from Modern college of Pharmacy, Nigdi, Pune.

Section II

Formulation and evaluation of niosomal in situ gel using newly synthesized bioadhesive polymer for ocular drug delivery.

The formulation and evaluation process was further divided into three subsections namely:

Subsection 1: Preformulation studies

Subsection II: Formulation and evaluation of niosomes

Subsection III: Formulation and evaluation of niosomal in situ gel

Subsection I: Preformulation studies

1. Characterization of Prednisolone Sodium Phosphate (PSP)

1.1. Description

The prednisolone sodium phosphate powder sample was manually analyzed for the organoleptic properties like color, odor, texture, appearance.

1.2. Melting point

Melting point of the prednisolone sodium phosphate was determined by using melting point apparatus (Veego, VMP 1). The readings were taken in triplicate.

1.3. Fourier transform infrared spectroscopy

Infrared spectrum of prednisolone sodium phosphate was determined on Fourier Transform Infrared Spectrophotometer (FT/IR 4100, Jasco) using KBr dispersion method. The base line correction was done using dried potassium bromide. The samples to be analyzed and KBr were previously dried in oven for 30 min and mixed thoroughly with potassium bromide in 1:300 (sample: KBr) ratio in a glass mortar. These samples were then placed in a sample holder and scans were obtained at a resolution of 2 cm-1 from 4000 to 400 cm-1.

1.4. Differential Scanning Calorimetry

DSC measurements were performed on a differential scanning calorimeter equipped with an intra-cooler (DSC Mettler STAR SW 9.20, Switzerland). Inert atmosphere was maintained by purging nitrogen gas at a flow rate of 50 ml/min. All accurately weighed samples (about 5-10 mg of samples) were placed in a sealed aluminum pan, and the samples were heated under nitrogen gas flow (20 ml/min) at a scanning rate of 10 ^{0}C per min from 40 to 340 ^{0}C. An empty aluminum pan was used as reference.

1.5. Particle size distribution

The average particle size of prednisolone sodium phosphate was measured by the method of laser light diffraction using Malvern Mastersizer Micro Ver. 2.19 (Malvern Instruments Ltd, UK). Prior to measurements, about 50 mg of each sample were dispersed with 100 ml of hexane. The particle size distributions were estimated by setting the intensity of the scattered light at wavelength of 750 nm and the scattering angle (θ) of 90.

2. Development and validation by UV spectrometry

Preparation of stock solution: The drug was accurately weighed (10mg) and dissolved in 100 ml of solution (distilled water/ Buffer pH 7.4/ STF). The resulting solution formed had concentration of 100µg/ml which was used was sock solution**.**

Preparation of working solution: From this stock solution, dilutions were made to get concentrations in the range of 2-10 µg/ml in water and 5-25 µg/ml in buffer pH 7.4 and STF repectively. The solution was scanned throughout the UV range (200-400 nm) to find the λmax of drug in that particular solution.

3. Compatibility Study of Drug-Excipients:

3.1. Differential Scanning Calorimetry (DSC):

Same as mentioned in Subsection I (1.4)

3.2. Fourier Transform-Infrared Spectroscopy:

Same as mentioned in subsection I (1.3)

Subsection II :Formulation and evaluation of niosomes

1. Method selection

1.1. Preparation of niosomes using Ethanol injection method

1. Niosomes containing PSP was prepared by modified ethanol injection method.
2. Surfactant and cholesterol were dissolved in a methanol. The resulting solution was slowly injected using micro syringe at a rate of 0.25 ml/min into 15 ml of Phosphate buffer pH 7.4.
3. The solution was stirred continuously on magnetic stirrer and temperature was maintained above 60^0C. As the lipid solution was injected slowly into aqueous phase which contain drug, Stirring continued for 1-1.5 hrs. Vaporization of solvent takes place, resulting in spontaneous vesiculation and formation of unilamellar spherical niosomes.

1.2. Preparation of niosomes using thin film hydration

Accurately weighed quantities of surfactant and cholesterol in different molar ratios (Table no. 3) were dissolved in chloroform and methanol mixture in a round bottom flask. The solvent mixture will evaporate in a rotary flash evaporator under a vacuum of 20 inches of Hg at a temperature of 25 ± 2 ^{0}C and the flask rotated at 100 rpm until a smooth, dry lipid film will be obtained. The film will hydrate with 10 ml of PBS 7.4 containing 25 mg prednisolone sodium phosphate drug for 45 minute at 60 ^{0}C with gentle shaking on a water bath. The niosomal suspension was further hydrated at 2-8 ^{0}C for 24 h.

Table no. 3 Composition of trial batches for method selection

Batch code	M1	M2	M3	M4	M5	M6	M7
Chol	25mg	25mg	25mg	25mg	25mg	25mg	-
Span 60	25mg	-	-	25mg	-	-	-
Tween 20	-	25mg	-	-	25mg	-	-
Tween 80	-	-	25mg	-	-	25mg	-
Span 80	-	-	-	-	-	-	25mg
Methanol	1ml	1ml	1ml	1ml	1ml	1ml	1ml
$CHCl_3$	4ml	4ml	4ml	4ml	4ml	4ml	4ml
Drug	25mg	25mg	25mg	25mg	25mg	25mg	25mg
Buffer	7.5ml	7.5ml	7.5ml	7.5ml	7.5ml	7.5ml	7.5ml
Technique	Ethanol injection method			Thin film hydration technique			

1.3Drug Content and Entrapment Efficiency

1.3.1. Drug content: Drug content was analyzed by taking 10 mg of PSP drug sample and dissolved in 100 ml of Phosphate Buffer pH 7.5. Each of these solutions was further diluted with phosphate buffer pH 7.4. Absorbance was measured on UV-visible spectrophotometer at 247nm. Drug content was determined by using the formula

$$\text{Percent drug content} = \frac{\text{Test abs.} \times \text{Standard conc.}}{\text{Standard abs.} \times \text{wt. of drug}} \times \text{Dilution factor} \times 100$$

1.3.2. Entrapment efficiency: The prepared PSP niosomes were separated from unentrapped drug by centrifugation at 2750 rpm for 60 minutes. Absorbance of supernatant was taken after appropriate dilution. The settled pellet was dispersed in ethanol to get a clear solution. Its appropriate dilutions were made and absorbance was recorded.

The entrapment efficiency was calculated through the following relationship,

$$\%\ \text{EE} = \frac{\text{Entrapped drug}}{\text{Entrapped drug} + \text{Drug in supernatant}} \times 100$$

2. Combination of surfactants

Table no. 4. Composition of trial batches for combination surfactants

	C1	C2	C3	C4	C5	C6
Chol	25mg	25mg	25mg	-	-	-
Span 60	12.5mg	-	-	12.5mg	12.5mg	-
Span 80	12.5mg	12.5mgmg	-	-	-	12.5mg
Tween 20	-	12.5mg	12.5mg	12.5mg	-	-
Tween 80	-	-	12.5mg	-	12.5mg	12.5mg
Methanol	1ml	1ml	1ml	1ml	1ml	1ml
$CHCl_3$	4ml	4ml	4ml	4ml	4ml	4ml
Drug	25mg	25mg	25mg	25mg	25mg	25mg
Buffer	7.5ml	7.5ml	7.5ml	7.5ml	7.5ml	7.5ml

2.1. Drug Content and Entrapment Efficiency

Same procedure as mentioned in subsection II(1.3)

3. Shortlisting of surfactants

Table no. 5. Composition of trial batches for shortlisting surfactant

	S1	S2	S3	S4
Chol	25mg	25mg	25mg	-
Span 60	25mg	-	-	-
Tween 20	-	25mg	-	-
Tween 80	-	-	25mg	-
Span 80	-	-	-	25mg
Methanol	1ml	1ml	1ml	1ml
$CHCl_3$	4ml	4ml	4ml	4ml
Drug	25mg	25mg	25mg	25mg
Buffer	7.5ml	7.5ml	7.5ml	7.5ml

3.1. Drug Content and Entrapment Efficiency

Same procedure as mentioned in subsection II(1.3)

4. Selection of surfactant

Table no. 6. Composition of trial batches for selection of surfactant

	CS1	CS2	CS3	CS4	CS5	CSS1	CSS2	CSS3	CSS4	CSS5
Chol (mg)	25	25	25	25	25	25	25	25	25	25
Span 60 (mg)	25	50	75	100	125	-	-	-	-	-
Span 80 (mg)	-	-	-	-	-	25	50	75	100	125
Methanol (ml)	1	1	1	1	1	1	1	1	1	1
$CHCl_3$ (ml)	4	4	4	4	4	4	4	4	4	4
Drug (mg)	25	25	25	25	25	25	25	25	25	25
Buffer (ml)	10	10	10	10	10	10	10	10	10	10
Chol: surfactant ratio	1:1	1:2	1:3	1:4	1:5	1:1	1:2	1:3	1:4	1:5

4.1. Drug Content and Entrapment Efficiency

Same procedure as mentioned in subsection II(1.3)

4.2 Particle size determination

Same procedure as mentioned in subsection I (1.5)

5. Selection of Cholesterol: surfactant ratio

Table no. 7. Composition of trial batches

	CS6	CS7	CS8	CS9	CS10	CS11	CS12	CS13	CS14	CS15	CS16	CS17
Chol (mg)	25	25	25	25	25	50	62.5	75	87.5	150	200	**250**
Span 60 (mg)	150	175	200	225	250	25	25	25	25	150	200	**250**
Methanol (ml)	1	1	1	1	1	1	1	1	1	1	1	**1**
$CHCl_3$ (ml)	4	4	4	4	4	4	4	4	4	4	4	**4**
Drug (mg)	25	25	25	25	25	25	25	25	25	25	25	**25**
Buffer (ml)	10	10	10	10	10	10	10	10	10	10	10	**10**
Chol:Surfactant ratio	1:6	1:7	1:8	1:9	1:10	2:1	2.5:1	3:1	3.5:1	1:1	1:1	**1:1**

5.1. Drug Content and Entrapment Efficiency

Same procedure as mentioned in subsection II(1.3)

5.2 Particle size determination

Same procedure as mentioned in subsection I (1.5)

5.3. In vitro release study

Drug release from niosomes was studied using a dialysis method. Dialysis bags were soaked before use in distilled water at room temperature for 12 hours to remove the preservative, followed by rinsing thoroughly in distilled water. In vitro release of PSP from niosomes was conducted by dialysis in a dialysis sac (12,000 MW cut off; Sigma-Aldrich) with 100 mL of phosphate-buffer (pH 7.4) at 37°C. Two ends of the dialysis sac were tightly bound with threads. The sac was hung inside a conical flask with the help of a glass rod so that the portion of the dialysis sac with the formulation dipped into the buffer solution. The beaker was kept on a magnetic stirrer (Matrex) and stirring was maintained at 100 rpm at 37°C with a thermostatic control. Samples were collected every at 15 min, 30 min, 45 min, 1hrs, 2hrs, 3hr and further 1hr interval over a period of 6 hours and assayed spectrophotometrically for drug content and the sampled volume of buffer maintained at the same temperature. The equal volume of fresh release medium was replaced at the same time intervals. The dissolution data was analyzed for calculating the amount of drug released and percentage cumulative drug released at different time intervals.

5.4. Release kinetics of drug

The kinetics of the drug release was evaluated by model fitting method using PCP Disso v3 software and the model with the highest correlation coefficient amongst them was considered to be the best model for particular formulation

6. Evaluation parameters

6.1. Optical Microscopy

Optical microscopy of the drug sample was carried out by using, Motic Digital Microscope. Very slight quantity of the niosomal sample solution was put on the glass slide. This slide was focused under various magnification lenses and the pictures were captured.

6.2. Particle size distribution

Same procedure as mentioned in subsection I (1.5)

6.3. Zeta potential determination

Niosomal dispersion (0.5 mL) was diluted to 50ml with distilled water in glass beaker with constant stirring. Zeta-potential of the resulting suspension was determined using the Zetasizer (model: Nano ZS, Malvern Instruments, Westborough, MA, USA) Electrophoretic mobility (μm/s) was measured using small volume disposable zeta cell and converted to zeta potential by in-built software using Helmholtz–Smoluchowski equation. All determinations were made in triplicate.

6.4. Polydispersity Index:

The PDI determination was using photon correlation spectroscopy with in-built Zetasizer (model: Nano ZS, Malvern Instruments, Westborough, MA, USA) at 633 nm. The polydispersity index was calculated by

$PDI = X_{90}\text{-}X_{10}/X_{50}$

6.5. TEM analysis

Transmission electron microscopy (TEM) was used to determine the morphology of the niosomal vesicles. Few drops of optimized niosomal formulation (CS17) were deposited on a carbon-coated copper grid and examined under transmission electron microscope.

6.6. Stability study

On the basis of the results of in vitro characterization of the developed niosomal formulation, CS17 (1: 1 cholesterol and surfactant ratio) formulation was selected for further stability study. Stability study of CS17 formulation was carried out by assessing the ability of vesicles to retain the drug (Drug Retention Behavior). CS17 niosomal formulation was kept at two different temperature conditions, that is, refrigeration temperature and room temperature (RT). Throughout the study, niosomal formulations were stored in aluminum foil-sealed glass vials. Samples were withdrawn at the 7th, 14th, 21st, 28th, and 30th day and were examined for physical changes such as color, particle size, and residual drug content spectrophotometrically.

Subsection III :Formulation and evaluation of in situ gel

1. Method of preparation

The ‘‘Cold method’’ was adopted for preparing poloxamer based gels as described in a previous report. The required amounts of P407 and P188 for each formulation were carefully weighed and placed in a flat bottomed vial. After addition of the required amount of 0.9% NaCl solution, the vial was placed at 4 C until P407 and P188 were dissolved completely and a clear solution was obtained. In the study, P407 and P188 concentrations in sols or gels were expressed as the weight percentage (% w/v).The equivalent amount of niosomal pellets obtained from freeze drying process were added into the gel formulation along with 100 mg of synthesized polymer to form final formulation of niosomal in situ gel. Preliminary batches were prepared blank without niosomes and bioadhesive polymer.

2. Evaluation parameters

2.1. Appearance

The appearance of the gels was examined for clarity. The clarity of various formulations was evaluated by visual inspection under black and white background.

2.2. pH

The pH of each formulation was examined by using digital pH meter. The pH meter was first calibrated using buffer solutions of pH 4 and pH 7. Then gel was taken in a beaker and their pH was measured.

2.3. Drug Content Determination

In this study, each formulation (1 ml) was taken in a 100-ml volumetric flask diluted with D.W. up to the mark. After suitable dilutions the amount of drug was measured in the formulation by using ultraviolet spectroscopy.

2.4. Gelation Time

The Tsol–gel of the formulation was determined by test tube inversion method. Niosomal in situ gel (2 ml) was transferred to a test tube and sealed with paraffin. This test tube was placed in the constant temperature water bath at 35 ± 1^0 C. The sample was examined for gelation.

2.5. Gelation Temperature and Gel Melting Temperature

Gelation temperature was determined as stated earlier. The obtained temperature is said to be T1. After attaining the temperature T1, further heating of gel causes liquefaction of gel and form viscous liquid and it starts flowing, this temperature is noted as T2 i.e. gel melting temperature.

2.6. Gelling capacity

Determination of *in-vitro* gelling capacity was done by visual method. Colored solutions (1% Congo Red solution in water) of *in-situ* gel forming drug deli-very system were prepared. The *in-vitro* gelling capacity of prepared formulations was measured by placing 5ml of the gelation solution (pH 7.2 buffer) in glass test tube and maintained at 37±1°C temperature. One ml of colored formulation solution was added with the help of pipette. As the solution comes in contact with gelation solution, it was immediately converted into stiff gel like structure. The gelling capacity of solution was evaluated on the basis of stiffness of formed gel and time period for which the formed gel remains as such. Color was added to give visualized appearance to formed gel. The *in-vitro* gelling capacity was graded in two categories on the basis of gelation time and time period for which the formed gel remains as such.

2.7. Texture analysis

Texture analysis of the prepared gel formulations was done by using brookefield texture analyser CT3. The formulations were evaluated for following parameters.

2.7.1. Gel strength

The gel strength, which is an indication for the viscosity of the nasal gel at physiological temperature, was measured by measuring the force required for the depression of gel at 35^0C temperature.

2.7.2. Mucoadhesive strength

The mucoadhesive force, detachment stress of the polymer was determined using a modification of mucoadhesive force measuring device. A section was cut from ocular mucosa of goat and instantly secured with the mucosal side out into each glass vial. The vials were stored at 36.5^0C for 10 min. One vial was connected to the balance and the other fixed with polymer gel and the height was adjusted so that the polymer is placed between mucosal sides of both vials. Water from the burette was allowed to fall drop by drop till the detachment of vials. Mucoadhesive force was determined from minimal weights of water that detached the vials.

Mucoadhesive strength (dynes/cm^2) = mg/A

Where,

m = Weight required for detachment in g

g = acceleration due to gravity (980 cm/s^2)

A = area of mucosa exposed (cm^2)

2.7.3. Spreadability

The spreadability was evaluated by measuring the distance to which the 10 ml formulation would spread under the influence of specified force applied on gel

2.7.4. Viscosity measurement:

The viscosity of prepared gel formulation was measured by using brookefield DV-II pro-plus viscometer (Brookefield engineering Labs Inc.Middleboro, USA) equipped with helipath stand and T bar spindle. Viscosity measurements were made at variable temperature and variable shear rate. For temperature dependent study, formulation was subjected to constant shear rate at different temperatures from 25 to 40^0C. During this testing, the temperature was raised gradually by 1^0C and the viscosity of sample was measured after attaining the set temperature. Measurements were done in triplicate. Steady shear sweep test was carried out by measuring the viscosity at constant temperature of 25^0C and 37^0C but varying the rotation speed of spindle from 10 to 100 rpm.

2.8. In-vitro drug diffusion study

In-vitro release studies were carried out using Franz diffusion cell and the temperature was adjusted to 37±0.5°C. The prehydrated dialysis membrane was used for study Samples were withdrawn at periodic intervals of 0.5,1,2,3,5 and 6 hours and replaced with fresh buffer solution to maintain sink conditions. The drug content was analyzed using UV-Visible Spectrophotometer at 247 nm using simulated tear fluid as blank.

2.9. Ex vivo diffusion study

Ex vivo drug diffused study was performed for the optimized formulation, marketed formulation and controlled formulation by using 24ml Franz diffusion cells containing simulated tear fluid. The goat conjunctival epithelium was used for the study. 1ml of sample was placed in donor compartment & diffusion study was conducted for 6 h at 37 ± 1^0C. 1ml sample was withdrawn at 1/2 hr. for an hour and then every 1 hr.& the same quantity of simulated tear fluid was added.

2.10. Pharmacodynamic study

2.10.1. Primary skin irritation test

Same procedure as mentioned in section I (8)

2.10.2. In vivo Draize eye irritation test

Same procedure as mentioned in section I (9)

2.11. Pharmacokinetic study

2.11.1. In Vivo Studies in Rabbits

The drug pharmacokinetics in the aqueous humor following ocular instillation of the optimized formulation (B) and the drug solution was evaluated on male New Zealand albino rabbits. The rabbits (2–2.5 kg) were accommodated in cages kept in a light-controlled (alternate night and day cycles, 12 h each) air-conditioned chamber under controlled humidity (45±5%). The feeding on standard laboratory diets and water ad libitum was ensured. Before the beginning of the study, a medical checkup by a veterinarian was conducted to check their physical state and ensure the lack of clinically observable abnormalities. All the experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) of Modern College of Pharmacy, Nigdi, Pune constituted under the guidelines of Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA, India) with protocol approval number MCP/IAEC/01/2016. The CPCSEA guidelines were adhered to during the housing and experimentation of the animals.

2.11.2. Estimation of drug pharmacokinetics in the aqueous humor of rabbits

HPLC Analysis of prednisolone sodium phosphate

The quantitative determination of prednisolone sodium phosphate pharmacokineticsin the rabbit aqueous humor was based on a previously validated HPLC method. A reversed-phase HPLC-UV method was used to quantify Prednisolone sodium phosphate in aqueous humor. The HPLC (Water 600) apparatus consisted of quaternary (gradient system), HPLC pump (isocratic) equipped with 30 w high resolution UV/Vis detector DATA ACE Chromatography Software (version 1.50) integrator software and a Grace smart RP C18 column (4.6 mm × 250 mm and 10mm particle size).

The mobile phase was prepared by mixing 250 ml of isopropanol with 2.0 ml ofH3P0+ and diluting with deionized water to 900 ml. The pH is adjusted to 3.0 with 1.0 M NaOH and then diluted further to 1.0 liter. The mobile phase was degassed and filtered through a 0.45 micron nylon filter. The flow rate of the mobile phase was set at 1.0 ml/min.

The detector was set at 245 nm (absorption maxima of prednisolone sodium phosphate).The injection volume was 10 to 20 µL; run time was 10 minutes.

The calibration concentration ranges for the standards were 0.050 to1.0 µg/ml. An internal standard 6-alpha-methylprednisolone was added to every standard and sample. Linear regression was performed on the calibration curves using peak height ratios of analyte to internal standard.

Study Design

A two treatment, non-blind, randomized, parallel design was adopted to compare the drug pharmacokinetics in the rabbit aqueous humor following ocular instillation of the optimized niosomal in situ gel (B).

Instillation of Drug Treatments and Sample Processing

Twelve rabbits were randomly divided into two groups. The niosomal in situ gel (B) was instilled (50 µl) in the lower conjunctival sac of the right eye of each rabbit belonging to the first group (Treatment A) while the same volume of the drug solution was applied to those of the second group (Treatment B). The rabbits were systemically anaesthetized with ketamine hydrochloride (intramuscular injection, 50 mg/ kg) along with xylazine as a muscle relaxant (intramuscular injection, 10 mg/kg). At 5 min intervals, the loading of formulae was performed in two instillations using amicropipette. Care was taken to avoid touching the eye surface (8). At 0.5, 1, 2, 4, and 8 h post-instillation, the rabbits were locally anaesthetized (benoxinate HCl, 0.4% (w/v)) and aqueous humor samples (0.15 ml) were withdrawn by anterior chamber paracentesis (30–32). Following each sampling point, an ophthalmologist examined the ocular surfaces via a slit lamp to detect the presence of any damage and/or inflammation based on a scoring system ranging from (0, no inflammation to +4, and severe inflammation/ damage). By the end of the study, half the number of rabbits showed moderate to severe inflammation/ damage (+2 to +4). Consequently, they were excluded from the study to avoid possible negative influences on the drug pharmacokinetics. The remaining rabbits (five per group) showed minor or little inflammation (+1) and were selected to be included in the study.

The aqueous humor samples were stored at −20°C until HPLC analysis. Prior to HPLC analysis, the thawed aqueous humor samples were spiked with 6 alpha methyl prednisolone. In order to precipitate the associated proteins, the spiked samples (0.10ml) were vortex mixed (30 s) with acetonitrile (0.20 ml). Following centrifugation (15min, 2,000×g), the drug content in the organic phase layer was determined by HPLC.

Pharmacokinetic Analysis

The concentration of prednisolone sodium phosphate (mean±SD) in the rabbit aqueous humor was plotted against time.The maximum drug concentration (Cmax, in micrograms per milliliter) and the time to reach Cmax (Tmax, in hours) were directly obtained from the individual subject curves. The mean residence time (MRT, in hours) was estimated using graph prism® software. The area under the aqueous humor concentration-time curve (AUC, in micrograms per hour per milliliter) was calculated using the trapezoidal rule method. The

results were statistically evaluated, using one-way ANOVA, at $P<0.05$. The increase in the ocular drug bioavailability (folds) was estimated by dividing the AUC of the niosomal in situ gel (B) over that of the drug solution.

2.12. Sterility test

The formulation was sterilized by membrane filtration method and was incubated with different culture media like soybean casein digest medium, fluid thioglycate medium etc., for a period of 14 days and observed for the absence of microbial growth.

2.13. Stability Study:

The purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product varies with the time under the influence of a variety of environmental factors such as temperature, humidity, & light, & to establish a re-test period for the drug substance or a shelf life for the drug product & recommended storage condition.

Drug and their formulations are exposed to variable storage condition throughout their shelf life, during storage, shipment & handling. In addition to this diversity of conditions with respect to temperature and humidity, in various countries, also propels to investigate the stability of drugs and their formulations under the influence of various storage conditions. Stability assessment started with studies on the substance to determine degradation products & degradation pathway. On the ICH, Harmonized Tripartite Guidelines on stability testing of New Drug substance & product, fundamental recommendations are summarized.

For the drug substances intended for storage in a refrigerator table no. 8

Table no. 8 ICH guidelines for the drug substances intended for storage in a refrigerator

Study	Storage condition	Minimum time period covered by data at submission
Long term	5°C ± 3°C	12 months
Accelerated	25°C ± 2°C/60% RH ± 5% RH	6 months

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In order to determine stability of gels, the samples were kept in air tight glass vials packed by aluminum foil. The solid dispersions were stored at 25 0C ± 2 0C / 60 % ± 5 % RH for 6 months [ICH guidelines: Q1A(R2)]. These samples were evaluated for drug content, gelation temperature and physical characteristics.

Samples were also stored at 5°C ± 3°C for 3 months. These samples were also evaluated for drug content, gelation temperature and physical characteristics.

RESULT AND DISCUSSION

Section I

Synthesis and evaluation of water insoluble but swellable polymer

1. Yield and density

Table no. 9 Yield and density of crosslinked swelling polymers

Sr. No.	Polymer	Yield (%)*	Density (g/ml)*
1.	DB1	72±0.208	1.458±0.237
2.	DB2	69±0.251	1.462±0.319
3.	DB3	78±0.372	1.483±0.456
1.	DG1	87±0.637	1.529±0.163
2.	DG2	81±0.432	1.572±0.089
3.	DG3	89±0.312	1.595±0.504
1.	HD1	94±0.583	1.622±0.275
2.	HD2	96±0.291	1.636±0.328
3.	HD3	92±0.726	1.641±0.461

*yield represent mean ± SD, n = 3 determinations

Polymerization occurred within a period of 30 min. While synthesis, extensive swelling of produced polymer was overcome by use of magnesium sulphate heptahydrate which served as suspending agent. Significant yields were obtained in case of all the polymers. The results indicated in Table no. 9 shows that the effect of concentration of crosslinking agent on density was insignificant. In addition to this, use of different crosslinking agent also had very small fraction of influence on density parameter.

2. Polymer hydration

Table No. 10 Polymer hydration of crosslinked swelling polymers

Sr. No.		1.	2.	3.	4.	5.	6.	7.	8.	9.
Time (min)		0	5	10	15	20	25	30	35	40
Vol (ml)*	DB1	0.7±0.2	1±0.3	1.2±0.6	1.4±0.4	1.5±0.4	1.5±0.3	1.6±0.4	1.6±0.5	1.6±0.1
	DB2	0.8±0.3	1.4±0.2	1.8±0.4	2±0.4	2.2±0.1	2.4±0.2	2.4±0.6	2.4±0.4	2.4±0.2
	DB3	1.2±0.	1.4±0.	1.5±0.	1.6±0.	1.8±0.	1.9±0.	2±0.4	2±0.1	2±0.3

		2	3	2	0	2	1			
	DG 1	0.6±0.4	1±0.1	1.1±0.5	1.3±0.4	1.4±0.3	1.5±0.3	1.5±0.2	1.6±0.6	1.6±0.4
	DG 2	0.7±0.5	1.2±0.6	1.5±0.2	1.9±0.3	2±0.4	2±0.3	2±0.1	2.1±0.5	2.1±0.5
	DG 3	1±0.2	1±0.5	1.3±0.1	1.4±0.2	1.7±0.5	1.9±0.4	2.2±0.3	2.2±0.4	2.2±0.6
	HD 1	0.7±0.1	0.9±0.4	1±0.5	1.2±0.4	1.3±0.4	1.5±0.2	1.5±0.5	1.6±0.3	1.6±0.3
	HD 2	0.7±0.5	1±0.3	1.2±0.5	1.6±0.5	1.8±0.3	1.9±0.5	2.2±0.5	2.2±0.2	2.4±0.1
	HD 3	1±0.2	1.2±0.1	1.3±0.3	1.5±0.4	1.9±0.2	1.9±0.1	2±0.6	2.1±0.1	2.2±0.6

*Polymer hydration represent mean ± SD, n = 3 determinations

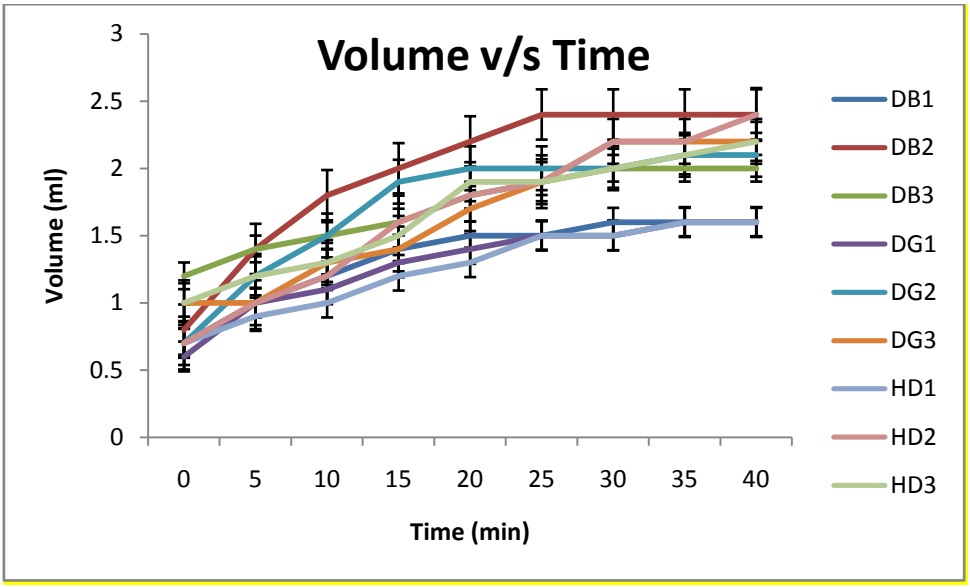


Figure No. 1 Graph of Hydration volume v/s time of crosslinked swelling polymers

The effect of different crosslinking agent on the extent of hydration is shown in Table no. 10. The rate and extent of hydration plays an important role in practical implications because swelling increases surface area. It was observed that hydration of polymer occurred quickly in water and equilibrium was achieved within 30-40 min (Figure No. 1). The degree of hydration was not significantly affected by change in either crosslinking agent or its concentration. Smaller-sized particles hydrate more quickly than larger-sized particles

because the penetration of water molecules through small particles is easy due to lesser thickness of small particles.

A) Effect of pH on swelling of polymer

Table No. 11 Hydrated volume of crosslinked swelling polymers at different pH

pH	Hydrated volume (ml)								
	DB1	DB2	DB3	DG1	DG2	DG3	HD1	HD2	HD3
1.2	5±0.15	6±0.15	5±0.37	1.5±0.4	1±0.63	1±0.21	2±0.41	2±0.2	2±0.22
2	5.4±0.2	6.2±0.5	6±0.16	2±0.25	2±0.45	1.5±0.4	3±0.23	4±0.41	3±0.38
3	5.6±0.4	6.4±0.4	6.5±0.2	2.5±0.2	2.6±0.3	2±0.36	4±0.5	4±0.3	4±0.44
4	8±0.47	8±0.19	7±0.43	4±0.28	4±0.32	3±0.29	5±0.19	5±0.05	6±0.28
5	8±0.62	8±0.36	9±0.61	5±0.38	4±0.27	3±0.17	7±0.24	8±0.1	7±0.42
6	8±0.25	9±0.41	10±0.3	7±0.41	8±0.44	5±0.21	13±0.14	9±0.24	8±0.16
7	9±0.31	10±0.2	12±0.1	11±0.3	9±0.18	10±0.2	18±0.33	12±0.11	15±0.34

Polymer hydration represent mean ± SD, n = 3 determinations

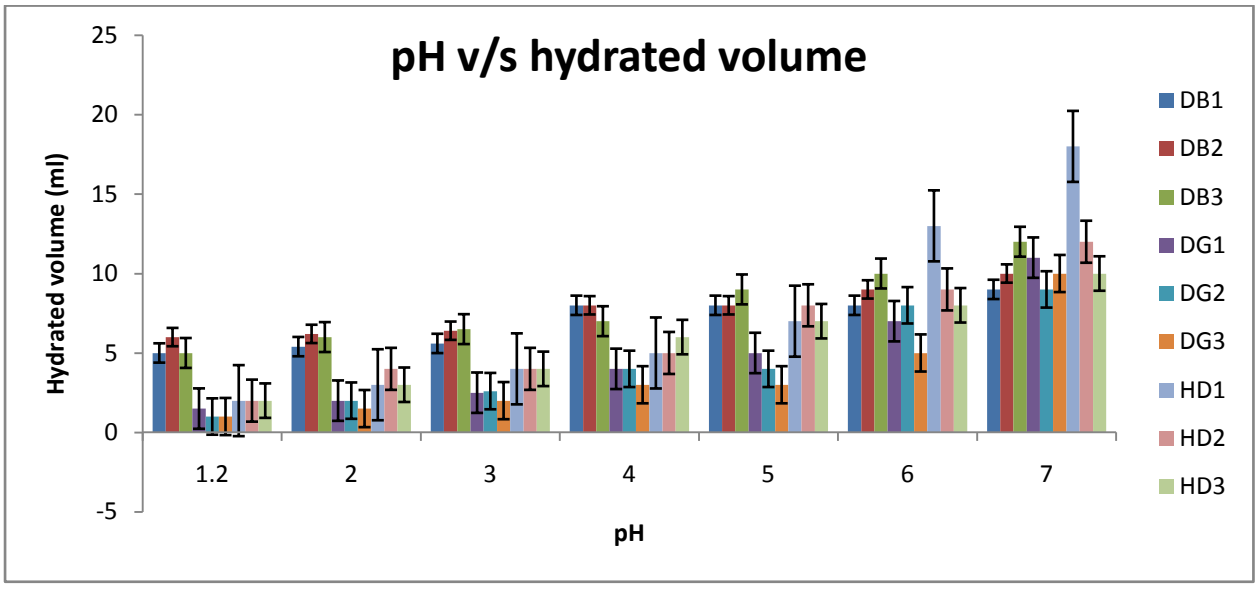


Figure No. 2 Graph of Hydrated volume v/s pH of crosslinked swelling polymers

Equilibrium swelling of polymer was measured at different pH values, and corresponding profiles are shown in Figure No.2. In acidic pH (upto 4), the increase in swelling was slight. As the pH started increasing above 4, significant degree of swelling was observed upto pH 7 (Table no. 11). In the process of polymer hydration, the dependence of water movement into the polymer network in the presence of electrolytes is known to be a characteristic typical of Donnan membrane equi1ibrium. Since the pKa of polyacrylic acid is 4.75, pH dependent

equilibrium swelling was expected. The polyacrylic acid consist of large number of carboxylic (COOH) groups along the polymer backbone which makes it pH sensitive, hydrophilic and capable of forming hydrogen bonds.

B) Effect of time on swelling ratio

Table No.12 Table of swelling ratios of polymers at different time intervals

Time (min)	Swelling Ratio (Ws-Wd/Wd)								
	DB1	**DB2**	**DB3**	**DG1**	**DG2**	**DG3**	**HD1**	**HD2**	**HD3**
0	1.18±0.02	1.06±0.042	1.06±0.041	1.01±0.038	1.06±0.027	1.07±0.019	0.06±0.025	1.64±0.042	1.2±0.038
5	1.37±0.031	3.33±0.041	2.02±0.037	3.5±0.033	2.72±0.025	2.6±0.012	1±0.037	2.26±0.028	1.6±0.042
10	1.8±0.026	5.83±0.031	0.73±0.049	4.9±0.016	5.08±0.042	3.2±0.032	1.2±0.015	2.46±0.028	1.720.052±
15	3±0.042	6.19±0.041	1.19±0.018	4.98±0.018	7.18±0.035	4±0.035	1.72±0.035	2.56±0.023	2±0.036
20	6.7±0.031	6.15±0.041	1.18±0.031	5.44±0.029	7.4±0.039	4±0.042	1.8±0.016	2.66±0.044	20.073.38±
25	6.2±0.012	7.53±0.037	2.44±0.026	5.46±0.034	7.4±0.037	4.2±0.031	2±0.035	3.36±0.016	2.8±0.042
30	7.25±0.016	7.30±0.042	1.53±0.02	5.88±0.043	7.44±0.055	5.32±0.035	2.26±0.037	3.4±0.027	3.72±0.033
35	6±0.037	7.94±0.036	1.85±0.045	6.72±0.031	7.56±0.042	5.4±0.038	2.4±0.019	3.7±0.033	4.06±0.038
40	6.4±0.031	7.85±0.042	1.74±0.016	7.26±0.052	7.8±0.042	6.3±0.047	3±0.014	3.9±0.036	4.34±0.063
50	9±0.027	7.96±0.038	4.92±0.019	7.4±0.037	9±0.031	6.68±0.031	3.4±0.046	4.2±0.031	4.5±0.047
60	12.45±0.014	7.62±0.042	6.17±0.031	8±0.011	9.74±0.047	7.86±0.037	4.1±0.042	5±0.025	5.04±0.037
90	12.72±0.05	8.53±0.031	5.56±0.039	8.84±0.035	10.54±0.032	7.9±0.039	5±0.018	5.8±0.011	5.6±0.041
120	12.13±0.042	9.45±0.028	4.9±0.032	10.52±0.042	10.78±0.063	8.52±0.036	5.98±0.042	6.2±0.029	5.94±0.038

150	9.1±0.029	10.7±0.04	4.6±0.039	10.54±0.036	11.6±0.045	8.88±0.028	6.96±0.037	6.6±0.035	6.36±0.038
180	11.14±0.016	8.60±0.031	4.7±0.048	10.56±0.043	12±0.032	9.4±0.037	7.84±0.038	7.36±0.033	6.9±0.019
210	12.4±0.01	8.9±0.021	4.57±0.042	10.66±0.022	12.8±0.041	11.6±0.035	9.04±0.027	7.64±0.047	7.02±0.035
240	12.5±0.03	9.98±0.038	4.67±0.043	11.18±0.035	13.06±0.027	16.92±0.036	10.22±0.051	7.92±0.058	7.2±0.036

Swelling ratio represent mean ± SD, n = 3 determinations

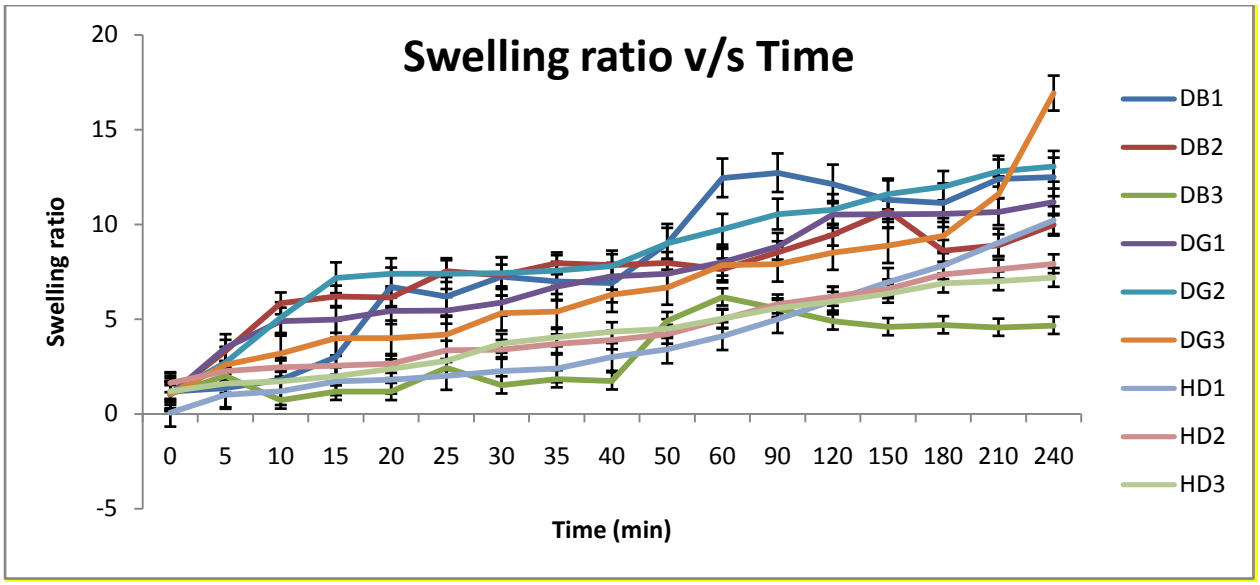


Figure No. 3 Plot of swelling ratio v/s time

Figure no. 3shows that all ofthe polymers tested hydrate quickly in water, reaching equilibriumin 20-40min. Once the equilibrium swelling was attained, the increase in swelling ratio was more or less constant (Table no. 12). Smaller-sized particles hydrated faster than larger-sized particles. The polymer molecules in dry state are highly coiled and tightly packed structures. When placed in water, they behave as anionic electrolytes. They dissociate and partially uncoil due to repulsion of negative charges generated along the polymer chains. The subsequent swelling is caused by difference in osmotic pressure inside the vicinity of polymer chains (cluster) and bulk medium.

C) Effect of ions on swelling ratio

Table no. 13 Effect of ions on swelling ratio of polymer

Type of solution (0.1 M)	Swelling Ratio (Ws-Wd/Wd)								
	DB1	DB2	DB3	DG1	DG2	DG3	HD1	HD2	HD3
HCl	1.29±0.05	3.55±0.021	0.73±0.044	1.5±0.025	0.95±0.017	0.01±0.053	0.5±0.016	0.9±0.042	0.1±0.035
KCl	4.23±0.032	3.05±0.055	1.75±0.032	0.1±0.049	0.08±0.036	2.49±0.025	1±0.029	0.2±0.037	0.5±0.017
NaCl	4.30.036 ±	4.36±0.031	3.09±0.036	0.6±0.046	0.7±0.048	2±0.036	0.6±0.034	0.5±0.037	0.7±0.025
NaOH	44.09±0.021	61.40±0.042	54.81±0.024	21.36±0.022	23.98±0.035	10.9±0.041	19.5±0.032	16.48±0.062	20.05±0.038
$NaHCO_3$	72.67±0.029	54.79±0.038	44.70±0.039	21.67±0.037	24.67±0.038	20.59±0.035	14.89±0.027	15.93±0.038	19.92±0.032
K_2HPO_4	33.35±0.044	32.50±0.027	13.50±0.021	1.81±0.014	1.04±0.043	1.94±0.038	1.5±0.038	1.22±0.027	6.31±0.048
$Na_2S_2O_3$	2.47±0.015	0.59±0.037	0.77±0.015	18.27±0.035	22.2±0.042	12.59±0.042	14.6±0.047	14.3±0.052	13.2±0.058
Na_2CO_3	6.4±0.031	14.75±0.041	11.21±0.032	0.26±0.017	0.3±0.049	1±0.039	3±0.035	0±	0.7±0.029
$CaCl_2$	2.5±0.062	0.38±0.033	0.54±0.016	0.6±0.015	4.5±0.052	4±0.042	2.5±0.041	1.23±0.049	1.08±0.038

Swelling ratio represent mean ± SD, n = 3 determinations

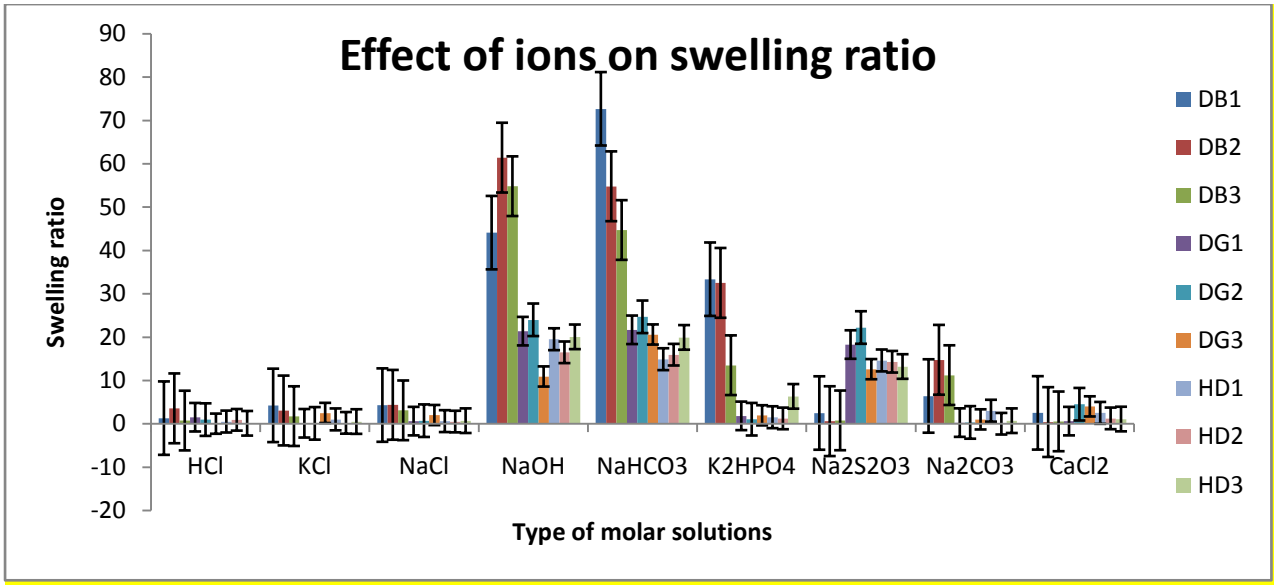
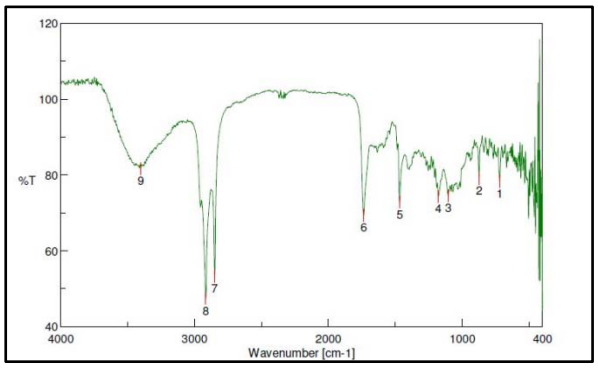


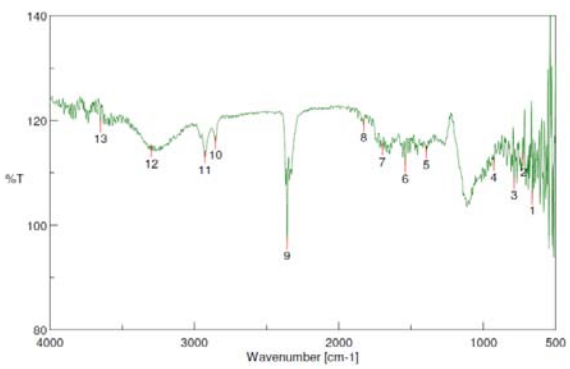
Figure No. 4 Plot of swelling ratio in different types of solution

Literature review indicated that the important influential factors were the ion strength of solution and the valency of cation. They considered that the electrostatic may be the main reason that the gel absorbs the water. The swelling characteristics of polymer at same ionic strength in acid, base and salt was measured. The study showed that the effect of different ions did not cause any significant change in the swelling of drug (Table no.13). Monovalent and divalent ions also did not affect the swelling characteristics of polymer. The increase in swelling for solutions like NaOH, NaHCO3, and K2HPO4 was attributed to change in swelling of reaction mixture solution (Basic solution) and not due to presence of ions. This was confirmed by use of other salts containing same ions but did not show swelling. Figure No.4 proves that electrostatic is not the main reason that the hydrogel absorbs water at room temperature

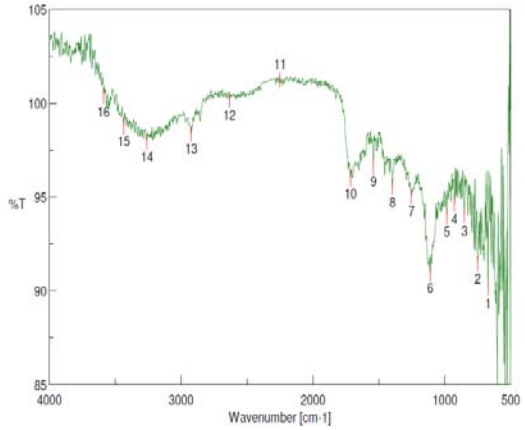
3. FTIR



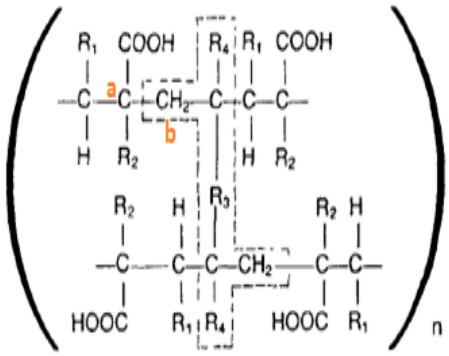
(A)



(B)



(C)



(D)

Polymer	R1	R2	R3	R4
DB	H	H	(p)-C_6H_4	H
DG	H	H	-CHOHCHOH-	H
HD	H	H	-CH_2CH_2-	CH_3

Figure no. 5 IR Spectra of DB1 (A), DG1 (B) and HD1(C) along with probable structure (D)

(A) The following wavenumbers 720.282, 1467.56, 1736.58, 2849.31, 3402.78 cm^{-1} denotes Rocking band –CH_2- bending (long chain band), -CH_2- bending, C=O carboxylic acid, Tertiary C-H symmetric stretching, sp =C-H stretching band respectively. The tertiary C-H (methine hydrogen) gives weak C-H absorption near 2890 cm-1. Methylene hydrogen (-CH_2-) gives rise to two C-H stretching bands representing the symmetric (sym) and asymmetric (asym) stretching modes of group. In effect, 2890 cm-1 methine absorption is split into 2 bands 2926 cm-1 (asym) and 2853 cm-1 (sym). The free carboxylic group was retained which is found to be responsible for bioadhesion. Four acrylic acid molecules are assumed to be bound together by one molecule of crosslinking agent viz divinyl benzene. Figure no. 5(A).

(B) The following wavenumbers 722.2, 1698, 2358.5, 2854, 3296, 3648.7 cm^{-1} denotes Bending motion associated with four or more CH_2 groups in an open chain (long chain band), C=O carboxylic acid stretching, O-H carboxylic acid stretching, -CH_2- stretching asymmetric, C-H alkane stretch, Free –OH group stretching respectively. The four acrylic acid groups are assumed to be bonded together by crosslinking agent viz divinyl glycol. Figure no. 5(B).

(C) The following wavenumbers 906.4, 1105, 1294, 1507.1, 1714.4, 2452, 2924.5, 3258.1 denotes H-bonded (O-H) out of plane bending, C-O stretch (2^0 alcohol saturated), C-O stretching band, C=C stretching, C=O carboxylic acid stretching, O-H stretching vibration of

carboxylic acid, C-H alkane stretching, O-H (H-bonded stretching) respectively. The crosslinking agent viz 2,5- dimethyl-1,5-hexadiene assume to bond four molecules of acrylic acid. Figure no. 5(C).

The change in concentration of crosslinking agent did not show any significant change in IR spectra of these polymers. Hence only one spectra of each crosslinked polymer is discussed here in detail as a representative of that class. The purpose of this study was to evaluate the effects of crosslinking agents and which functional groups are responsible for these effects was suggested by IR spectroscopy

3. Ex Vivo Mucoadhesive strength

Table no. 14Mucoadhesive strength of polymer

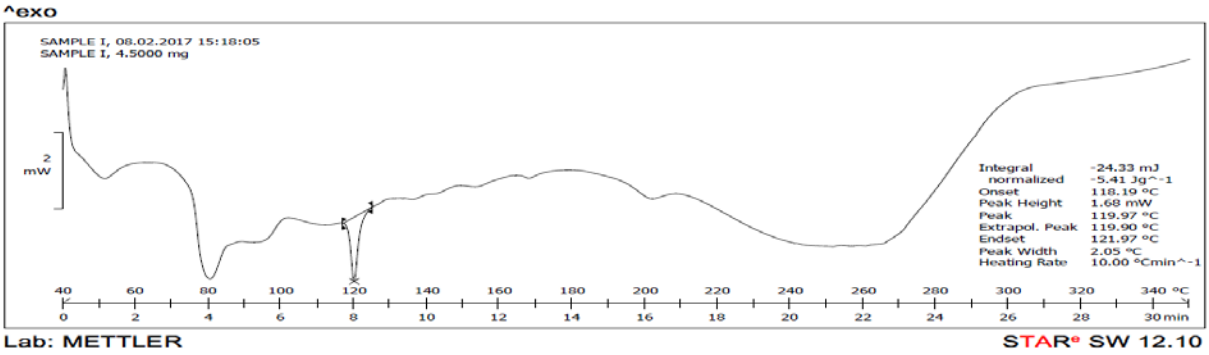
Sr. No.	Polymer Code	Weight required for detachment in g	Mucoadhesive strength (dynes/cm^2)
1.	DB1	2.2±0.057	686.92±0.042
2.	DB2	2.43±0.063	749.04±0.031
3.	DB3	. 2.57±0.039	780.25±0.053
1.	DG1	1.49±0.041	436.94±0.046
2.	DG2	1.62±0.026	499.36±0.018
3.	DG3	2.0±0.037	624.20±0.044
1.	HD1	2.63±0.046	811.46±0.039
2.	HD2	3.87±0.028	1185.98±0.027
3.	HD3	3.34±0.016	1029.93±0.016
3.	HD3	3.3±0.021	1029.93±0.011

Mucoadhesive strength represent mean ± SD, n = 3 determinations

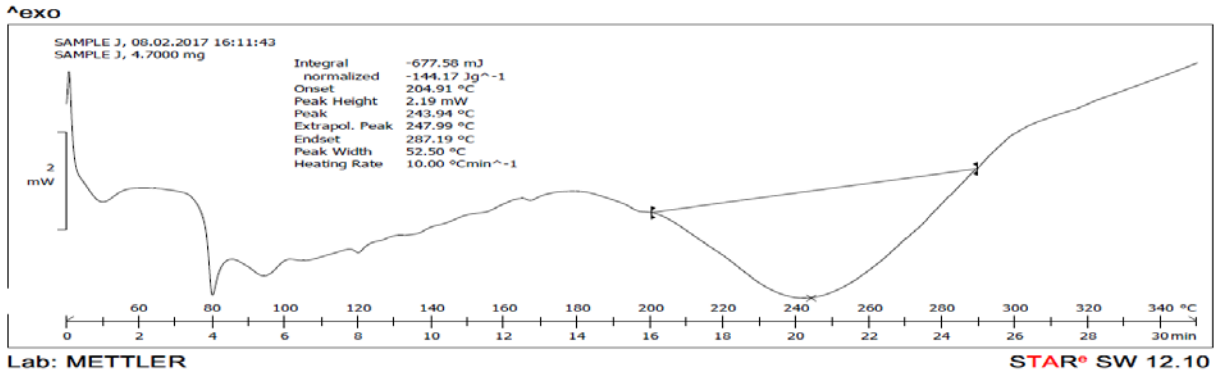
The modified surface tensiometer method proved to be successful method to measure polymer adhesion to animal tissue unless the test conditions are maintained constant for all test samples. A primary mechanism of bioadhesion involves interpolation of the polymer with the mucin. The thickness of conjunctival mucin thickness is considerably less and thus it becomes difficult for the polymer to have intimate contact. The adhesive force of the acrylic polymers was found to be sufficient for the conjunctival surface to maintain long contact time. The density of carboxyl group is important for mucoadhesion. The carboxyl groups present in polymer were found to be in protonated form and hence caused mucoadhesion to occur by hydrogen bonding. In addition, the density of the cross-linking agent significantly affects mucoadhesion. As the density of the cross-linking agent is lowered, the

mucoadhesive strength increases (table no. 14). It is concluded that for mucoadhesion to occur, polymers must have functional groups that are able to form hydrogen bonds above the critical concentration and the polymer chains should be flexible enough to form as many hydrogen bonds as possible.

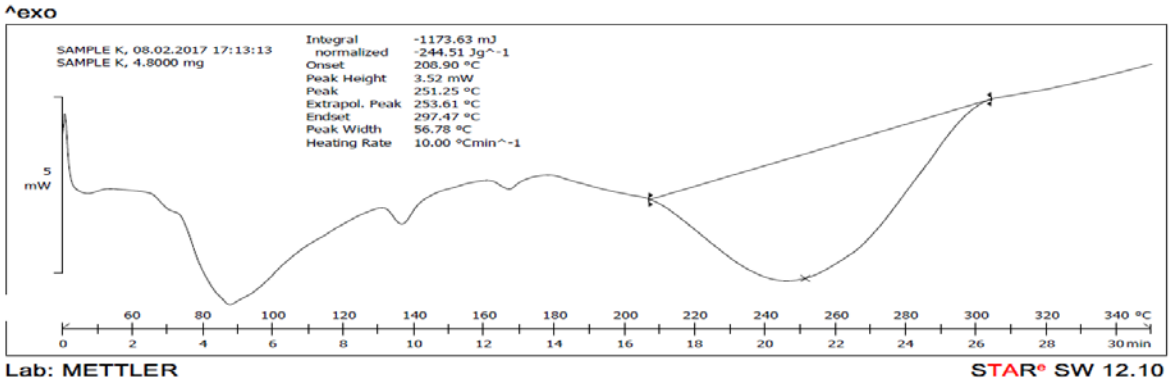
5. Differential Scanning Calorimetry



(A)



(B)



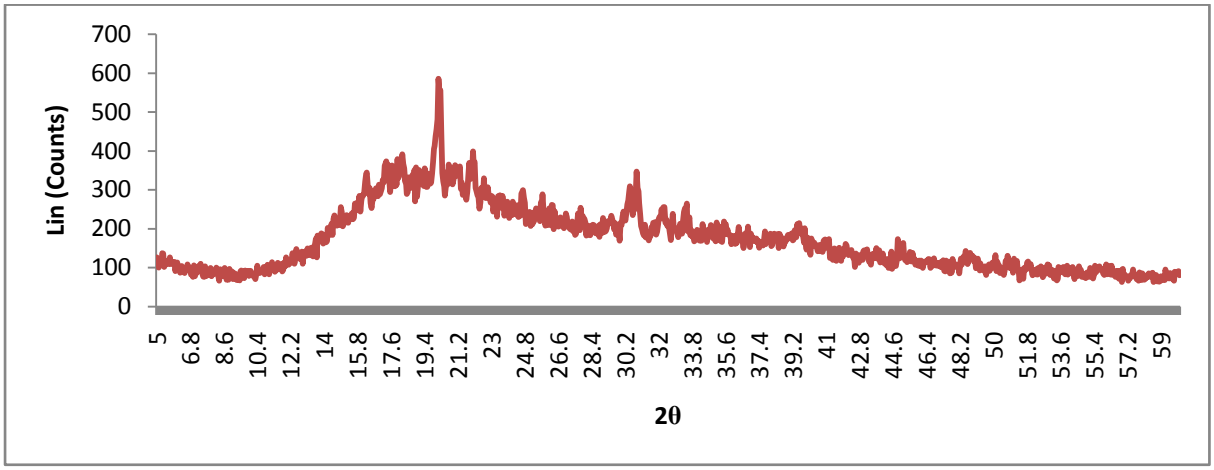
(C)

Figure No. 6 DSC spectra A) DB B) DG C)HD

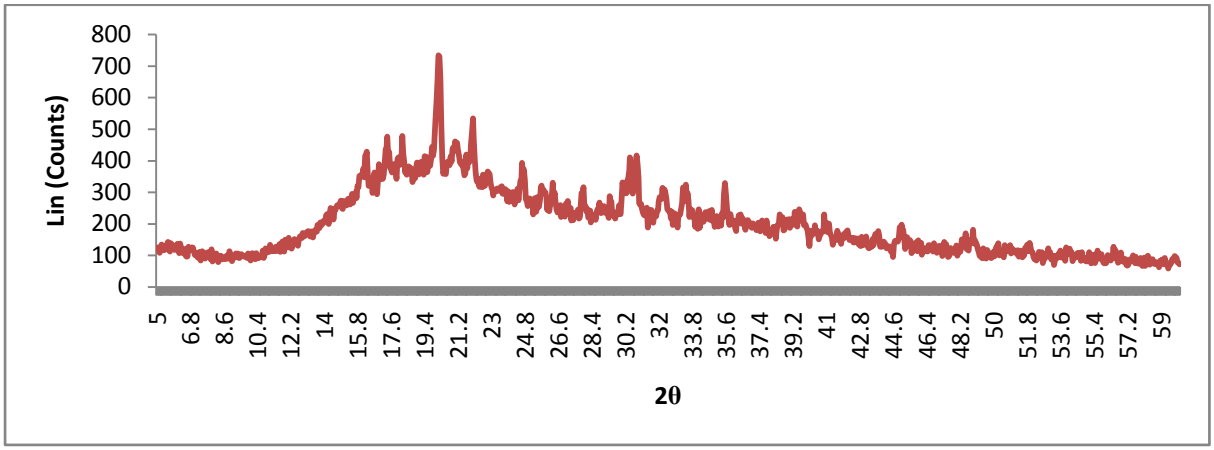
The thermograms (Figure no. 6)for all three polymers exhibited two endothermic peaks. The first endothermic peak between 80-85^{0}C is short and narrow peak assigned to the evaporation of water from hydrophilic groups in the polymers. The second one between 240-250^{0}C

corresponds to a thermal degradation through intermolecular anhydride formation and water elimination (32). There was no significant difference found in thermograms of individual polymer except that the temperature of both the endothermic peak in HD was increased. The most probable reason behind this increase could be presence of hydrophobic moiety in the form of crosslinking agent which requires more energy for breaking the bond in thermal degradation process.

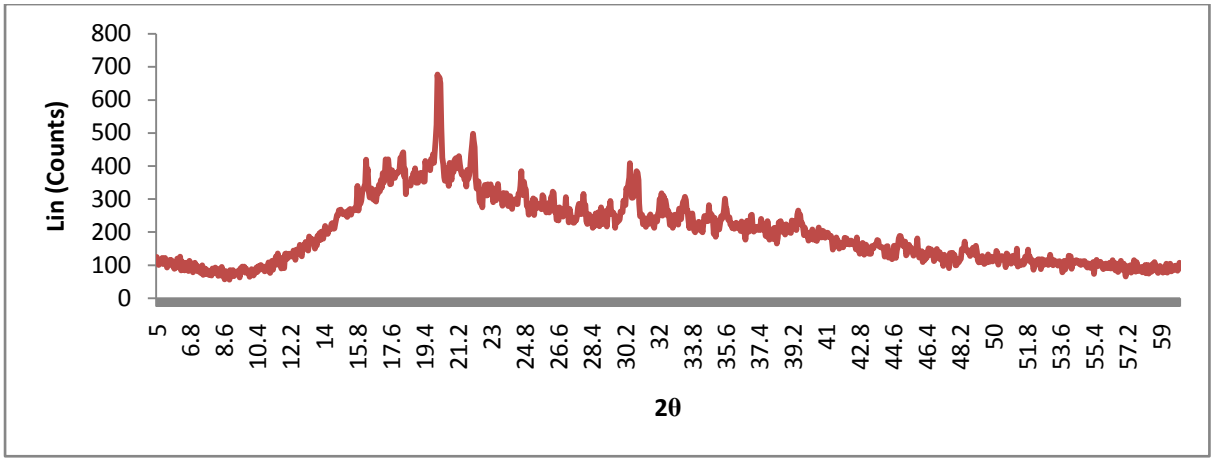
6. XRD analysis



(A)



(B)



(C)

Figure No. 7 XRD spectra A) DB B) DG C) HD

The X-ray diffraction pattern of polymer showed prominent diffraction peak at 19° (2θ) and minor peak appears at 30° (2θ) (Figure no.7). These are the typical peaks of polyacrylic. The XRD spectra of all three polymers indicate that the samples are semi crystalline. The inference can be validated by the intensity and number of peaks. There are few peaks which are sharp. Most of the spectra cover broad peak region indicating that the samples are semicrystalline.

7. HET CAM TEST

(A) **(B)** **(C)**

Figure no. 8 Developmental stages of growing embryo (A) embryo with intact yolk sac and CAM (B) dead embryo with intact yolk sac CAM (C) embryo with broken yolk and intact CAM

Figure no. 8 outlines the development stages of the growing embryos Embryos with intact yolk and viable CAM were only further incubated for 10 days. The test samples were applied on these ten days old CAMs. The temperature and relative humidity kept 37.5 ± 0.5 ◦C and 67± 5% RH were found to be the optimum conditions for CA growing (23,33).Initially the embryo were grown in the egg shell itself and later on to view the results, hole was drilled in

the egg shell. The problems associated with it were limited visibility through the hole and chances of pieces of egg shell falling inside on surface of embryo while drilling the hole. Hence a modified method was reported in the literature, where the chick embryo was grown in a Petri dish from day 3 onwards to allow ready access to the entire CAM surface for better visibility and convenience (33) Thus the process of cracking and proper temperature and humidity conditions are necessary for the survival of the embryos and therefore the number of CAMs available for testing.

Fig. 9 shows the cumulative HET-CAM scores for the controls and synthesized polymers. The average cumulative scores calculated for DG polymers were found to be <0.9. These results reveal that the DG polymers are practically non-irritant when applied to the surface of the CAM. Application of DB and HD polymers in a powder form developed minimal irritation potential in the form of hyperaemia after 3 min. This indicates that DB and HD polymers are slight irritant when applied on the surface of the CAM.

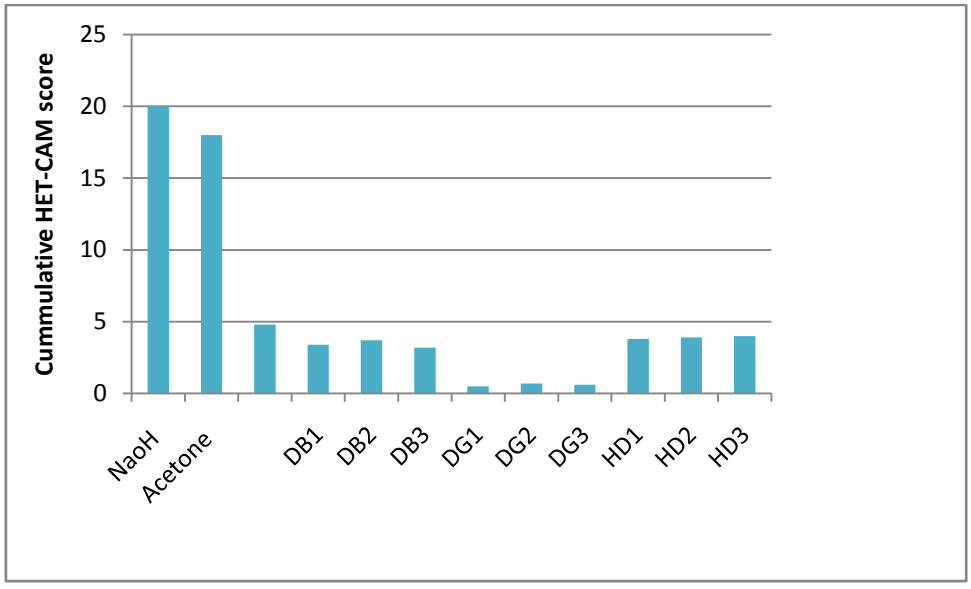


Figure no. 9 Cummulative HET-CAM score of polymers

8. Primary skin irritation test

The primary Irritation index of the test sample was calculated to be 0.00; No irritation was observed on the skin of the rabbits/ rats. Individual results of derma scoring appear in table no. 15

Table no. 15 Reaction scores of animals to irritation potential

Animals	**Reaction**	**24 Hours**	**72 Hours**
Rabbit 1	Erythema	0	0

	Edema	0	0
Rabbit 2	Erythema	0	0
	Edema	0	0

The scores for erythema and edema were summed for intact and abraded skin for rabbits at 24 and 72 hours. The primary irritation index (P.I.I) was calculated. Based on the sum of the scored reactions divided by 32 (two scoring intervals multiplied by two test parameters multiplied by 8 animals).

Primary Irritation Index: 0/32 = 0.00. Under the conditions of this test, the test sample would not be considered a primary skin irritant since the primary Irritation Index was less than 5.00.

9. In vivo Draize eye irritation test:

The results of the ophthalmic irritation studies were given in Table. 16

Table no. 16 Irritation scores of animals to polymer

Days	**Observations**			
	Cornea	**Iris**	**Conjunctivae**	**Total**
1	0	0	2	2
2	0	0	2	2
3	0	0	3	3
4	0	0	2	2
5	0	0	2	2
6	0	0	0	0

The possibility of eye irritation due to polymer administration was evaluated in rabbits. The rabbits were observed for ocular lesions, and no symptoms of ocular irritation such as redness, tearing, inflammation, or swelling were observed after polymer administration. No ophthalmic damage or abnormal clinical signs to the cornea, iris or conjunctivae were visible. Thus, the developed ocular drug delivery systems are apparently free from any ocular irritation potential and can be safely administered to humans. The scores were calculated according to Draize scale.

Section II

Formulation and evaluation of niosomal in situ gel for ocular delivery

Subsection I : Preformulation study

Characterization of Drug

1.1.Description

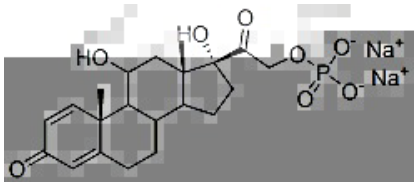
The PSP sample was found white, amorphous, odourless and smooth to touch.

1.2. Melting Point

Melting point of a substance is defined as the temperature at which the solid phase exists in equilibrium with its liquid phase. This property is of great value as characterization tool since its measurement require relatively little material, only simple instrumentation is needed for its determination, and information can be used for compound identification or in estimation of purity. It is general rule that pure substances will exhibit sharp melting points, while impure materials (or mixtures) will melt over a broad range of temperature. Melting point of PSP was found to be 212-214°**C.** The reported value for PSP is 216 °C.

1.3. Fourier Transform Infra-Red spectroscopy (FTIR)

The FTIR studies for the pure drug was carried out the observed peaks are noted as given in the table 17. The peaks are compared with standard drug peaks and the comparison of all peaks is given in the table 1. From the Figure 10, it was found that all the standard peaks of the PSP are present in the IR graph of the standard and this confirms the purity of the drug.



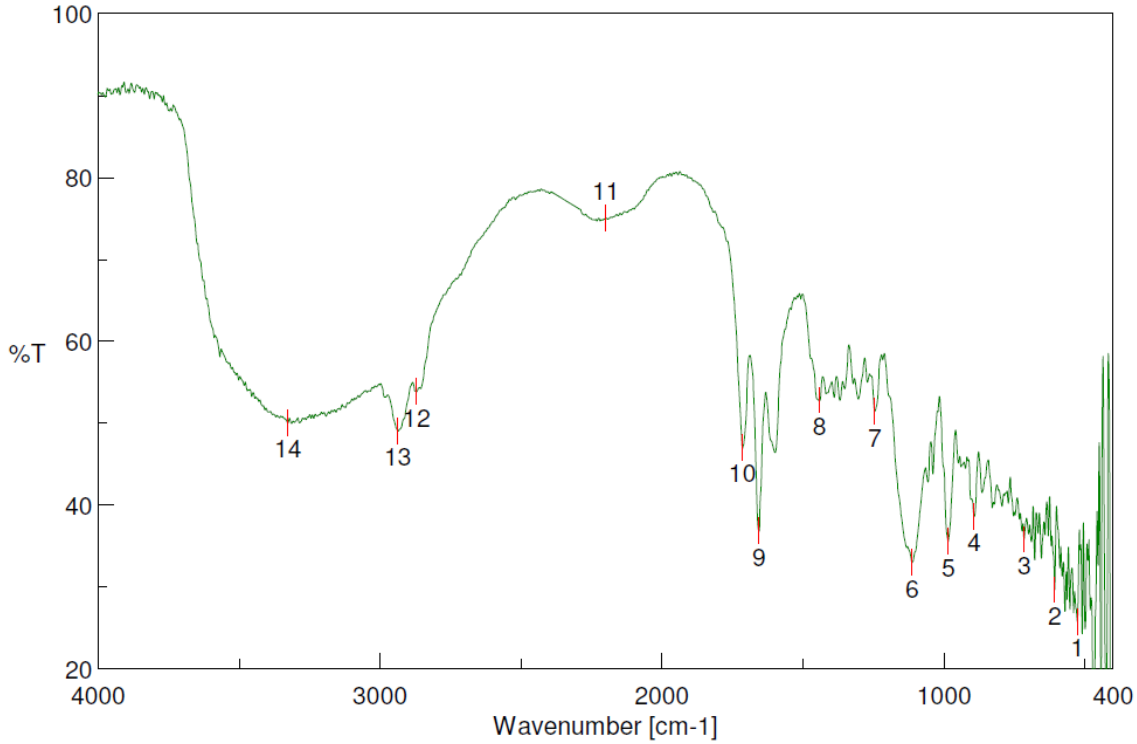


Figure no. 10 FTIR spectra of PSP

Table no. 17. Interpretation of FTIR

Peak No.	**Remarks**	**Peak cm^{-1} (Observed)**	**Peak cm^{-1} (Standard)**
3	=C-H out of plane bending	715.5	700
4	C-H aromatic out of plane bending	892.8	900
5	C-H stretching of alkyl substituted alkenes	984.5	960
6	C-H in plane bending	1113.7	1100
7	C-O stretching	1245.8	1200
8	C=C aromatic stretching	1441.5	1475
9	C=C aromatic stretching	1656.5	1600
10	C=O ester stretching	1715.4	1725
11	Weak combination & overtone of –C-H indicating monosubstitution on aromatic ring	2200.4	2100
12	$-CH_2-$ symmetrical stretching	2870.5	2850
13	C-H aromatic stretching	2937.06	3050
14	O-H (free) alcoholic stretching	3327.6	3300

1.4. DSC

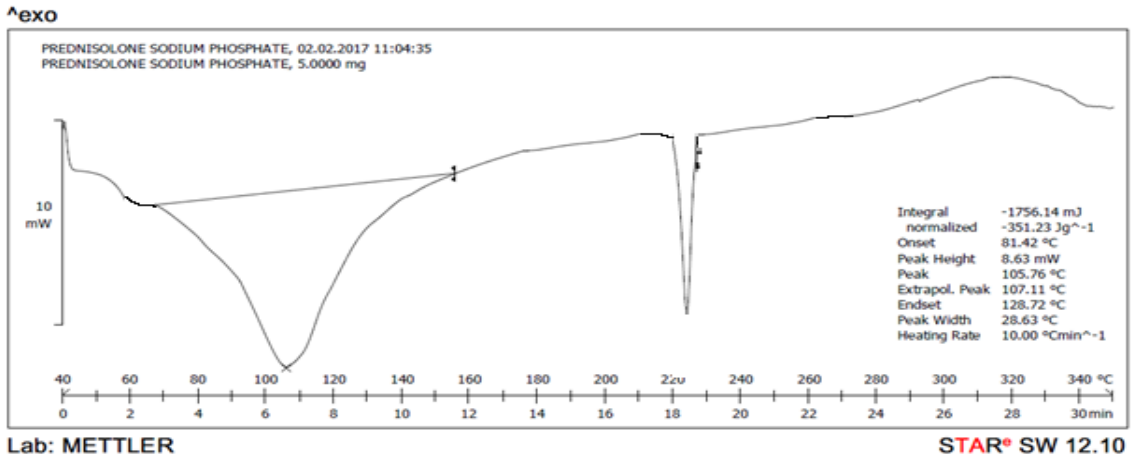


Figure no. 11 DSC spectra of PSP

DSC of drug was performed to evaluate the thermal profile of drug (Figure no. 11). The onset of peak observed at 80^0C represents peak of evaporation of water molecules due to heating. A sharp endothermic peak was observed at 220^0C indicating melting point of prednisolone sodium phosphate (reported value 216^0C). PSP crystals exhibit an endothermic peak at 320°C due to decomposition of PSP.

1.5. Particle size distribution

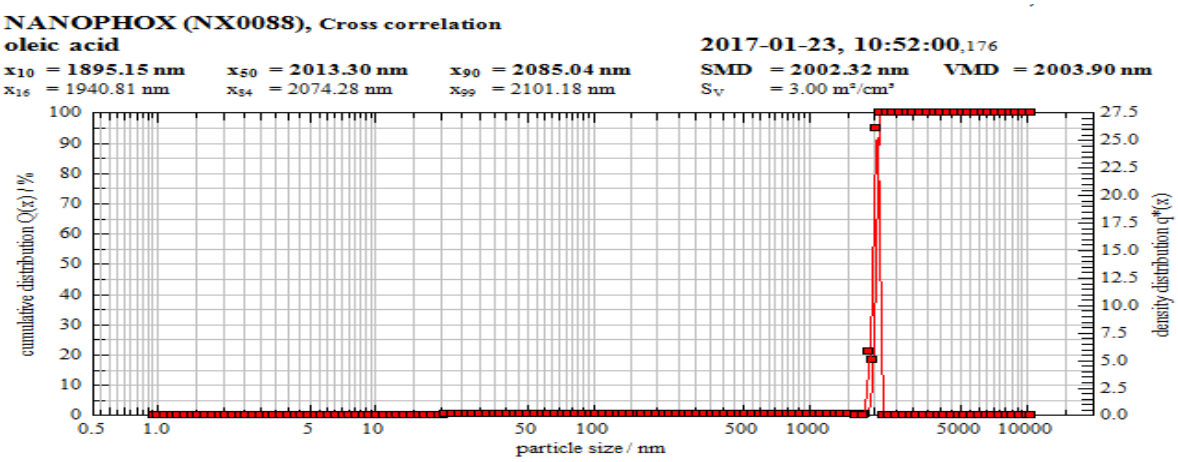


Figure no. 12 Particle size distribution of PSP

The particle size distribution was carried out by Nanophox (NX0088) to find the mean particle size and particle size distribution (Figure no. 12). The mean particle size was found to be 2002.32nm. The PSP powder being amorphous in nature was found to possess small particle size.

2. Development of UV method

2.1.Determination of λmax of PSP

Table no.18λmax of PSP in different solvents

Sr. No.	Solvent	Λmax (nm)
1.	Water	247.5
2.	Phosphate buffer pH 7.4	247.5
3.	Simulated tear fluid (STF)	247

2.2. Calibration curve of PSP in water, phosphate buffer pH 7.4 and STF

For Water:

0.2-1ml of 100 µg/ml solution was diluted and the volume was made up to 10 ml using distilled water to produce 2-10 µg/ml solutions respectively and absorbance was measured. Absorbance calibration curve was plotted with concentration on x-axis and absorbance on y-axis which produced a straight line (in figure no. 13.). This straight line obeyed linearity in the concentration range of 2-10µg/ml. The correlation was found to be 0.9947.

For Phosphate buffer pH 7.4 and STF:

0.5-3ml of 100 µg/ml solution was diluted and the volume was made up to 10 ml using buffer pH 7.4 and STF to produce 5-30 µg/ml solutions respectively and absorbance was measured. Absorbance calibration curve was plotted with concentration on x-axis and absorbance on y-axis which produced a straight line (in figure no. 14 and 15). This straight line obeyed linearity in the concentration range of 5-30µg/ml. The correlation was found to be 0.9992 and 0.9939 respectively.

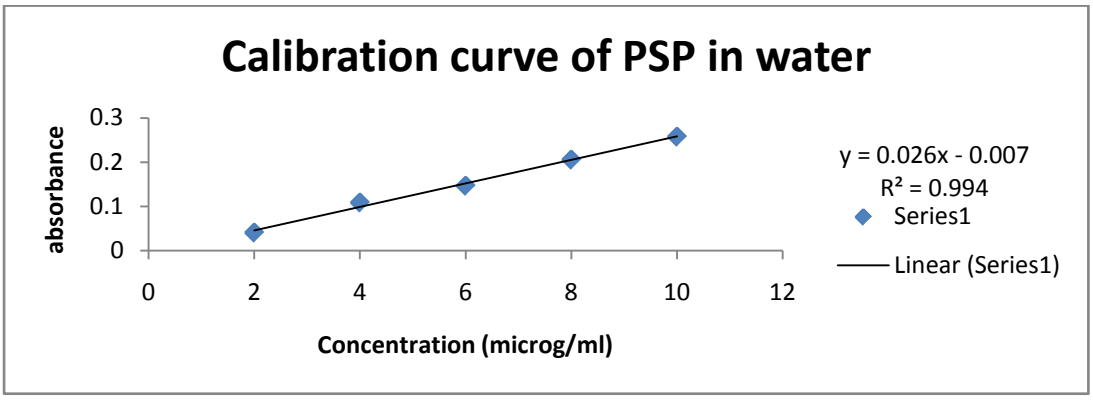


Figure no. 13. Calibration of PSP in water

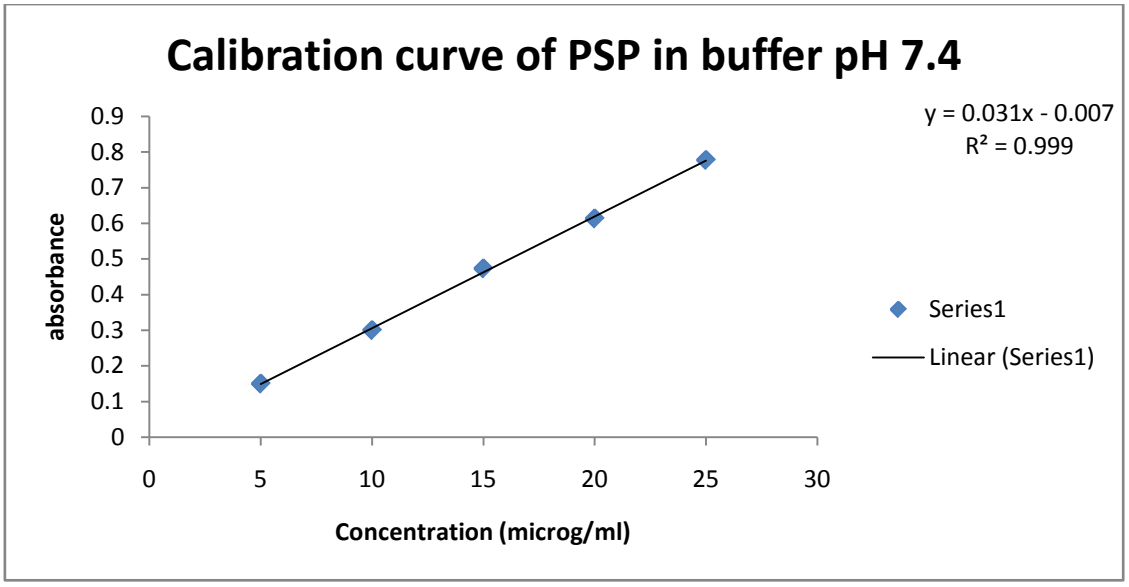


Figure no.14 . Calibration of PSP in Buffer pH 7.4

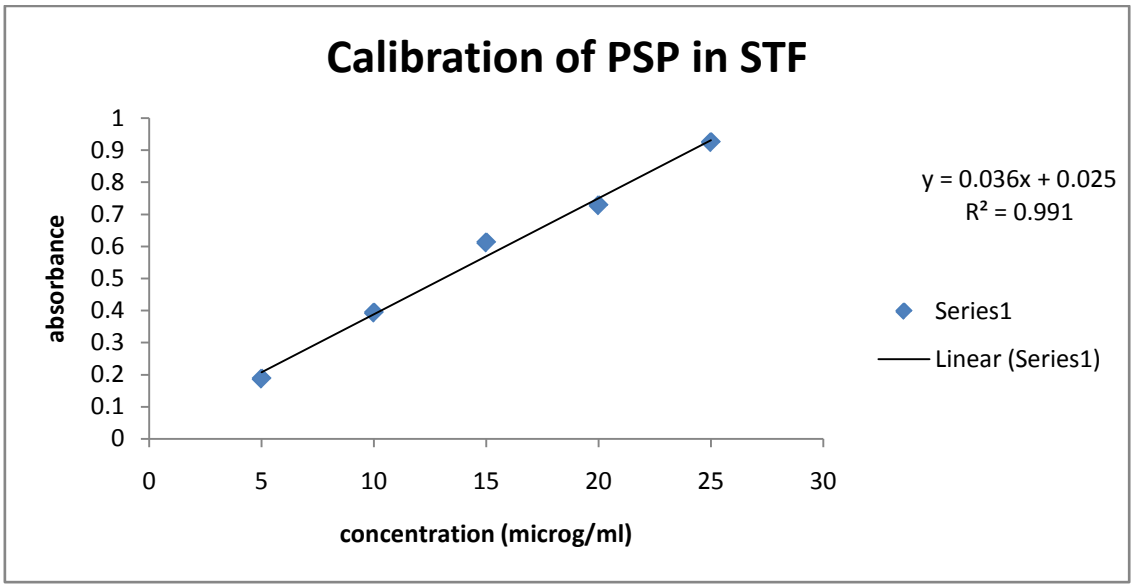


Figure no. 15 . Calibration of PSP in STF

Linearity

It was found that the selected drug shows linearity between the ranges of 2-10 µg/ml in water, 5-25 µg/ml in buffer pH 7.4 and simulated tear fluid (table 19).

Table No.19. Summary of calibration curve

Statistical Terms	Water	Buffer pH 7.4	STF
Beer's law limit(µg/ml)	2-10 µg/ml	5-25 µg/ml	5-25 µg/ml
Correlation coefficient	0.9947	0.9992	0.9939

Regression equation(Y*)	0.0266x - 0.0071	0.0313x- 0.0075	0.0362+0.0257
Slope(a)	0.0266	0.0313	0.0362
Intercept(b)	0.0071	0.0075	0.0257

3. Compatibility study

3.1. Differential Scanning calorimetry

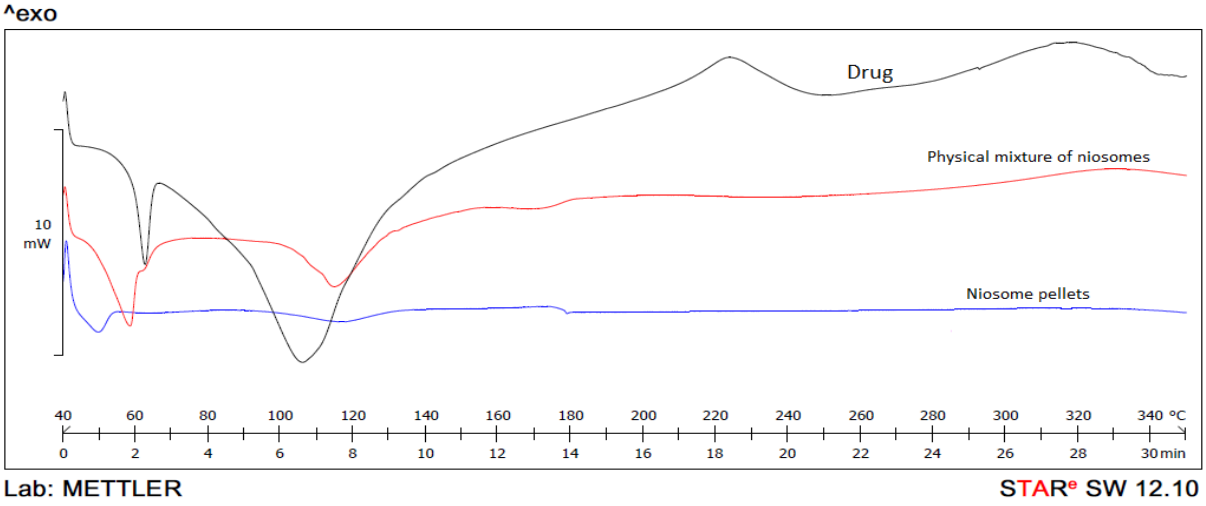


Figure no. 16.Overlay of drug, physical mixture of niosomes, and noisome pellets

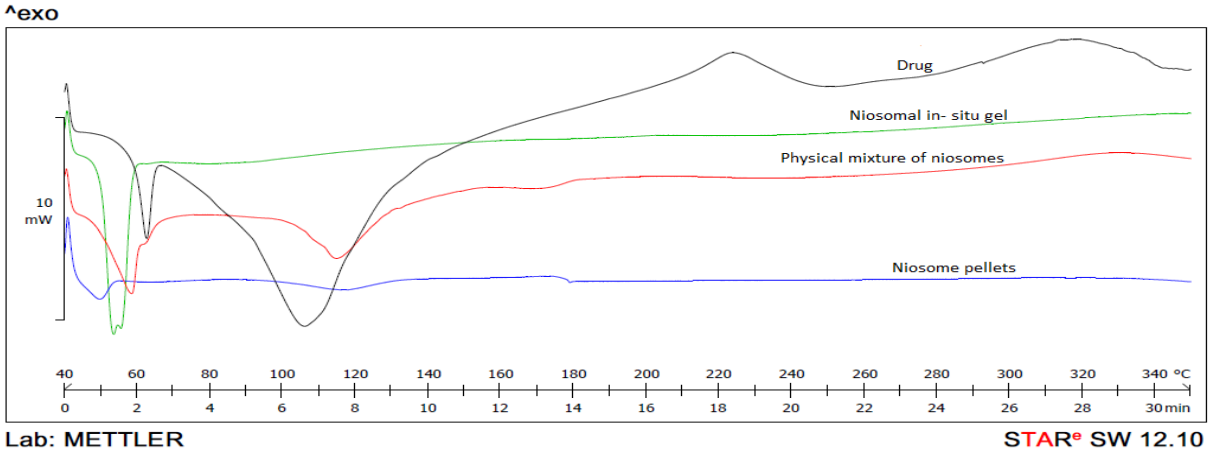


Figure no.17**.** Overlay of drug, physical mixture of niosomes, niosome pellets and niosomal in-situ gel

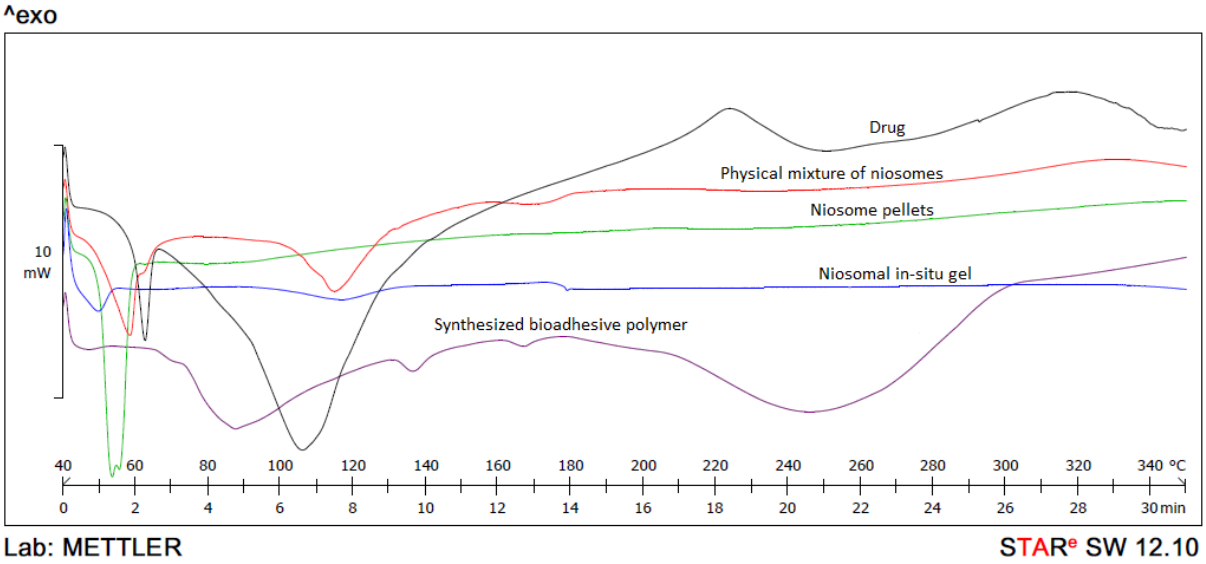


Figure no.18. Overlay of drug, physical mixture of niosomes, niosome pellets, niosomal in-situ gel and synthesized bioadhesive polymer.

The DSC thermograms of pure drug, physical mixture of niosomes, nisosme pellets and niosomal in situ gelalong with synthesized polymer were obtained (Figure no. 16,17 and 18).The peaks observed between 50-60^{0}C indicated melting points of maximum excipients used in the formulation like span 60, poloxamer 407 and poloxamer 188. The cholesterol has melting point of 148^{0}C which was shifted to 120^{0}C due to evaporation of water absorbed by the poloxamer during formulation of in situ gel. The broad peak observed at 240^{0}C indicates the melting point of synthesized bioadhesive polymer and drug. The broad peak indicates formation of amorphous product.

3.2. FTIR spectrometry

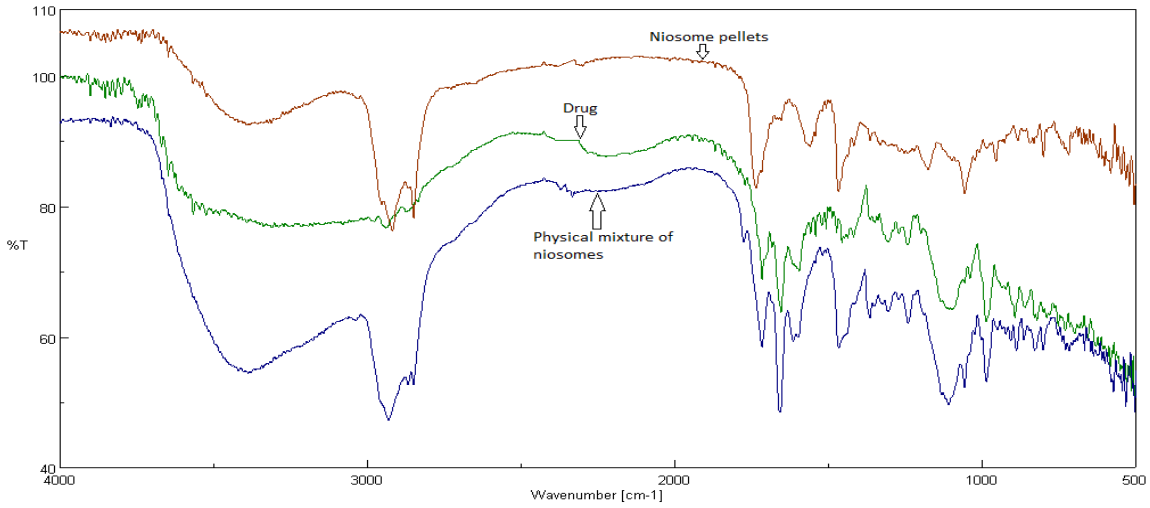


Figure no.19. Overlay of drug, physical mixture of niosomes and noisome pellets

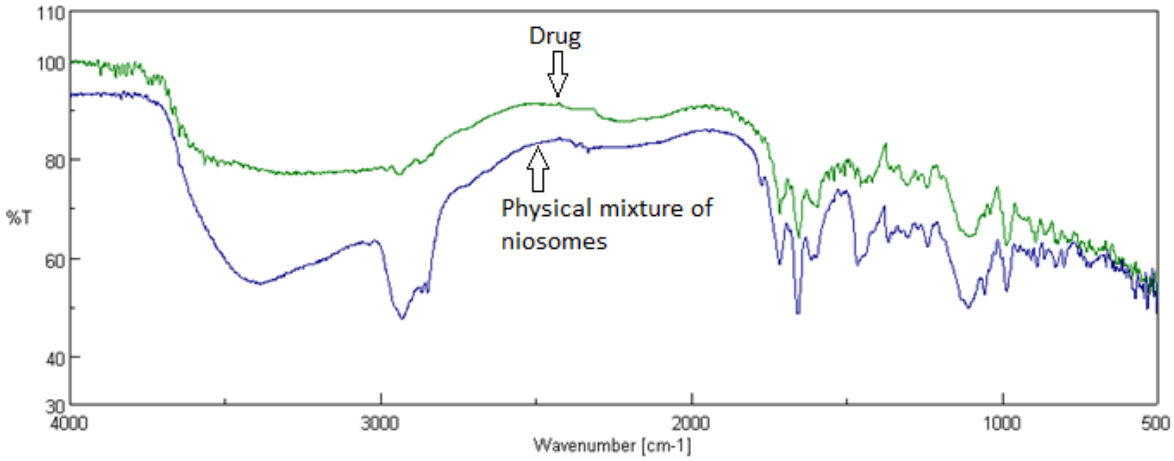


Figure no 20 Overlay of drug and physical mixture

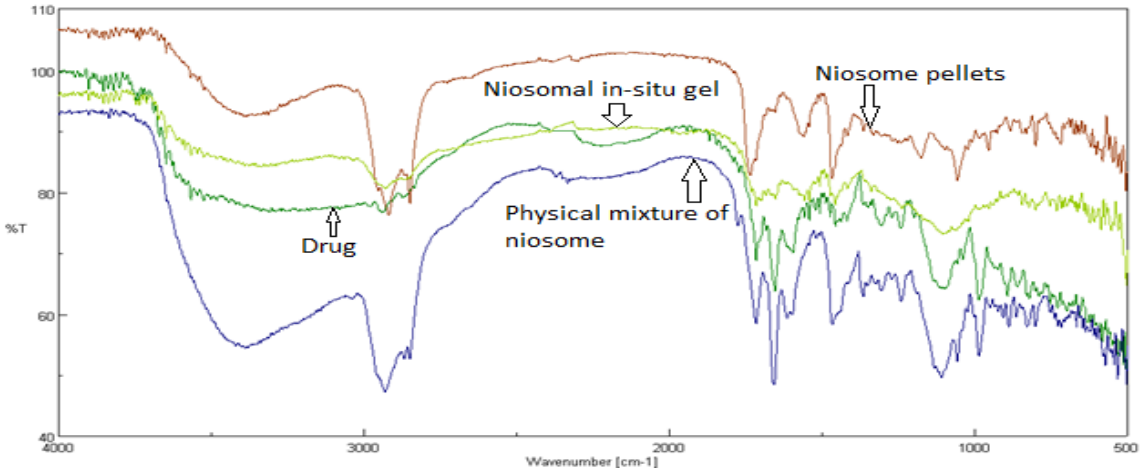


Figure no.21. Overlay of drug, physical mixture of niosomes, niosome pellets and niosomal in-situ gel

FTIR spectra of pure drug, physical mixture of niosomes, noisome pellets and niosomal in situ gel were evaluated (Figure no. 19, 20 and 21). An overlay of all the spectra confirmed that all the peaks of drug (Table no. 17) were retained in physical mixture and formulation as in pure drug which indicates drug and excipients are compatible with each other.

Subsection II

Formulation and evaluation of niosomes

1. Method selection

1.3. DC and EE

	M1	M2	M3	M4	M5	M6	M7
DC (%)	23.8±0.34	22.4±0.32	16.8±0.23	50.21±0.43	39.41±0.11	31.36±0.23	25.27±0.39
EE (%)	30.7±0.19	28.3±0.27	21.4±0.18	66.25±0.25	46.46±0.14	37.54±	59.72±0.44

Table No.20 DC and EE

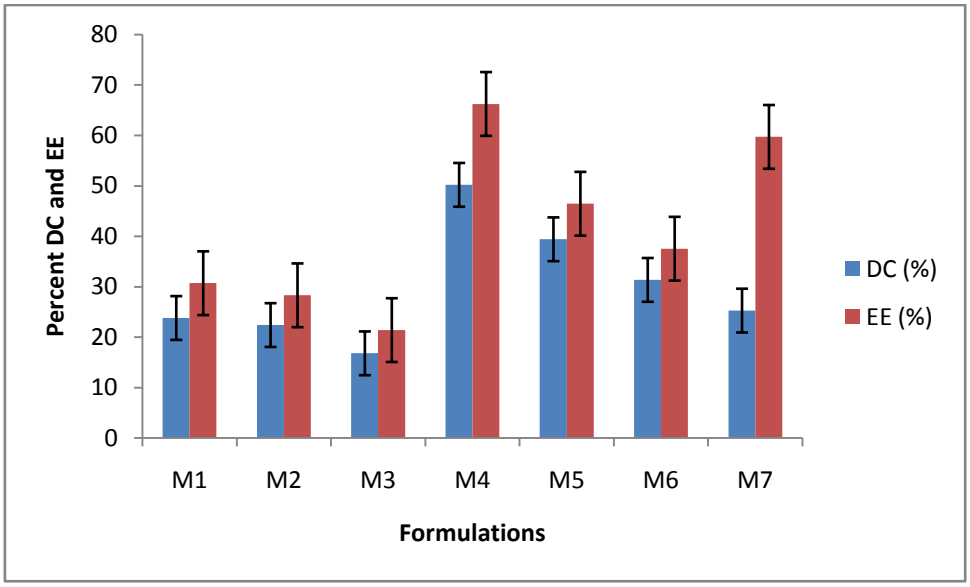


Figure no.22 Graph of DC and EE of preliminary batches for method selection

The niosomes were prepared by two methods namely thin film hydration and ethanol injection method. It was observed that the drug content and entrapment efficiency of noisome prepared by thin film hydration method was higher than of niosomes prepared by ethanol injection method (Figure no. 22 and table no. 21). In addition to this, the niosomes were also evaluated on the basis of their morphology and appearance by motic digital microscopy. The niosomes prepared by thin film hydration technique were spherical and uniform in size. Hence thin film hydration technique was selected for niosomes preparation.

2. Combination of surfactants

2.1. DC and EE

Table no. 21. DC and EE

	C1	C2	C3	C4	C5	C6
DC (%)	12.19±0.23	20.3±0.56	18.28±0.07	16.36±0.14	20.76±0.48	17.37±0.16
EE (%)	61.71±0.41	54.32±0.19	35.52±0.37	22.6±0.29	47.76±0.33	47.53±0.28

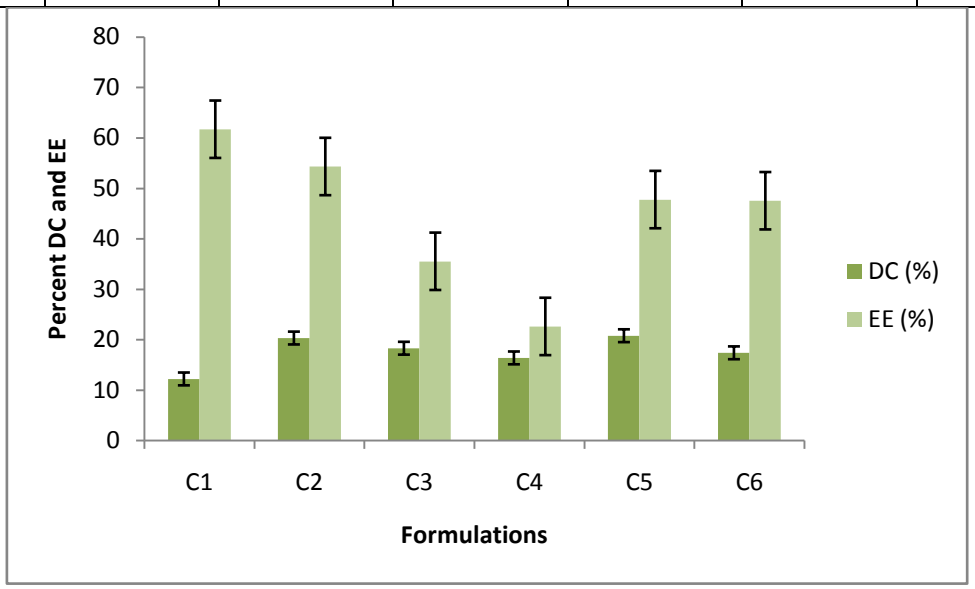


Figure no.23. Graph of DC and EE of trial batches for combination of surfactants

Niosomes were evaluated for combination effect of surfactants (Table no. 21). By preparing niosomes using single surfactant and in combination, drug content and entrapment efficiency was calculated (Figure no. 23). It was observed that the use of surfactants in combination did not show significant effect on drug content and entrapment efficiency. Hence single surfactant niosomes were prepared instead of combination to prevent interaction.

3. Shortlisting of surfactants

3.1. DC and EE

Table no.22. DC and EE

	S1	S2	S3	S4
DC (%)	87.38±0.23	69.78±0.39	83.92±0.37	70.09±0.25
EE (%)	77±0.19	76±0.05	53.88±0.44	87±0.17

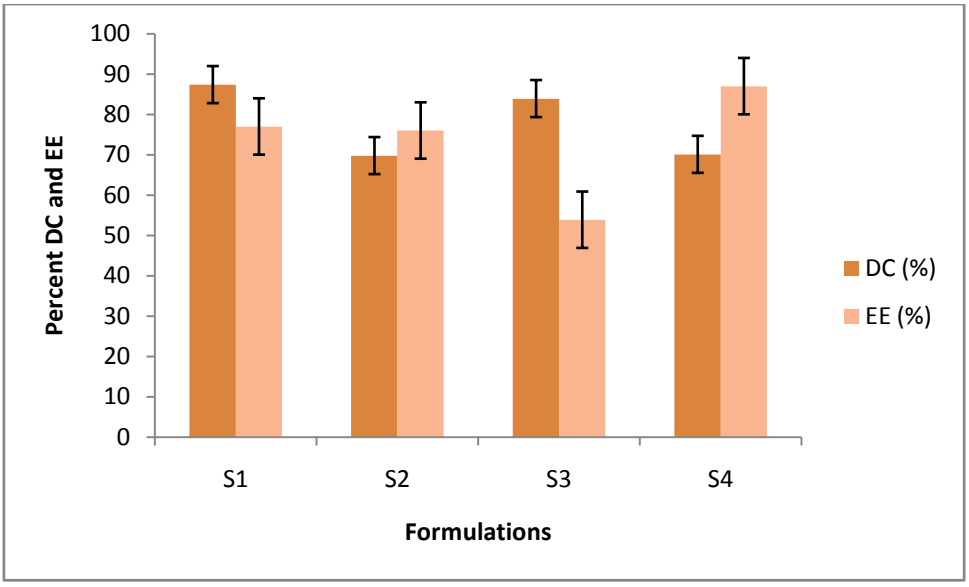


Figure no. 24. Graph of DC and EE of trial batches for shortlisting of surfactants

The four surfactants used during formulation were span 60, span 80, tween 20 and tween 80 to prepare niosomes (Table no. 22). It was observed that, use of Spans showed higher drug content and entrapment efficiency over tweens (Figure no. 24). The probable reason behind this is the HLB value. HLB is a dimensionless parameter, which is the indication of the solubility of the surfactant molecule. The HLB value describes the balance between the hydrophilic portion to the lipophilic portion of the nonionic surfactant. The HLB range is from 0 to 20 for nonionic surfactants. The lower HLB refers to more lipophilic surfactant and the higher HLB to more hydrophilic surfactant. Surfactants with a HLB between 4 and 8 can be used for preparation of vesicle. Hydrophilic surfactants with a HLB value ranging from 14 to 17 are not suitable to form a bilayer membrane due to their high aqueous solubility. However with the addition of an optimum level of cholesterol, niosomes are indeed formed from Tween 20 (HLB value = 16.7)The spans possess lower HLB which makes the drug entrapment of steroid drug moiety more efficient. Tween 20 forms stable niosome in the presence of equimolar cholesterol concentration. The interaction occurs between the hydrophobic part of the amphiphile next to head group and the 3-OH group of cholesterol at an equimolar ratio and this interaction could explain the effect of cholesterol on the formation and hydration behavior of Tween 20 niosomal membranes.

4. Selection of surfactant

4.1. DC, EE and particle size

Table no.23. DC, EE and particle size for selection of surfactant

Batch code	**DC (%)**	**EE (%)**	**Particle size (nm)**
CS1	60.16±0.12	72.4±0.23	423±0.03
CS2	51.59±0.16	86.95±0.36	401±0.43
CS3	57.98±0.47	86.06±0.41	494±0.52
CS4	41.96±0.14	90±0.33	485±0.16
CS5	59.33±0.05	91.54±0.16	469±0.38
CSS1	54.4±0.27	87.19±0.36	356±0.26
CSS2	61.29±0.62	91.31±0.22	407±0.14
CSS3	42.12±0.29	80±0.43	396±0.49
CSS4	43.47±0.32	62.33±0.62	404±0.04
CSS5	55.57±0.38	81.61±0.04	375±0.55

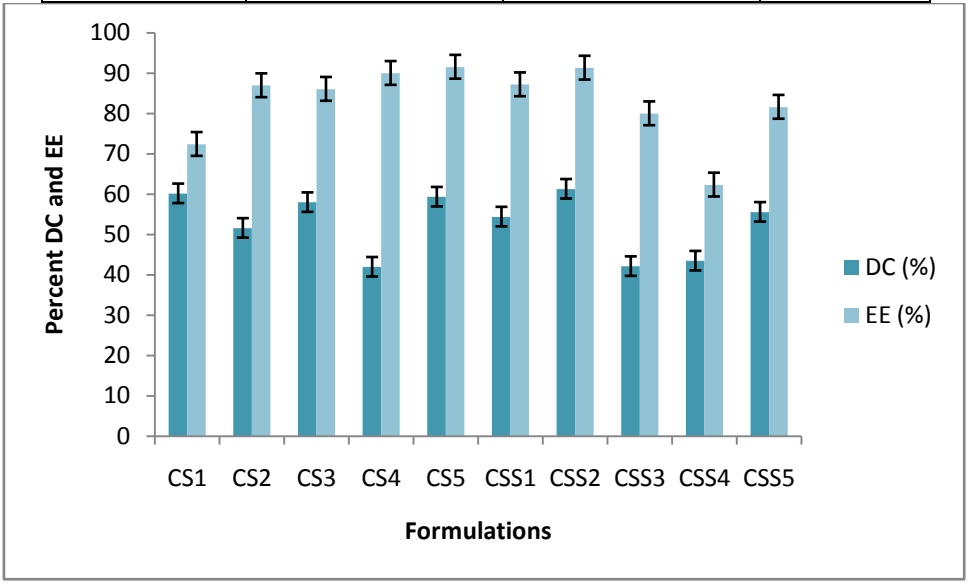


Figure no. 25. Graph of DC and EE of trial batches for selection of surfactant

As discussed in selection of surfactant section, HLB value plays an important role for formation of stable niosomes. The hydrophilic-lipophilic balance (HLB) system, which is a measure of the relative contributions of the hydrophilic and lipophilic regions of the surfactant molecules, is more commonly used as an indicator on potential niosomes formation. The HLB value of span 60 is 4.7 and HLB value of span 80 is 4.3 respectively. So considering the HLB value, Span 80 having lower HLB value compared to Span 60 must be

able to incorporate steroid moiety more efficiently than span 60. But there is an exception. Sorbitan monooleate (Span 80, HLB of 4.3) cannot assemble into niosomes (on their own) due to their inadequate geometry, hence packing properties. The oleate moiety of this surfactant molecule has a double bond (with relatively high electron density) at the C9 which repels adjacent hydrocarbon chains resulting in the characteristic "kink" in the structure. The particle size of niosomal dispersion containing span 80 was less as compared to that of span 60. This might be due to the increase in the hydrophobicity of the surfactant from Span 60 to Span 80. The decrease in surface free energy with increasing the hydrophobicity of surfactants may be the major attribute of reduction in the particle size of niosomes. Since the drug content of niosomes obtained by using span 80 was significantly less due to kink in the structure as compared to span 60, the span 60 was selected as surfactant (Figure no. 25 and table no. 23).

5. Selection of cholesterol:surfactant ratio

5.1. DC, EE and particle size

Table no.24. DC, EE and particle size for selection of cholesterol:surfactant ratio

Batch code	**DC (%)**	**EE (%)**	**Particle size (nm)**
CS6	64.6±0.12	63.9±0.16	324±0.05
CS7	55.8±0.35	69.2±0.26	357±0.15
CS8	66.5±0.41	70.9±0.38	373±0.22
CS9	57±0.55	79.4±0.41	410±0.29
CS10	56.3±0.26	73.2±0.64	428±0.45
CS11	42.1±0.09	66.9±0.28	385±0.62
CS12	68±0.034	70.1±0.36	448±0.54
CS13	53.5±0.49	53.8±0.05	424±0.32
CS14	68.5±0.54	61.4±0.19	401±0.41
CS15	72.4±0.06	80±0.32	436±0.38
CS16	78.1±0.42	80.7±0.46	417±0.61
CS17	86.3±0.39	83.4±0.22	465±0.24

In order to find the optimum concentration ratio of cholesterol: surfactant, different batches of niosomes were prepared (Table no. 24). The ratio value (Cholesterol: span 60) ranged from 1 to 10. Reverse order of ratio (Cholesterol: span 60) from 2 to 3.5 was also used to prepare niosomes. The amount of cholesterol to be added depends on the HLB value of the

surfactants. As the HLB value increases above 10, it is necessary to increase the minimum amount of cholesterol to be added in order to compensate for the larger head groups. Entrapment efficiency decreases as the HLB value decreases from 8.6 to 1.7. For HLB>6, cholesterol must be added to the surfactant in order to form a bilayered vesicle and for lower HLB values, cholesterol enhances stability of vesicles. It is also seen that the addition of cholesterol enables more hydrophobic surfactants to form vesicles, suppresses the tendency of the surfactant to form aggregates, and provides greater stability to the lipid bilayer by promoting the gel liquid transition temperature of the vesicle[37]. The entrapment efficiency is affected by the phase transition temperature (Tc) of the surfactant. Thus Span 60 with a high Tc exhibits the highest entrapment efficiency. The particle size was also not affected with change in ratio of cholesterol: surfactant. The DC and EE of different ratios did not show significant difference (Figure no. 26). Hence the batch was selected on the basis of in vitro drug release.

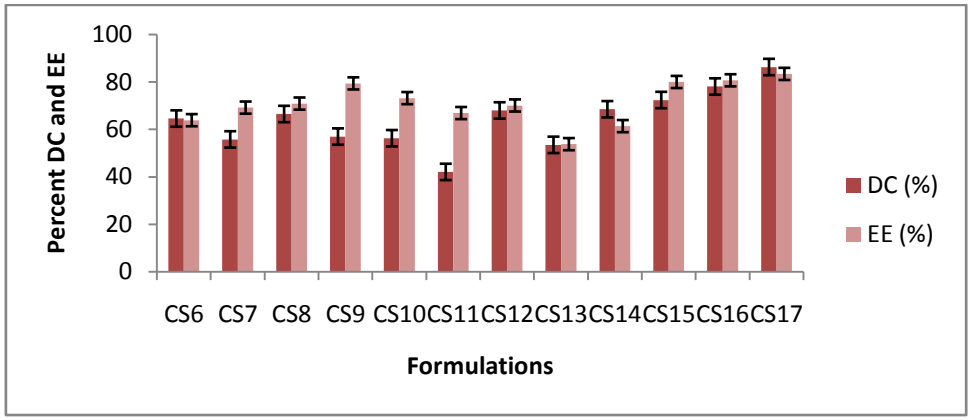


Figure no.26. Graph of DC and EE of trial batches for selection of cholesterol:surfactant ratio

5.2. In vitro drug release

In vitro dissolution of niosomal batches was carried out by dialysis bag method. The value of t90 played an important role in determining the optimized niosomal batch. Our main purpose of this study was to sustain the release of drug and hence t90 was expected to be higher of optimized batch. The batch CS17 (cholesterol:span 60 ratio was 1) exhibited t90 of 490 min that is the release was sustained upto 8 hours of drug by this formulation (Figure no.27 and 28). The change in cholesterol: span60 ratio did not showed linear correlation with either drug content, entrapment efficiency or in vitro drug release. The values differed randomly without showing any correlation. The niosomal batches CS1, CS15,CS16,CS17 all contained cholesterol: span 60 ratio 1 i.e. both the ingredients were in equal quantities but still there t90 values were variable viz 112 min, 370 min, 435 min and 490 min respectively. The difference in these batches was the change in concentration with respect to whole composition. The

amount of surfactant used in CS1 was ten times lesser than that used in CS17. Hence the value of t90 was shifted from 112 to 490. The surfactant concentrations were within the limits in accordance with safety guidelines that are the concentration of surfactant does not exceed by 1-2.5 % w/w. In batch CS17 the amount of span is 1% w/v. The primary function of surfactant is to improve the solubility of substance but PSP being water soluble, this function need not has to be achieved. The surfactants in higher concentrations act as sustained release polymers which cause the drug release at controlled rate.

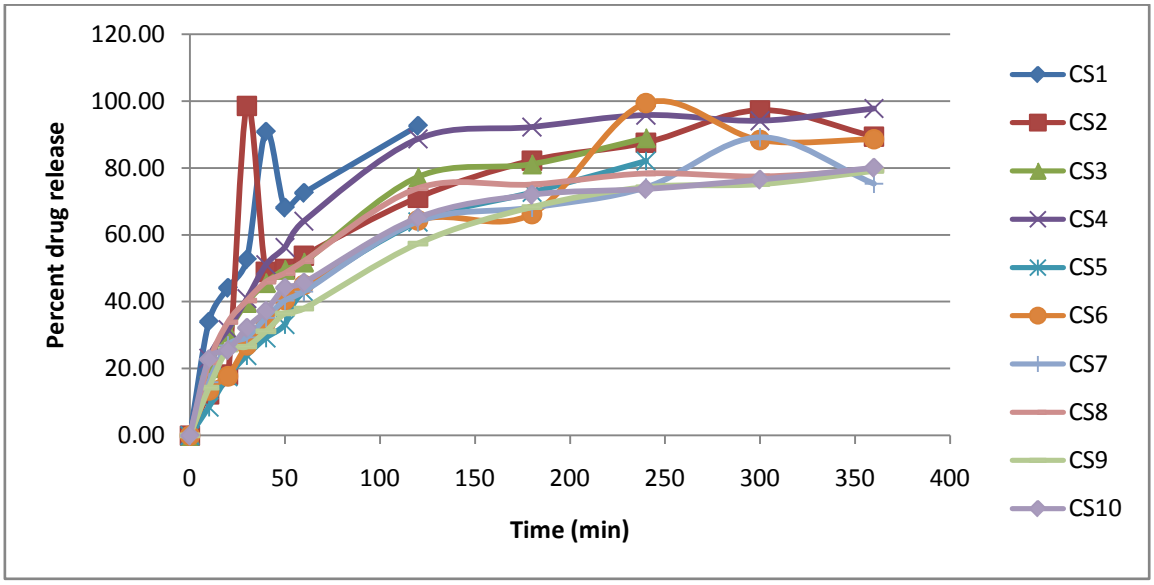


Figure no. 27. Dissolution drug profile of batches CS1 to CS10

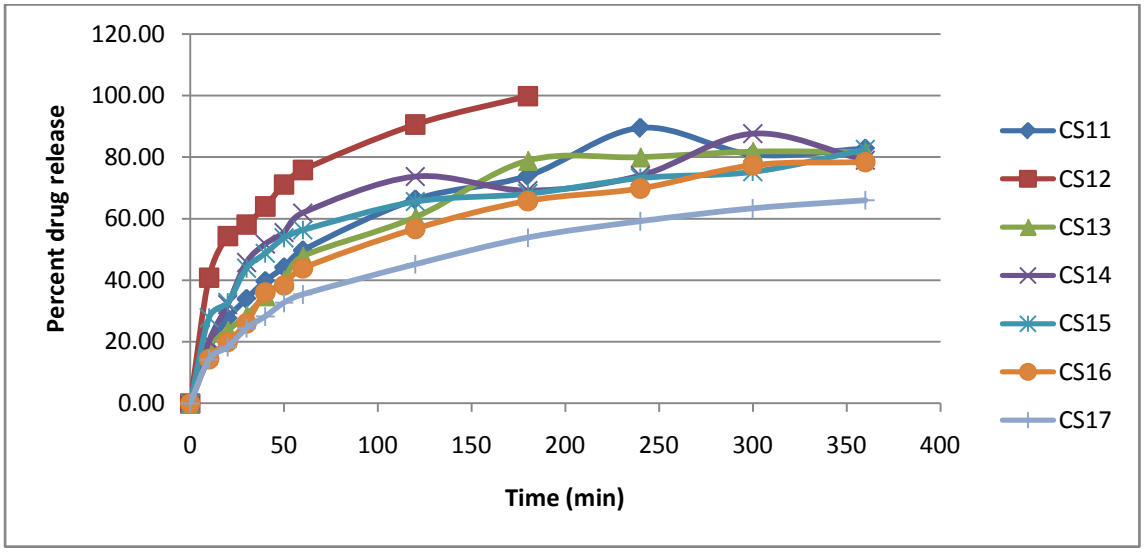


Figure no. 28. Dissolution drug profile of batches CS11 to CS17

Release kinetics of drug

The kinetics of the drug release was evaluated by model fitting method using PCP Disso v3 software and the model with the highest correlation coefficient amongst them was considered to be the best model for particular formulation

Table no. 25. Release kinetics of niosomal batches

Batch Code	Zero order model	First order model	Higuchi/Matrix model	Hixson Crowell model	Korsmeyer–Peppas model			Best fit kinetic model	$t_{90\%}$
	R	R	R	R	R	n	K		Min
CS1	0.6148	0.9860	0.9877	0.9286	0.9987	0.4034	13.37	**KP**	112.8
CS2	0.6615	0.9251	0.9646	0.8872	0.9526	0.5329	4.8165	**M**	271.9
CS3	0.6229	0.9635	0.9817	0.9027	0.9820	0.4598	7.8542	**KP**	201.2
CS4	0.5982	0.9818	0.9726	0.9250	0.9832	0.4718	8.3280	**KP**	155.2
CS5	0.8932	0.9926	0.9849	0.9742	0.9866	0.6975	2.0973	FO	307.9
CS6	0.8892	0.8520	0.9785	0.9333	0.9867	0.6135	3.3073	KP	218.1
CS7	0.6320	0.8788	0.9653	0.8419	0.9836	0.4461	6.6215	KP	347.1
CS8	0.6321	0.7365	0.8837	0.5983	0.9683	0.3460	11.909	KP	345.8
CS9	0.7173	0.9369	0.9842	0.8888	0.9891	0.4683	5.5747	KP	379.7
CS10	0.4564	0.8718	0.9504	0.7877	0.9860	0.3827	9.1718	KP	390.7
CS11	0.5505	0.8696	0.9561	0.8192	0.9815	0.4330	7.6207	KP	299.5
CS12	0.2242	0.9414	0.9506	0.8244	0.9946	0.3199	19.98	KP	110.4
CS13	0.6615	0.9251	0.9646	0.8872	0.9526	0.5329	4.8165	KP	271.9
CS14	0.1567	0.7936	0.8777	0.6439	0.9339	0.3713	11.318	KP	266
CS15	0.4219	0.7193	0.8344	0.5055	0.9733	0.2875	15.69	KP	370.8
CS16	0.6864	0.9312	0.9800	0.8766	0.9834	0.4759	5.3902	KP	435.2
CS17	**0.6671**	**0.8889**	**0.9826**	**0.8347**	**0.9935**	**0.4352**	**5.846**	**KP**	**491.9**

Different t90 values were observed for different batches. The batch CS17 showed (Cholesterol:span 60 – 1:1)maximum t90 value of 491.9 min (Table no. 25). This indicates that the 90% drug release occurred in 490 min thus sustaining the release to upto 8 hours. The kinetics of the drug release was evaluated by model fitting method using PCP Disso v3 software and the model with the highest correlation coefficient amongst them was considered

to be the best model. The release kinetics indicated that the optimized batch (CS17) followed korsmeyer peppas kinetics where R is 0.9935, n is 0.4352 and k is 5.846. The Korsemeyer – Peppas release model equation is, F = (M t /M) = k m t n Where, F = fraction of drug release at time t; Mt = amt of drug release at time t; M = total amt of drug in dosage form; K = constant. 'n' is estimated from linear regression of log (Mt/M) Vs log t. If n = 0.45, it indicates Fickian diffusion; n<0.45 and >0.89 indicates non fickian diffusion. Non fickian diffusion relates to combination of both diffusion and erosion controlled release rate.

6. Evaluation parameters

6.1. Optical Microscopy

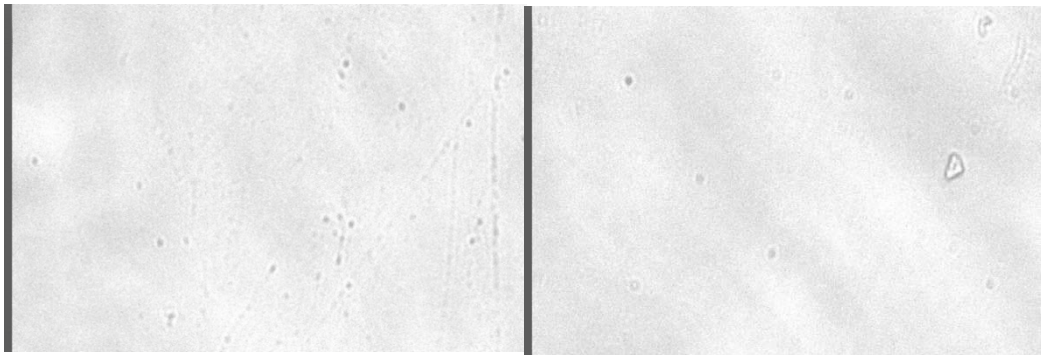


Figure no.29 Optical microscopic image of niosomes.

The niosomes were observed under digital motic microscope (Figure no. 29). The Vesicle size of niosomes was found to be in 0.2-0.5 micrometer range. The vesicles were circular in shape with uniform particle size distribution.

6.2. Particle size distribution

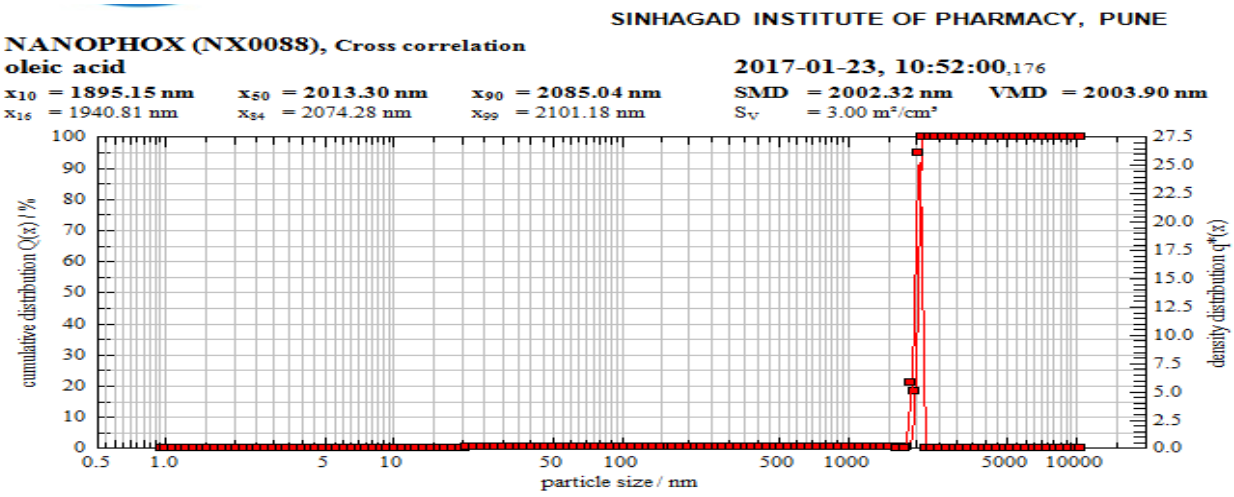


Figure no 30 Particle size distribution of pure drug

The particle size of pure drug and niosomal dispersion was measured by Nanophox NX0088 (Figure no. 30 and 31). The particle size of pure drug was found to be 2002 nm which was

reduced to 465 nm for niosomal dispersion. Thus the particle size was reduced by 4 times making the formulation feasible for ocular use to enhance their penetration through different biological barriers of the eye. According to previous studies of ophthalmological applications, the size of complex drug particles should be less than 10 µm to avoid a foreign body sensation after administration. Especially for ocular drug delivery, larger sized particles (>1 µm) may potentially cause ocular irritation. Based on these results, delivery of ocular therapeutics via niosomes can be used to reduce the sensation and irritation of the eye.

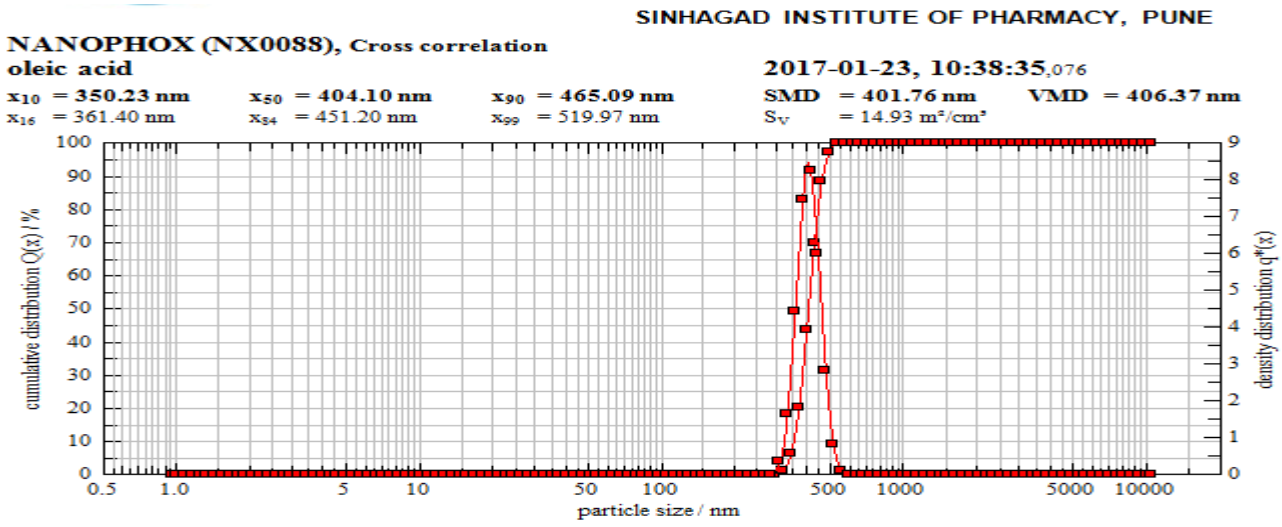


Figure no.31.Particle size distribution of niosomal dispersion

6.3. Zeta potential determination

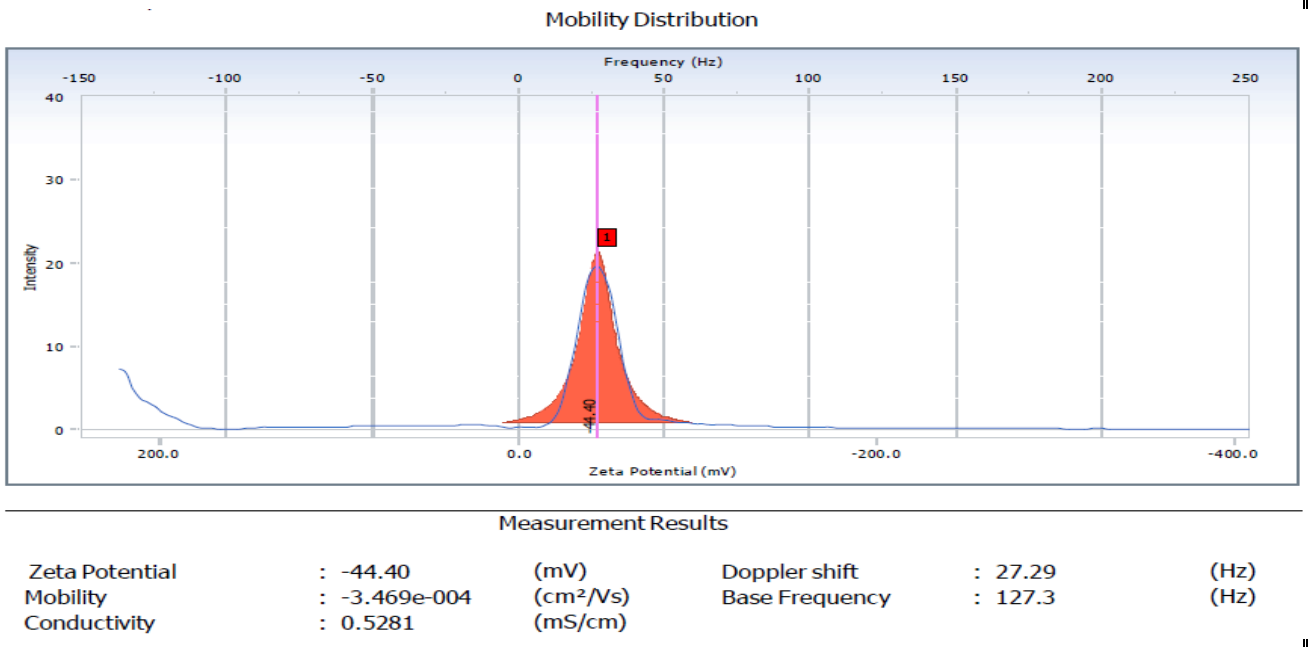


Figure no.32. Zeta potential of niosomal formulation

The zeta potential for niosomal formulation was found to be -44mV (Figure no. 32). Thus it indicates that the formulation is stable and particle size will not increase due to aggregation or coagulation even after the formulation is kept for long time**.**

6.4. Polydispersity Index:

Table.no. 26 PDI of pure drug and niosomal formulation

Sr.No.	Sample Name	PDI
1.	Pure drug	0.095
2.	Niosomal Formulation	0.284

The PDI values are within standard range i.e. less than 1 indicating that the formulation is monodisperse (Table no. 26). Homogenity of niosomal dispersions was indicated by the PDI values.

6.5.TEM analysis

Morphological characteristics of niosomal formulations were further confirmed by TEM analysis. TEM photomicrograph of (CS17) niosomal formulation at 40,000x (Figure no. 33) and 45,000x (Figure) magnification revealed the spherical shape and morphology of the niosomes. Further, it was observed from the TEM images that niosomes are with hollow vesicular structure.

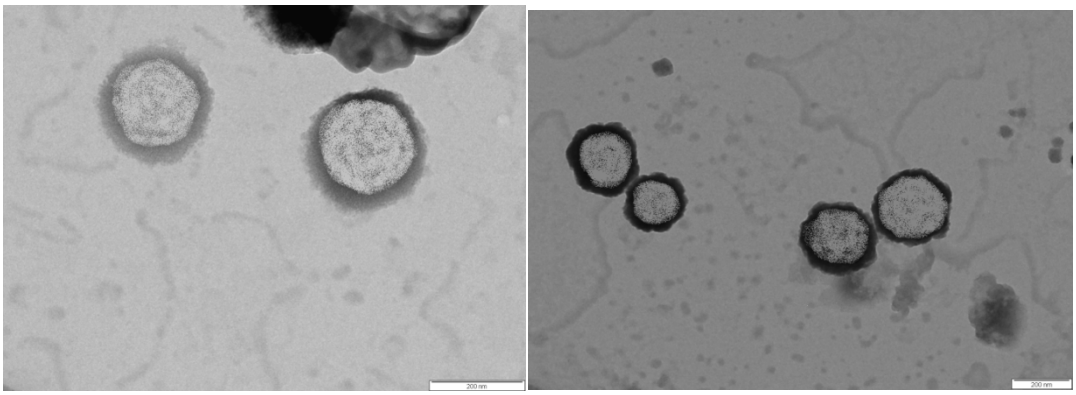


Figure no.33 TEM images of optimized niosomal batch (CS17)

6.5. Stability study

Table no.27 Effect of storage on particle size at refrigerated and room temperature

Formulation code	Days interval	Particle size (nm)		
		initial	Refrigerated temperature	Room temperature
CS17	7^{th} day	465±0.24	470±0.36	480±0.31

	14th day		478±0.51	489±0.46
	21st day		482±0.28	497±0.25
	28th day		486±0.64	506±0.08
	30th day		490±0.33	517±0.17

Table no.28 Effect of storage on drug content

Formulation code	Days interval	Initial drug content (%)	Residual drug content (%)	
			Refrigerated temperature	Room temperature
CS17	7th day	86.3±0.23	85.73±0.28	82.64±0.13
	14th day		84.62±0.34	80.18±0.27
	21st day		83.84±0.41	79.53±0.74
	28th day		81.49±0.15	77.29±0.63
	30th day		80.66±0.06	76.95±0.58

The particle size of the niosomes was slightly increased at room temperature while there was no significant change observed at refrigerated temperature as shown in Table no. 27. Further drug content slightly decreased at room temperature, whereas, in case of refrigerated temperature, no significant change was observed in drug content as shown in Table no. 28 . CS17 formulation was found to be stable at the end of the study on storage condition.

Subsection III

Formulation and evaluation of in situ gel

1. Preliminary batches of in situ gel

Table no.34. Formulation batches of in situ gel batches along with gelation time, gel capacity and gel temperature

Batch code	**Poloxamer 407 (%w/v)**	**Poloxamer 188 (%w/v)**	**Gelation temperature (^{0}C)**	**Gel capacity**	**Gelation time (min)**
G1	20	-	>60	-	-
G2	18	-	>60	-	-
G3	16	-	>60	-	-
G4	21	-	>60	-	-
G5	22	-	>60	-	-
G6	23	-	>60	-	-

G7	20	14	>60	-	-
G8	20	15	>60	-	-
G9	20	16	>60	-	-
G10	23	14	>60	-	-
G11	22	15	>60	-	-
G12	21	16	>60	-	-
G13	15	15	>60	-	-
G14	20	10	>56	-	-
G15	17	17	>60	-	-
G16	10	11	>57	+	-
G17	11	10	>57	+	-
G18	11	11	>57	+	-
G19	10	12	>52	-	-
G20	12	20	>52	-	-
G21	12	12	>52	-	-
G22	20	13	48-52	-	-
G23	13	20	48-52	-	-
G24	13	13	48-52	-	-
G25	10	15	43	+	-
G26	15	20	43	-	-
G27	15	15	42	-	<1
G28	10	16	43	+	<1
G29	17	10	42	+	<1
G30	17	17	43	++	<1
G31	10	18	42	+	<1
G32	18	20	42	++	<1
G33	20	18	40	++	<1
G34	**10**	**25**	**37-38**	**+++**	**<1**
G35	**10**	**27**	**37-38**	**+++**	**<1**
G36	**10**	**30**	**36-37**	**+++**	**<1**

(–): The solutions which did not undergo phase transition at all. (+): The solutions which exhibited phase transition only after 60 sec. and the formed gels which collapsed within 1-2 hrs. (++): The solutions which formed the gels after 60 sec. however, the

gels formed did not remain stable for more than 3 hrs. (+++): The solutions which exhibited phase transition within 60 sec. and the gels so formed remained stable for more than 7-8 hrs.

Preliminary batches were prepared varying the concentrations of poloxamer 407 and poloxamer 188 indifferent ratios and evaluated for gelation temperature (Table no. 34). The concentrations of poloxamer was varied from 10 to23% w/v whereas the concentration of poloxamer 188 was varied from 10-30% w/v. while defining the ratios, care was taken that the total poloxamer concentration should not exceed 40%w/v. It was found that with increase in concentration of poloxamer 188, the gelation temperature was equivalent to physiological temperature that is 36-37^{0}C.

2. Evaluation parameters of in situ gel

Table no. 30. Evaluation results of shortlisted formulations

Formulations	**G34 (A)**	**G35 (B)**	**G36 (C)**
Appearance	Translucent	Translucent	Translucent
pH	6.8±0.1	6.5±0.1	6.7±0.1
Drug content (%)	88.45±0.37	91.37±0.26	86.13±0.15
Gelation temperature (^{0}C)	37.7±0.5	37.2±0.5	36.9±0.5
Gel strength (sec)	29±0.36	32±0.42	35±0.18
Gelation time (min)	<1	<1	<1
Mucoadhesive strength (dynes/cm^2)	1837±0.35	2043±0.26	2465±0.14
Spreadability (cm)	2.3±0.33	2.1±0.52	1.8±0.08

The appearance of formulation was translucent since niosomal pellets equivalent to drug dose were introduced into the gel formulation (Table no. 30). The pH of the solution was found to be in the range of 6.5 to 6.8 since the pH of formulation was maintained by addition of 0.1 M NaOH. The effect of polymer concentration on drug content was negligible because the drug was incorporated into niosomes and the drug was not directly dissolved into the gel formulation. Thus drug content was affected by niosomal formulation parameters and not by gel formulation parameters. With increase in concentration of poloxamer 188, the gelation temperature was decreased and thus the optimum temperature of 37.2±0.2 was obtained. Gel strength is the indication of viscosity of gel formulation. It was observed that with increase in

polymer concentration, gel strength was also increased. The mucoadhesive strength limits the total clearance of drug from ocular surface. The bioadhesive strength was not significantly affected by polymer concentration because the amount of addition of synthesized biaodhesive polymer was constant in all the three formulations. The bioadhesive strength of formulation was increased with increase in concentration of polymer forming in situ gel formulation. The spreadability of the gel formulation was measured by texture analyser. It was found that with increase in polymer concentrations, the spreadability decreased due to increase in viscosity of formulation.

2.7.4. Viscosity measurements

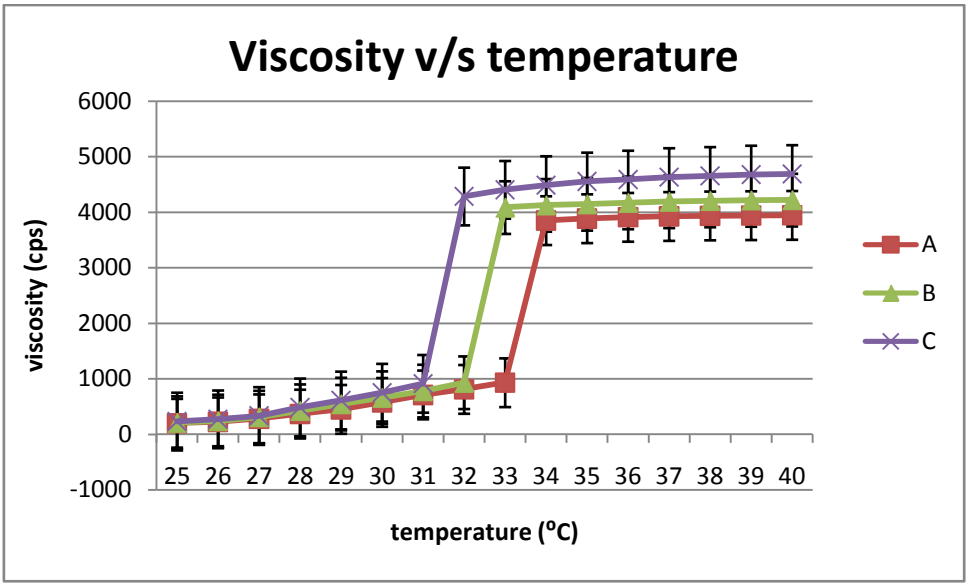


Figure no.35 Effect of temperature on viscosity of in situ gel

Viscosity of the optimized formulation was measured at different temperature from 25 to 40^0C (Figure no.35). It was observed that, the viscosity of gel was suddenly increased between temperature 35-37^0C which indicated sol to gel transformation. The viscosity of gel formulation was found to be in the range of 800-900 cps below 35^0 C which increased to about 4000-5000 cps above 35^0 C indicating gelation temperatures. The increase in concentration of poloxamer 188 caused decrease in the gelation temperature.

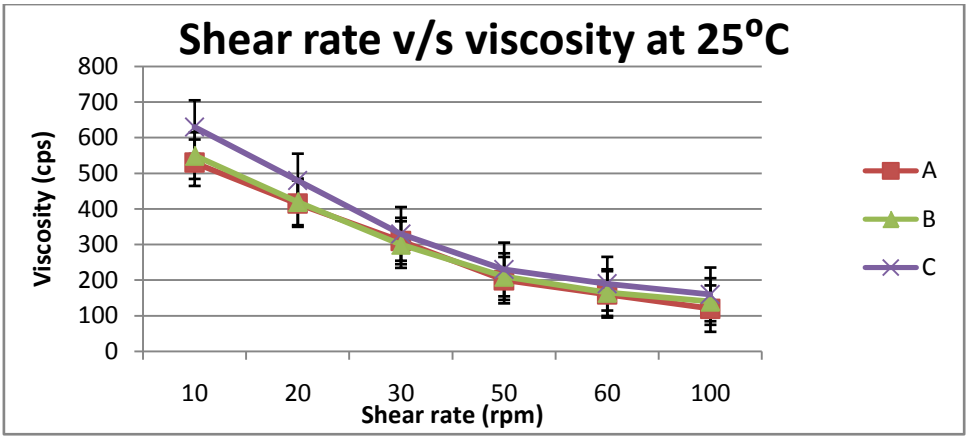


Figure no.36 Comparison of viscosity change with change in shear rate at 25^0C for different formulations

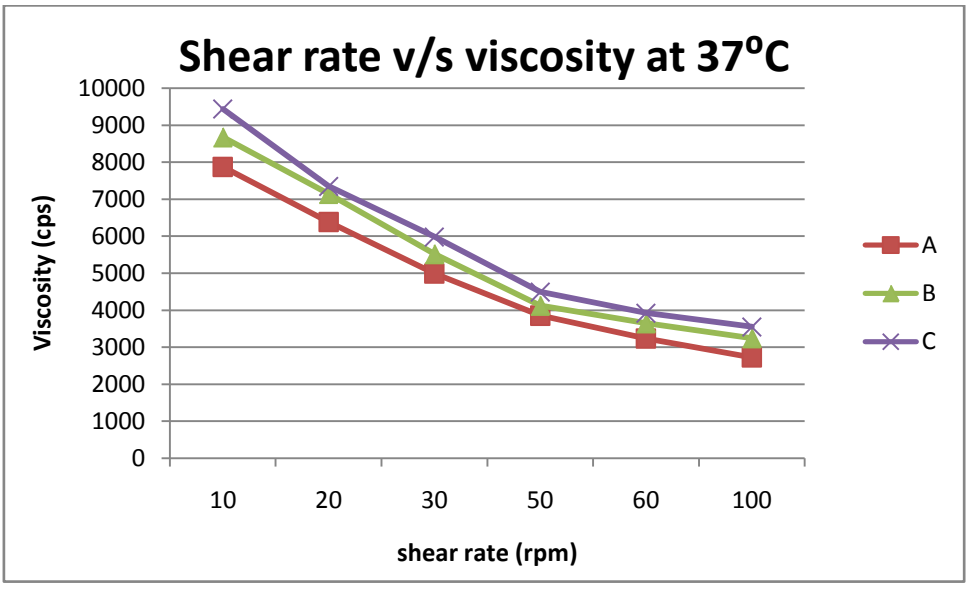


Figure no.37 Comparison of viscosity change with change in shear rate at 37^0C for different formulations.

All the formulations showed pseudoplastic rheological flow after studying at various temperatures, as evidenced by shear thinning and increase in shear stress with increased angular velocity. It was found that the rheological parameter was directly dependent on polymer concentration of formulation. At 25^0C, all formulations were having low viscosity (Figure no. 36) and at 37^0C, the formulations showed high viscosity (Figure no.37). This indicates conversion of these formulations from sol to gel. It was also observed that viscosity of all formulations was decreasing with increase in shear rate. The non-newtonian formulations with pseudoplastic properties can acquire a viscosity decrease with increasing shear rate, creating blinking and ocular movement. Pseudoplasticity is thus interesting

because it offers significantly less resistance to blinking and shows much greater acceptance than viscous newtonian formulations.

2.8. In vitro drug diffusion study

The in vitro drug release profile of gel formulations was calculated by PCP disso software where the percent drug release along with dissolution kinetics and best fit model was found out. T90 value was found out using this software and comparison was done on its basis (Figure no. 38). T90 value is the time at which 90% of drug is released (Table no. 31). A, B and C batch exhibited t90 value of 523 min (8 hours and 43 min), 596 min(9 hours 56 min) and 555 min (9 hours 15 min) respectively . The release kinetics indicated that it followed zero order which means drug release does not depend on initial concentration. The B batch (poloxamer 407:poloxamer 188- 1:2.7) was found to be the optimized batch with maximum t90 value thus sustaining action for a long period of time. The marketed formulation and the pure drug showed 20-23% drug diffusion in 6 hours. Thus the amount of drug diffused was increased by niosomal in situ gel formulation.

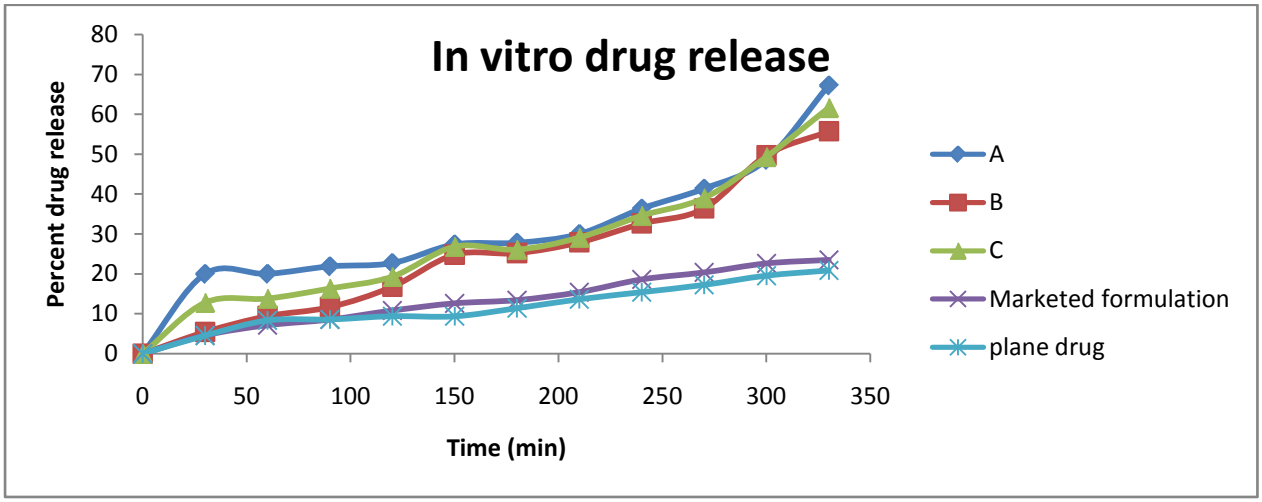


Figure no.38. In vitro drug release profile of insitu niosomal batches

Table no. 31 Release kinetics of insitu niosomal batches

Batch Code	**Zero order model**	**First order model**	**Higuchi/Matrix model**	**HixsonCrowell model**	**Korsmeyer–Peppas model**			**Best fit kinetic model**	**$t_{90\%}$**
	R	R	R	R	R	n	K		Min
A	0.9146	0.883	0.892	0.902	0.8526	0.4819	2.7082	**ZO**	523.4

B	**0.9777**	**0.946**	**0.888**	**0.959**	**0.9728**	**0.9384**	**0.2028**	**ZO**	**596.1**
C	0.9631	0.928	0.904	0.944	0.9353	0.6425	1.0740	**ZO**	555.6

2.9. Ex vivo drug release studies

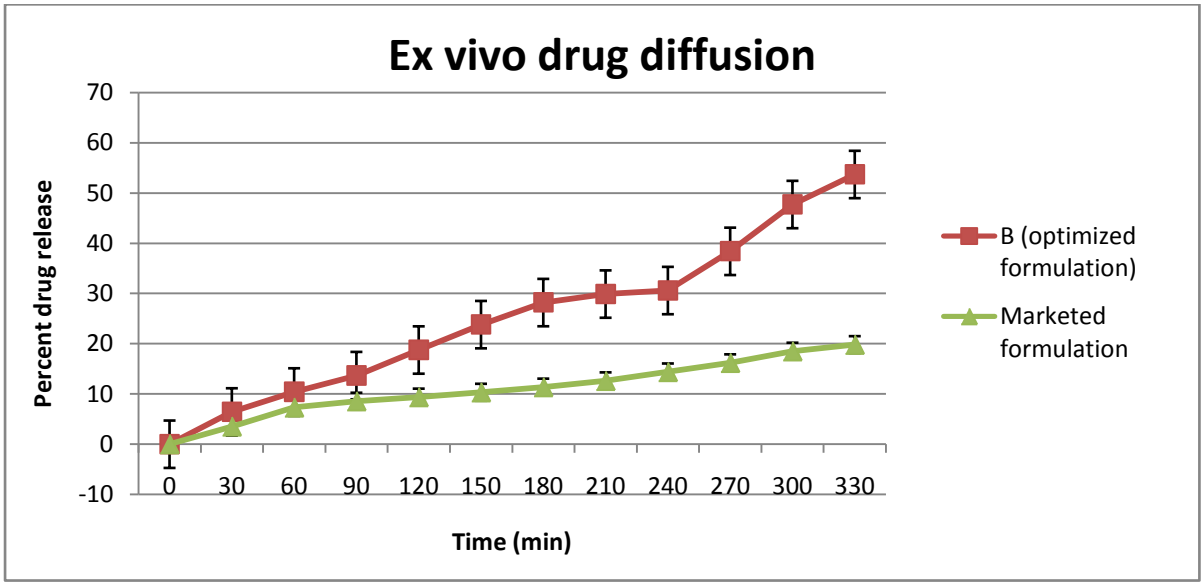


Figure no.39 Ex vivo drug diffusion of optimized formulation

The ex vivo drug diffusion study of optimized formulation shown that about 90% of drug is going to release upto 10 hours providing sustained effect (Figure no. 39). The marketed formulation shown just 20% drug release after 6 hours which may be due to its larger molecule weight and thus large molecule size which indicates there may be problem in the conjunctival absorption of drug. Thus this problem was overcome by niosomal in situ gel formulation. The size of particle was reduced due to its incorporation into niosomes and the release was sustained by in situ gel formulation.

2.10. Pharmacodynamic study

2.10.1. Primary skin irritation test

The primary Irritation index of the test sample was calculated to be 0.00; No irritation was observed on the skin of the rabbits/ rats. Individual results of derma scoring appear in table no. 32

Table no. 32 Reaction scores of animals to irritation potential of niosomal in situ gel

Animals	Reaction	24 Hours	72 Hours
Rabbit 1	Erythema	0	0
	Edema	0	0

Rabbit 2	Erythema	0	0
	Edema	0	0

The scores for erythema and edema were summed for intact and abraded skin for rabbits at 24 and 72 hours. The primary irritation index (P.I.I) was calculated. Based on the sum of the scored reactions divided by 32 (two scoring intervals multiplied by two test parameters multiplied by 8 animals).

Primary Irritation Index: 0/32 = 0.00. Under the conditions of this test, the test sample would not be considered a primary skin irritant since the primary Irritation Index was less than 5.00.

2.10.2. In vivo Draize eye irritation test:

The results of the ophthalmic irritation studies were given in Table.no. 33

Table no. 33 Irritation scores of animals to niosomal in situ gel

Days	Observations			
	Cornea	Iris	Conjunctivae	Total
1	0	0	1	1
2	0	0	2	2
3	0	0	3	3
4	0	0	2	2
5	0	0	1	1
6	0	0	0	0

The possibility of eye irritation due to polymer administration was evaluated in rabbits. The rabbits were observed for ocular lesions, and no symptoms of ocular irritation such as redness, tearing, inflammation, or swelling were observed after polymer administration. No ophthalmic damage or abnormal clinical signs to the cornea, iris or conjunctivae were visible. Thus, the developed ocular drug delivery systems are apparently free from any ocular irritation potential and can be safely administered to humans. The scores were calculated according to Draize scale.

2.11. Pharmacokinetic study

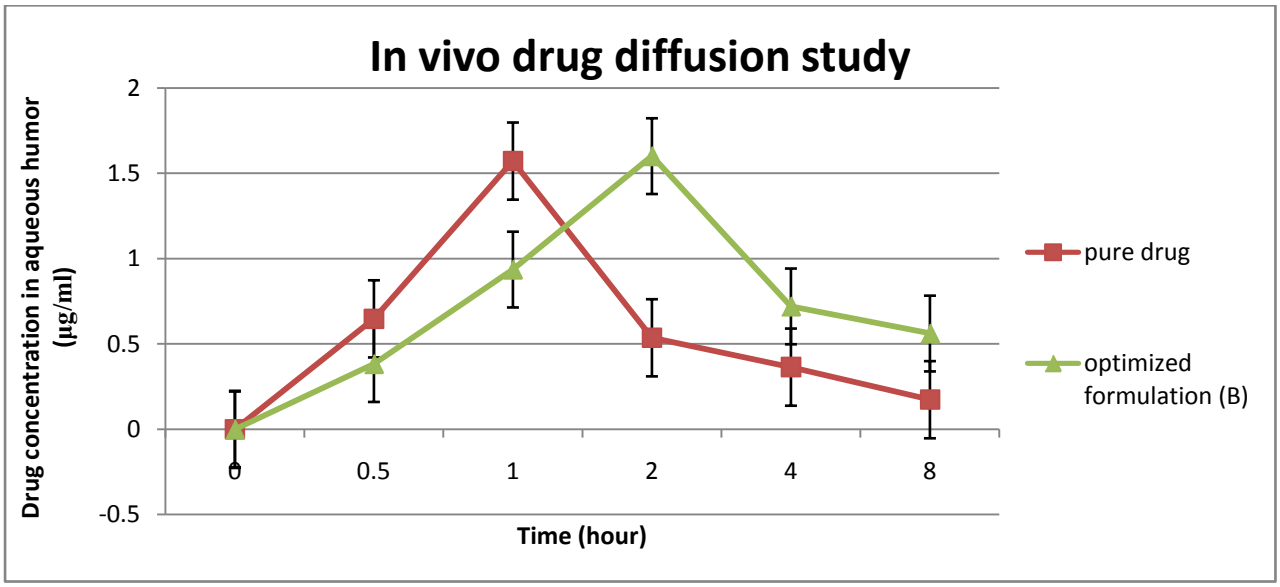


Figure no.40. Aqueous humor concentration-time profiles of prednisolone sodium phosphate following ocular instillation of the drug solution and optimized B suspension to rabbits (mean±SD, n=3)

Table no. 34. The Pharmacokinetic Parameters of prednisolone sodium phosphate following Ocular Instillation of the Drug Solution and optimized formulation B Suspension to Rabbits (Mean±SD, n=3)

Batch	**C_{max} (µg/ml)**	**T_{max} (h)**	**$AUC_{(0-8)}$ (µg $h^{-1}ml^{-1}$)**	**$MRT_{(0-8)}$ (h)**	**Increase in bioavailability (folds)**
Pure drug solution	1.573±0.345	1	3.75225±0.254	3.583±0.526	-
Optimized formulation (B)	1.602±0.427	2	6.584±0.127	5.349±0.035	1.754

The aqueous humor concentration (mean±SD) time profiles of prednisolone sodium phosphate following ocular instillation of optimized niosomal in situ gel (B) and the drug solution in rabbits are depicted in Figure no.40. The differences between the estimated drug pharmacokinetic parameters (Cmax, Tmax, andMRT(0–8h)) of the two treatmentsare illustrated in Table no. 34. The mean (±SD) Cmax of the drug solution and that of optimized formulation (B) NP suspension were found to be equivalent. The delay in the median Tmax (from 1 to 2 h) as well as the prolongation in the MRT(0–8h) (from 3.583±0.526 to

5.349±0.035 h) for the drug solution and the optimized formulation, respectively could indicate the sustained-release characteristics of the latter.Based on the calculated AUC(0–8) values, the increase in the ocular bioavailability was found to be 1.657-fold. The drug penetration enhancement following the instillation of the optimized formulation, could be attributed to the presence of surfactant (span 60) in niosomal formulation which also acts as penetration enhancer. In addition to this, prednisolone sodium phosphate being hydrophilic in nature can easily pass the epithelial barrier and thus contribute to increased penetration. The bioadhesive synthesized polymer macromolecular hydrocolloids with numerous hydrophilic functional groups (carboxylic acid). The cornea and conjunctiva have a negative charge where these mucoadhesive polymers may interact intimately with these extra ocular structures, would increase the concentration and residence time of the associated drug. A general conclusion is that charged polymers both anionic and cationic demonstrate a better mucoadhesive capacity.

2.12. Sterility test

In order to ensure the sterility of the finished product, the final formulation GS36 was subjected to sterility test. The formulation sterilized by membrane filtration method and incubated with different culture media like soybean casein digest medium; fluid thioglycate medium etc., for a period of 14 days of incubation did not show growth of organism on the culture medium. This indicated that the formulation was sterile.

2.13. Stability study:

2.13.1 Accelerated stability study:

2.13.1.1 Appearance:

All the formulations were found to be translucent but there was a little formation of gel at the bottom of the container. The gel could be converted into a sol with slight shaking.

2.13.1.2 Percent drug content determination:

Table no.35 Stability testing by percent drug content determination at regular time interval.

Time period	**Percent drug content of optimized formulation**
Initial	91.36±0.36
After 3 months	90.53±0.27
After 6 months	88.45±0.47

The results obtained shown that all the formulations were found to contain almost same amount of drug after 6 months. There was a little decrease in percent drug content but it was less than 5% for each formulation after 6 months (Table no. 35).

2.13.1.3.Gelation temperature

Table no.36 Stability testing by gelation temperature measurement at regular time interval.

Time period	**Gelation temperature (^{0}C)**
Initial	37.2±0.23
After 3 months	37.0±0.41
After 6 months	37.0±0.05

The results obtained showed little decrease in the gelation temperature of the formulation with increase in time but that was so less that it can be neglected (Table no. 36).

2.13.2 Long term stability study:

2.13.2.1 Appearance:

All the formulations were found clear and stable.

2.13.2.2 Percent drug content determination:

Table no. 37 Stability testing by percent drug content determination at regular time interval.

Time period	**Percent drug content of optimized formulation**
Initial	91.36±0.36
After 3 months	89.53±0.27

The results obtained shown that all the formulations were found to contain almost same amount of drug after 3 months. So it can be concluded that there wasn't any drug loss from formulation during storage (Table no.37).

2.12.2.3. Gelation temperature

Table no. 38: Stability testing by gelation temperature measurement at regular time interval.

Time period	**Gelation temperature (^{0}C)**
Initial	37.2±0.23
After 3 months	37.1±0.41

The results obtained showed there wasn't any significant change in the gelation temperature of the formulations after 3 months. So, it can be predicted that the formulation would be stable during storage (Table no. 38).

CONCLUSION

Monomer acrylic acid was subjected to polymerization by using three different crosslinking agent namely divinylbenzene, divinyl glycol and 2,5-dimethyl-1,4-hexadiene. These crosslinking agents were used in different concentrations (0.3,0.6,1 g)with respect to 100 g monomer. The effect of different crosslinking agents on polymer swelling and hydration property was found to be insignificant. Similar results were obtained with respect to different concentrations of crosslinking agent. But the effect of different crosslinking agent on bioadhesion was significant. With decrease in density of crosslinking agent, the mucoadhesive strength was increased. The carboxylic groups present in polymer were responsible for bioadhesion process because the free protons would cause bioadhesion by hydrogen bonding. Thus more the number of free carboxylic groups present in the polymer, more is the mucoadhesive strength. The XRD spectra indicated that all the polymers were found to be in semicrystalline in nature. The irritation potential measured by ex vivo HET-CAM test indicated that DG polymer were non irritant and did not show any signs of inflammation and swelling. Thus DG polymer was further tested by in vivo draize skin and eye irritation test. The results coincided with the ex vivo test proving that the DG polymer was found to be non-irritant polymer. Niosomes were prepared by using varying concentrations of cholesterol and span 60. The preliminary batches were prepared for method selection, surfactant selection and the ratio selection of cholesterol and surfactant. These niosomes were spherical in nature and with high drug content and entrapment efficiency. The particle size was found to be in the range of 400-500 nm thus feasible for ocular use without causing irritation to eye. The in vitro drug release showed t90 value of 8 h thus sustaining the release upto 8 h. The niosomes were converted into solid pellets by process of freeze drying. The in situ gel batches were prepared using various concentration of poloxamer 188 and poloxamer 407 respectively in order to obtain gelation temperature in the range of 36-37^{o}C. The optimized batch was further evaluated for gelling properties and rheogram was studied which proved that the system was shear thinning in nature. The in vitro drug release showed sustained action of drug upto 10 h which coincided with the results of ex vivo study. Pharmacokinetic study in rabbits proved that the total concentration drug in aqueous humor was higher as compared to that of pure drug solution. The Mean residence time and shift in tmax value indicated sustained release of drug. Thus niosomal in situ system proved to be very useful system for ocular drug delivery with promising results.

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Research Publication

On

Research Project

Research Article

Synthesis and Evaluation of Water Insoluble but Swellable Bioadhesive Polymer for Ocular Drug Delivery

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ABSTRACT

Introduction: Our main purpose of present study was to find out the effect of different crosslinking agents along with its concentration during polymer synthesis and to find the most suitable polymer for ocular drug delivery with optimum bioadhesive strength and less irritation potential. It was expected that the synthesized polymer will remain adhered to the conjunctival mucin layer thus preventing loss of drug by precorneal factors. **Materials and methods:** Acrylic acid procured from Loba chemicals was polymerized with Divinyl glycol from Merck specialities Private limited, Divinylbenzene and 2, 5-dimethyl-1, 5-hexadiene from Alfa Aesar to form water insoluble swellable polymer. Different parameters like density, polymer hydration, Differential Scanning Calorimetry, Infra-Red spectroscopy, X ray diffraction and mucoadhesive strength were determined. Polymer hydration was studied with respect to time, pH and ionic strength. HET-CAM test and Draize skin irritation test was performed to evaluate the degree of irritation caused by these polymers. **Results and Discussion:** It was found that as more hydrophobic groups were introduced in the polymer structure, hydration potential was reduced. The effect of concentration of crosslinking agent on bioadhesive strength was significant. With increase in density of crosslinking agent, the bioadhesive strength decreased. Polymers were found to be non-irritant to slight irritant in nature.

Key words: Bioadhesion, Ocular, Water Insoluble polymer, Acrylic acid, Hydration.

INTRODUCTION

The most probable reasons responsible for low ocular bioavailability of drugs include the precorneal loss factors which include tear dynamics (blinking reflex and tear turn-over),[1] non-productive absorption, transient residence time in the cul-de-sac, relative impermeability through corneal epithelial membrane, rapid precorneal elimination, drainage by gravity, frequent instillation, enzymatic metabolism, nasolacrimal drainage and the absence of controlled release.[2-9] Only ≤1 % of administered drug dose is absorbed ocularly because of the factors mentioned above.[10,11] Thus increasing the dosing frequency of drugs becomes the requirement in ocular drug delivery to achieve therapeutic concentration of drug which may lead to surge in local and systemic side effects like gastrointestinal disorders.[12] The side effects can be overcome by opting for systemic route, but the blood brain barrier and blood aqueous barrier further leads to high loading dose. The difficulties involved in conventional ocular therapy can be overcome by various approaches like liposomes, niosomes, nanoparticles, micro-particles, gel based drug delivery system, ocuserts and bioadhesive systems.[3,13,14]

Use of bioadhesive polymer proves to be the solution where polymeric substances remains attached to precorneal surface through non covalent bonds.[15] Literature survey[16,17] showcased two points after thor-ough study of polymer's binding affinity to

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mucin epithelial surface for bioadhesion process. First point was the polyanionic and water insoluble polymer would offer more advantages over neutral drug delivery system/ polycationic polymer and water soluble polymer respectively. Second point was that presence of carboxylic acid would be preferred over other functional groups as it relates with bioadhesion.[16] Acrylic acid polymers are example of water insoluble swellable polymer which has wide water holding capacity.[17] Following properties should be exhibited by the bioadhesive polymer: a) non toxic b) non absorbable at target site c) should adhere to the conjunctival mucin/epithelial surface by non covalent bond d) quick adhesion e) easy inclusion of drug without any interference to its release f) cost effective. The objectives behind the present study was to synthesize series of polymers by using different crosslinking agents, to investigate its properties physical properties and to find out best suitable polymer for ocular drug delivery with optimum bioadhesion and less irritation potential to animals. Monomer acrylic acid was crosslinked with three different cross linking agents namely divinyl glycol, divinyl benzene and 2,5-dimethyl-l,5-hexadiene to produce series of polymers and evaluated for its suitability for ocular drug delivery.

MATERIALS AND METHODS

Materials

Acrylic acid and benzoyl peroxide were obtained from Loba Chemicals. Magnesium sulfate heptahydrate and Divinyl glycol were procured from Merck Specialities Private Limited. Divinylbenzene and 2,5-dimethyl-1,5-hexadiene were purchased from Alfa Aesar. All of the above chemicals were of analytical grade.

Method

In 100 gm acrylic acid, one gram of benzoyl peroxide (initiator) was dissolved. The cross-linking agent (divinely benzene/ divinyl glycol/ 2,5-dimethyl-1,5-hexadiene) was added in three different concentrations (0.3/0.6/1 g) with stirring in to a solution containing 800 g of magnesium sulfate (MgS04. 7H20) in 100 mL of distilled water and refluxed at temperature of 95°. Polymerization was achieved within 30 min of reflux. After polymerization, the mixture was maintained at the same temperature with stirring for 2 h of curing time. At the end of the reaction, the mixture was diluted with 150 mL of hot water and then repeatedly washed with equal portions of water. The washed cross-linked polymer was dried in a hot air oven at 90°C for a specified time before being ground to the required size (30-40 mesh).[18]

Physicochemical Properties of Polymers

Yield and Density

The density of each polymer was determined in a 2 ml specific gravity bottle at 25°C. Benzene of known density (0.874 g/ml) was used as the medium because no swelling of the polymer in benzene was observed.[17]

Polymer hydration

Each polymer was weighed 0.2g and allowed to hydrate in 10 mL of distilled water at 25°C in a 25-mL graduated cylinder. The volume of the hydrated polymer was measured at 5-min intervals until the rise in hydrated volume was constant.[17]

Effect of pH on swelling of polymer

In 100 ml test solution, 50 mg polymer was introduced and allowed to hydrate at 37°C for 24 h with occasional stirring to remove trapped air bubbles. The pH of the solution was constantly checked and adjusted with saturated sodium hydroxide solution, if required, to maintain pH. After 24 h, the fully hydrated polymer was transferred to a 10-mL graduated cylinder and allowed to settle. After several h, the increase in hydration volume was measured. The test solutions were HCL of pH 1.2 and 2, 0.1 M monobasic potassium phosphate of pH 3,4,5,6,7.[18]

Effect of time on swelling ratio[18]

Polymer (0.1 g) was placed in 10 ml distilled water. At different time intervals like 5, 10, 15, 20, 25,30,35,40, 50,60,90,120,150,180,210,240 min, weight of swollen polymer was noted. The swelling ratio was calculated as

Swelling ratio = weight of swollen polymer- initial weight of polymer/ initial weight of polymer

Effect of ions on swelling ratio

Polymer (0.1 g) was dissolved in 0.1 M different solutions for 2 hrs. The solutions were prepared in a manner where polymer was exposed to different monovalent and divalent ionic solutions. The hydrated polymer was weighed after 2 hrs and the swelling ratio was determined by formula as mentioned in above procedure.[17,18]

FTIR spectrometry

Infrared spectrums of polymers were determined on Fourier Transform Infrared Spectrophotometer (FTIR 4100, Jasco) using KBr dispersion method. The base line correction was done using dried potassium bromide. The samples to be analysed and KBr were previously dried in oven for 30 min and mixed thoroughly with potassium bromide in 1:300 (sample: KBr) ratio in a glass mortar. These samples were then placed in a

sample holder and scans were obtained at a resolution of 2 cm-1 from 4000 to 400 cm-1.

Ex vivo Mucoadhesive strength determination

The mucoadhesive force which is defined as detachment stress of the polymer was determined by using a modification of weighing balance. Fresh goat lower eyelids of both eyes were bought from slaughter house and placed in an aerated saline solution until used. One of the eyelids was secured over a weighted glass vial using a rubber band so that the conjunctiva faced outwards. The vial was then placed in a jacketed beaker thermostated at 37°C containing 500 ml pH 7.4 isotonic Sorensen buffer and positioned under the scale. Another eyelid was placed on rubber stopper of another vial which was hanged on the balance. One vial was connected to the balance and the other fixed with polymer gel and the height was adjusted so that the polymer is placed between mucosal sides of both vials. Water from the burette was allowed to fall drop by drop till the detachment of vials. Mucoadhesive force was determined from minimal weights of water that detached the vial.[17,19]

Mucoadhesive strength (dynes/cm2) = mg/A
Where, m = Weight required for detachment in g
g = acceleration due to gravity (980 cm/s^2)
A = area of mucosa exposed (cm^2)

Differential Scanning Calorimetry

DSC measurements were performed on a differential scanning calorimeter (DSC Mettler STAR SW 9.20, Switzerland). Nitrogen gas was purged at a flow rate of 50ml/min in order to maintain inert atmosphere. In a sealed aluminium pan, all accurately weighed samples were placed and the heating of samples was carried out at the rate of 10°C/min under nitrogen gas flow (20 ml/min) for 40-340°. An empty aluminum pan was used as reference.

X Ray Diffraction analysis

X-ray diffraction patterns of the polymer samples were recorded using Philips PW3710 Analytical XRD B. V. X-ray diffractometer using Cu K 2α rays with a voltage of 40 kV and a current of 25 mA. Samples were scanned for 2θ from 5 to 500. Diffraction patterns of polymers were obtained using an X-ray diffractometer (mod. D8 Discover, Bruker, USA).

Conjunctival (HET-CAM) test[20]

Fertilized hen's eggs were freshly bought from poultry farm and were kept in incubation chamber at temperature 37.5± 0.5 ◦C and 66 ± 5% relative humidity (RH) for a period of three days. On third day, eggshells were broken and inner content was exposed on petri plate. The main criterion for this test was only viable embryo with intact CAM and yolk sacs were used and further incubated. The conjunctival irritation potential of the polymers was investigated. On tenth day, 0.1 g (solid) of each polymer was placed on the membrane. The test was carried out in triplicates. Following were the chemicals used as standards in order to compare the degree of irritation. Sodium hydroxide (0.5M) was used as positive control strong irritant, acetone as moderate irritant, propylene glycol as slight irritant and normal saline solution as negative control.[21] The blood vessels and capillaries were inspected for irritation effects. The irritation effects considered in order to classify the polymers on degree of irritation after instillation for 5 min were hyperaemia, haemorrhage and clotting.[22] Based on how much time is required for each effect to occur by each polymer, a time-dependent numerical score was assigned (Table 1). A single numerical value was calculated by addition of numerical values obtained for each irritant response and which ultimately determined degree of irritation potential (Table 2). The classification system used to determine irritation potential was similar to that used in Draize test.[23]

Table 1: Synthesis scheme for polyacrylic acid along with the concentrations.

Monomer (100 gm)	Cross-Linking agent	Quantity (gm)	Polymer code	Initiator
Acrylic acid	Divinyl glycol	0.3	DG1	Benzoyl peroxide
	Divinylbenzene		DB1	
	2,5,-Dirnethyl-l,5hexadiene		HD1	
Acrylic acid	Divinyl glycol	0.6	DG2	
	Divinylbenzene		DB2	
	2,5,-Dirnethyl-l,5hexadiene		HD2	
Acrylic acid	Divinyl glycol	1	DG3	
	Divinylbenzene		DB3	
	2,5,-Dirnethyl-l,5hexadiene		HD3	

Table 2: Irritation scores and interpretations used in HET-CAM test.

	Score			Cumulative score	Irritation assessment
Effect/time (min)	0.5	2	5	0-0.9	None
Hyperemia	5	3	1	1.0-4.9	Slight
Haemorrhage	7	5	3	5.0-8.9	Moderate
Clotting/ coagulation	9	7	5	9.0-21.0	Severe

Primary skin irritation test

Two healthy albino rabbits were used for the experiment. Animal husbandry was conducted in accordance with the "Guide for the Care and use of Laboratory Animals," NIH publication No.85-23.

Methods: The backs of the animals were cleaned free of fur with a razor atleast 4 hrs before application of the sample. One ml sample of the least irritant polymer obtained from *ex vivo* test was then applied to the particular site to an area of skin approximately 1" × 1" (2.54 × 2.54 cm) square. The sample applied site was covered with a nonreactive tape. Animals were returned to their cages. After a 24 hrs exposure, the tape was removed and the test sites were wiped with tap water to remove the test sample. At 24 and 72 hrs after test sample application, the test sites were examined for dermal reactions in accordance with the FHSA-recommended Draize scoring criteria (Appendix 1). The Primary Irritation Index (P.I.I.) of the test sample was calculated following test completion.[24,25]

In vivo Draize eye irritation test

The Draize test was performed on white albino rabbits. In this test 100 µg test sample was placed into the lower cul-de-sac of rabbit's right eye (1.5-2 kg, 13 week of age). Left eye was treated as a control. Rabbits' eyes were observed periodically for redness, swelling and watering of the eye at 1 h, 4 h and every 24 h for 7 days. Three rabbits were used for test substance. These parameters were calculated from weighted scores for each part of the rabbit eye such as (cornea, iris and conjunctiva) and also from the sum of these scores. The maximal average Draize total scores (MAS) are classified into non-irritants (0<MAS<0.5), slight irritants (0.5<MAS<15), mild irritants (15<MAS<25), moderate irritants (25<MAS<50) and severe irritants (50<MAS).[26] Approval of the institutional animal ethics committee (Approval No. MCP/IAEC/01/2016) was obtained prior to the commencing of the study from Modern college of Pharmacy, Nigdi, Pune.

RESULTS AND DISCUSSION

Yield and density

Polymerization occurred within a period of 30 min. While synthesis, extensive swelling of produced polymer was overcome by use of magnesium sulphate heptahydrate which served as suspending agent. Significant yields were obtained in case of all the polymers. The results indicated in Table 3 shows that the effect of concentration of crosslinking agent on density was insignificant. In addition to this, use of different crosslinking agent also had very small fraction of influence on density parameter.

Table 3: Yield and density of crosslinked swelling polymers.

Sr. No.	Polymer	Yield (%)	Density
1.	DB1	72±0.208	1.458±0.237
2.	DB2	69±0.251	1.462±0.319
3.	DB3	78±0.372	1.483±0.456
1.	DG1	87±0.637	1.529±0.163
2.	DG2	81±0.432	1.572±0.089
3.	DG3	89±0.312	1.595±0.504
1.	HD1	94±0.583	1.622±0.275
2.	HD2	96±0.291	1.636±0.328
3.	HD3	92±0.726	1.641±0.461

Polymer hydration

The effect of different crosslinking agent on the extent of hydration is shown in Figure 1. The rate and extent of hydration plays an important role in practical implications because swelling increases surface area. It was observed that hydration of polymer occurred quickly in water and equilibrium was achieved within 30-40 min (Figure 1). The degree of hydration was not significantly affected by change in either crosslinking agent or its concentration. Smaller-sized particles hydrate more quickly than larger-sized particles because the penetration of water molecules through small particles is easy due to lesser thickness of small particles.

Effect of pH on swelling of polymer

Equilibrium swelling of polymer was measured at different pH values and corresponding profiles are shown in Figure 2. In acidic pH (upto 4), the increase in swelling was slight. As the pH started increasing above 4, significant degree of swelling was observed upto pH 7. In the process of polymer hydration, the dependence of water movement into the polymer network in the presence of electrolytes is known to be a characteristic typical of Donnan membrane equi1ibrium. Since the pKa of polyacrylic acid is 4.75,[17] pH dependent equilibrium swelling was expected. The polyacrylic acid consists of large number of carboxylic (COOH) groups along the polymer backbone which makes it pH sensitive, hydrophilic and capable of forming hydrogen bonds.[27]

Effect of time on swelling ratio

Figure 3 shows that all of the polymers tested hydrate quickly in water, reaching equilibrium in 20-40 min. Once the equilibrium swelling was attained, the increase in swelling ratio was more or less constant. Smaller-sized

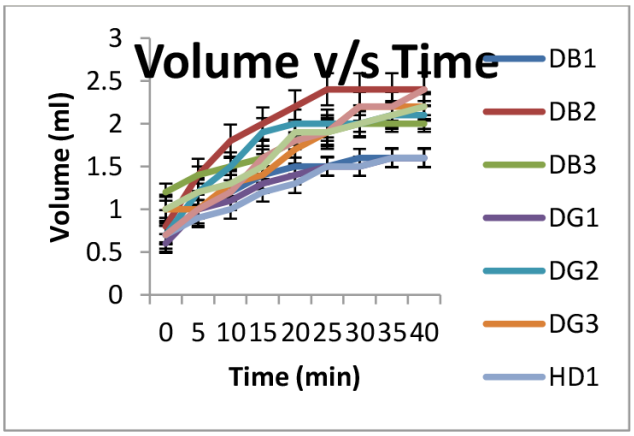

Figure 1: Graph of Hydration volume v/s time of cross linked swelling polymers.

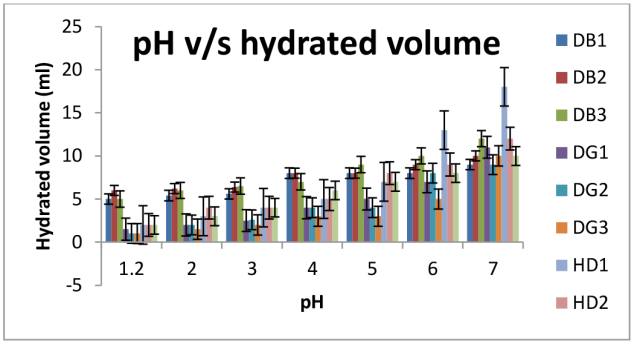

Figure 2: Graph of Hydrated volume v/s pH of cross linked swelling polymers.

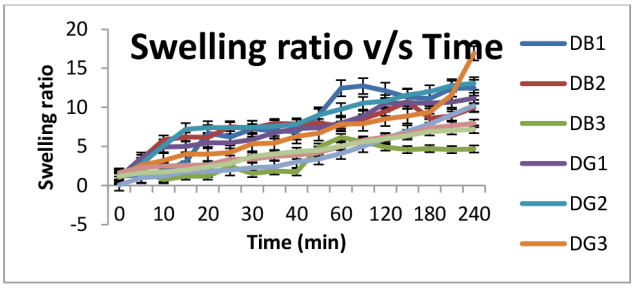

Figure 3: Plot of swelling ratio v/s time.

particles hydrated faster than larger-sized particles. The polymer molecules in dry state are highly coiled and tightly packed structures. When placed in water, they behave as anionic electrolytes. They dissociate and partially uncoil due to repulsion of negative charges generated along the polymer chains. The subsequent swelling is caused by difference in osmotic pressure inside the vicinity of polymer chains (cluster) and bulk medium.[28]

Effect of ions on swelling ratio

Literature review[29] indicated that the important influential factors were the ion strength of solution and the

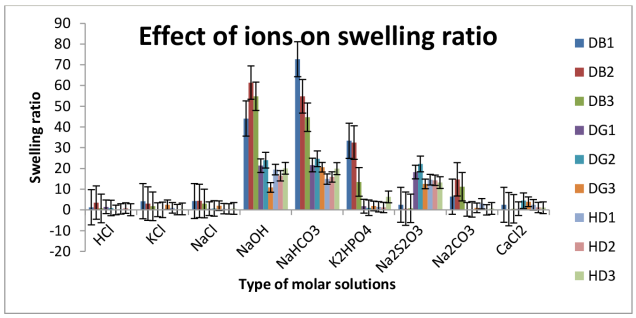

Figure 4: Plot of swelling ratio in different types of solution.

valency of cation. They considered that the electrostatic may be the main reason that the gel absorbs the water. The swelling characteristics of polymer at same ionic strength in acid, base and salt was measured. The study showed that the effect of different ions did not cause any significant change in the swelling of drug (Figure 4). Monovalent and divalent ions also did not affect the swelling characteristics of polymer. The increase in swelling for solutions like NaOH, NaHCO3, K2HPO4 was attributed to change in swelling of reaction mixture solution (Basic solution) and not due to presence of ions. This was confirmed by use of other salts containing same ions but did not show swelling. Figure 4 proves that electrostatic is not the main reason that the hydrogel absorbs water at room temperature. The effect of temperature on swelling ratio showed similar results where there was no significant effect observed with respect to change in temperature (from 25 °C to 60 °C). Thus ultimately it proved that the bioadhesive property was not affected by presence of ions or with change in temperature.

FTIR

(A) Figure 5(A) exhibits following wavenumbers 720.282, 1467.56, 1736.58, 2849.31, 3402.78 cm^{-1} denotes Rocking band $–CH_2$- bending (long chain band), $-CH_2$- bending, C=O carboxylic acid, Tertiary C-H symmetric stretching, sp =C-H stretching band respectively. The tertiary C-H (methine hydrogen) gives weak C-H absorption near 2890 cm-1. Methylene hydrogen ($-CH_2$-) gives rise to two C-H stretching bands representing the symmetric (sym) and asymmetric (asym) stretching modes of group. In effect, 2890 cm-1 methine absorption is split into 2 bands 2926 cm-1 (asym) and 2853 cm-1 (sym). The free carboxylic group was retained which is found to be responsible for bioadhesion. Four acrylic acid molecules are assumed to be bound together by one molecule of crosslinking agent viz divinyl benzene (Figure 5(D)).

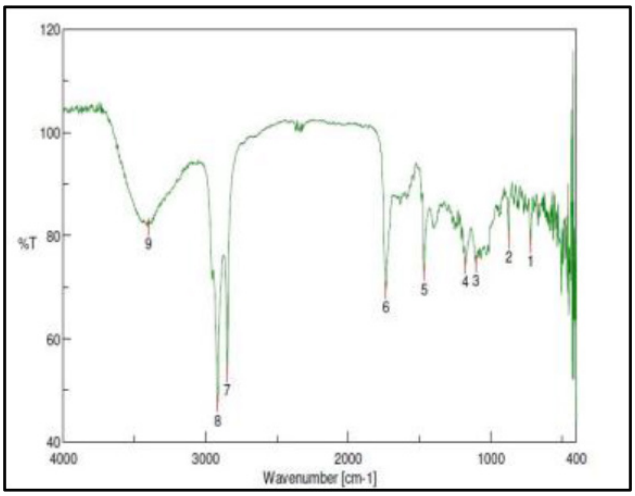

Figure 5(A): IR Spectra of DB1.

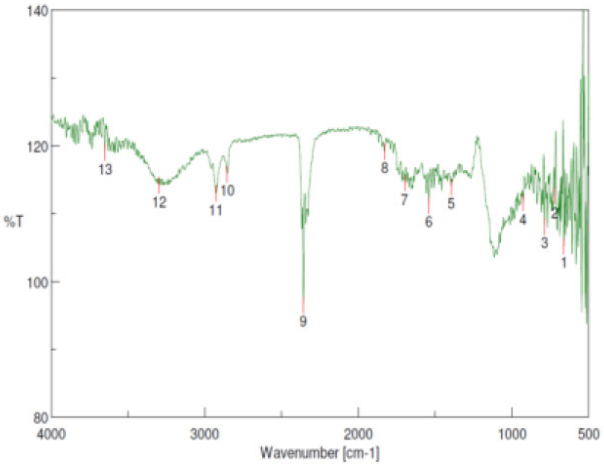

Figure 5(B): IR Spectra of DG1.

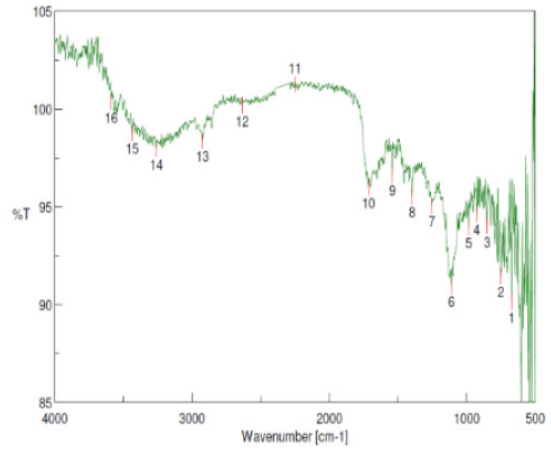

Figure 5(C): IR Spectra of HD1.

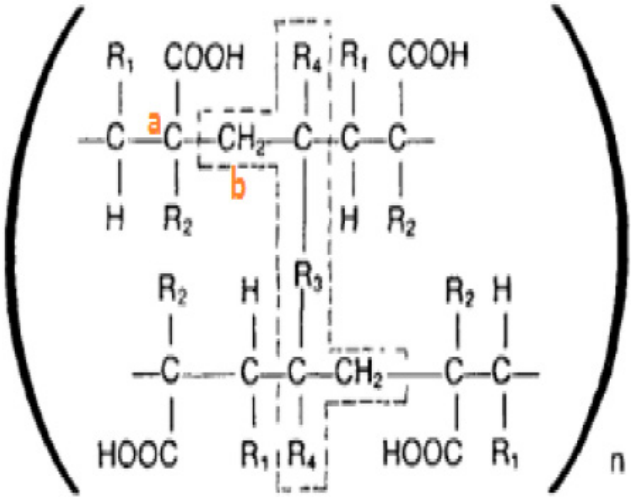

Figure 5(D): Probable structure of polymers.

Table 4: Mucoadhesive strength of polymer.

Polymer	R1	R2	R3	R4
DB	H	H	(p)-C_6H_4	H
DG	H	H	-CHOHCHOH-	H
HD	H	H	$-CH_2CH_2-$	CH_3

(B) Figure 5 (B) exhibits following wavenumbers 722.2, 1698, 2358.5, 2854, 3296, 3648.7 cm^{-1} denotes Bending motion associated with four or more CH_2 groups in an open chain (long chain band), C=O carboxylic acid stretching, O-H carboxylic acid stretching, $-CH_2-$ stretching asymmetric, C-H alkane stretch, Free –OH group stretching respectively. The four acrylic acid groups are assumed to be bonded together by crosslinking agent viz divinyl glycol (Figure 5(D)).

(C) Figure 5 (C) exhibits following wavenumbers 906.4, 1105, 1294, 1507.1, 1714.4, 2452, 2924.5, 3258.1 denotes H-bonded (O-H) out of plane bending, C-O stretch (2° alcohol saturated), C-O stretching band, C=C stretching, C=O carboxylic acid stretching, O-H stretching vibration of carboxylic acid, C-H alkane stretching, O-H (H-bonded stretching) respectively. The crosslinking agent viz 2,5- dimethyl-1,5-hexadiene assume to bond four molecules of acrylic acid (Figure 5(D)).[30] Table 4 represents different substitution present in polymer structure due to different crosslinking agents.

The change in concentration of crosslinking agent did not show any significant change in IR spectra of these polymers. Hence only one spectra of each crosslinked polymer is discussed here in detail as a representative of that class. The purpose of this study was to evaluate the effects of crosslinking agents and which functional groups are responsible for these effects was suggested by IR spectroscopy

Table 5: Reaction scores of animals to irritation potential.

Sr. No.	Polymer Code	Weight required for detachment in g	Mucoadhesive strength (dynes/cm²)
1.	DB1	2.2±0.057	686.92±0.042
2.	DB2	2.43±0.063	749.04±0.031
3.	DB3	. 2.57±0.039	780.25±0.053
1.	DG1	1.49±0.041	436.94±0.046
2.	DG2	1.62±0.026	499.36±0.018
3.	DG3	2.0±0.037	624.20±0.044
1.	HD1	2.63±0.046	811.46±0.039
2.	HD2	3.87±0.028	1185.98±0.027
3.	HD3	3.34±0.016	1029.93±0.016
3.	HD3	3.3±0.021	1029.93±0.011

Ex vivo Mucoadhesive strength

The modified surface tensiometer method proved to be successful method to measure polymer adhesion to animal tissue unless the test conditions are maintained constant for all test samples. A primary mechanism of bioadhesion involves interpolation of the polymer with the mucin. The thickness of conjunctival mucin thickness is considerably less and thus it becomes difficult for the polymer to have intimate contact. The adhesive force of the acrylic polymers was found to be sufficient for the conjunctival surface to maintain long contact time. The density of carboxyl group is important for mucoadhesion. The carboxyl groups present in polymer were found to be in protonated form and hence caused mucoadhesion to occur by hydrogen bonding. In addition, the density of the cross-linking agent significantly affects mucoadhesion. As the density of the cross-linking agent is lowered, the mucoadhesive strength increases (Table 5). It is concluded that for mucoadhesion to occur, polymers must have functional groups that are able to form hydrogen bonds above the critical concentration and the polymer chains should be flexible enough to form as many hydrogen bonds as possible.[31]

Differential Scanning Calorimetry

The thermograms (Figure 6) for all three polymers exhibited two endothermic peaks. The first endothermic peak between 80-85°C is short and narrow peak assigned to the evaporation of water from hydrophilic groups in the polymers. The second one between 240-250°C corresponds to a thermal degradation through intermolecular anhydride formation and water elimination.[32] There was no significant difference found in thermograms of individual polymer except that the temperature of both the endothermic peak in HD was increased. The most

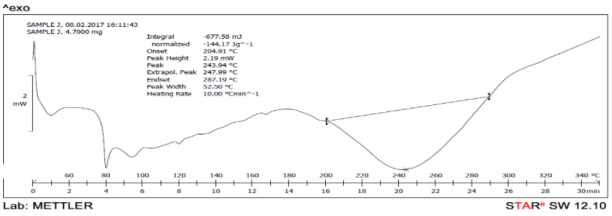

Figure 6(A): DSC spectra of DB1.

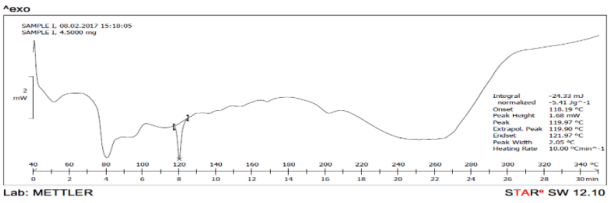

Figure 6(B): DSC spectra of DG1.

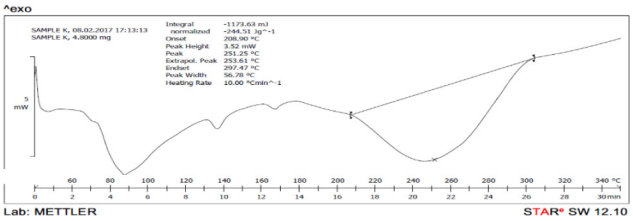

Figure 6(C): DSC spectra of HD1.

probable reason behind this increase could be presence of hydrophobic moiety in the form of crosslinking agent which requires more energy for breaking the bond in thermal degradation process.

XRD analysis

The X-ray diffraction pattern of polymer showed prominent diffraction peak at 19° (2θ) and minor peak appears at 30° (2θ) (Figure 7). These are the typical peaks of polyacrylic. The XRD spectra of all three polymers indicate that the samples are semi crystalline. The inference can be validated by the intensity and number of peaks. There are few peaks which are sharp. Most of the spectra cover broad peak region indicating that the samples are semicrystalline.[32]

HET CAM test

Figure 8 outlines the development stages of the growing embryos. Embryos with intact yolk and viable CAM were only further incubated for 10 days. The test samples were applied on these ten days old CAMs. The temperature and relative humidity kept 37.5 ± 0.5°C and 67± 5% RH were found to be the optimum conditions for CAM growing.[23,33] Initially the embryo were grown in the egg shell itself and later on to view the results, hole was drilled in the egg shell. The problems associated with it

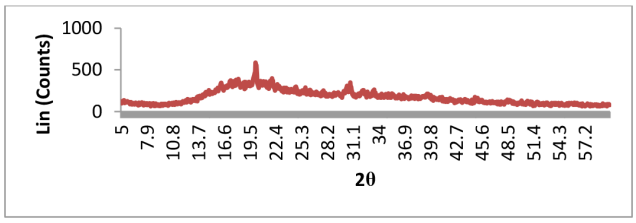

Figure 7(A): XRD spectra of DB1.

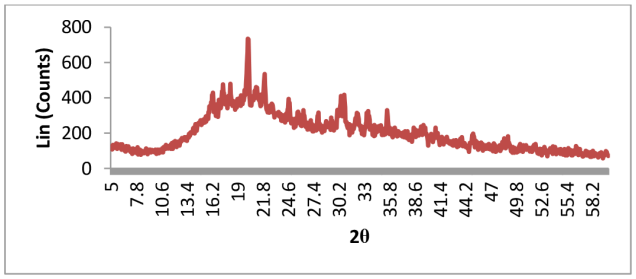

Figure 7(B): XRD spectra of DG1.

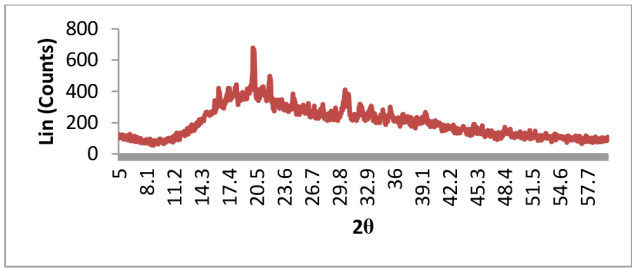

Figure 7(C): XRD spectra of HD1.

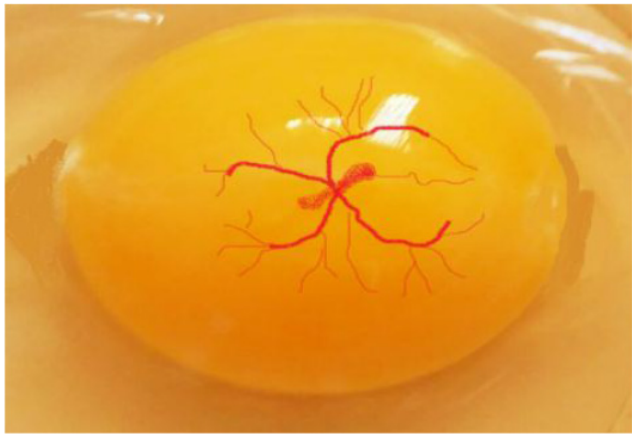

Figure 8: Developmental stages of growing embryo (A) embryo with intact yolk sac and CAM.

Figure 8(B): dead embryo with intact yolk sac CAM.

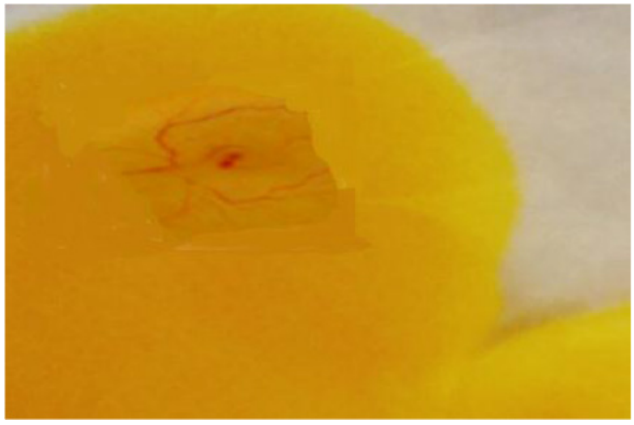

Figure 8(C): embryo with broken yolk and intact CAM.

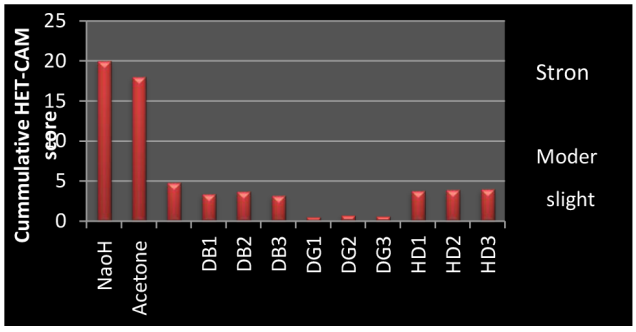

Figure 9: Cummulative HET-CAM score of polymers.

were limited visibility through the hole and chances of pieces of egg shell falling inside on surface of embryo while drilling the hole. Hence a modified method was reported in the literature, where the chick embryo was grown in a Petri dish from day 3 onwards to allow ready access to the entire CAM surface for better visibility and convenience.[33] Thus the process of cracking and proper temperature and humidity conditions are necessary for the survival of the embryos and therefore the number of CAMs available for testing.

Figure 9 shows the cumulative HET-CAM scores for the controls and synthesized polymers. The average cumulative scores calculated for DG polymers were found to be <0.9. These results reveal that the DG polymers are practically non-irritant when applied to the surface of

Table 6: Irritation scores of animals to polymer.			
Animals	**Reaction**	**24 Hrs**	**72 Hrs**
Rabbit 1	Erythema	0	0
	Edema	0	0
Rabbit 2	Erythema	0	0
	Edema	0	0

Table 7: xxxx				
Days	**Observations**			
	Cornea	**Iris**	**Conjunctivae**	**Total**
1	0	0	2	2
2	0	0	2	2
3	0	0	3	3
4	0	0	2	2
5	0	0	2	2
6	0	0	0	0

the CAM. Application of DB and HD polymers in a powder form developed minimal irritation potential in the form of hyperaemia after 3 min. This indicates that DB and HD polymers are slight irritant when applied on the surface of the CAM.

Primary skin irritation test

The primary Irritation index of the test sample was calculated to be 0.00; No irritation was observed on the skin of the rabbits/ rats. Individual results of derma scoring appear in Table 5. The scores for erythema and edema were summed for intact and abraded skin for rabbits at 24 and 72 hrs. The primary irritation index (P.I.I) was calculated. Based on the sum of the scored reactions divided by 32 (two scoring intervals multiplied by two test parameters multiplied by 8 animals). Primary Irritation Index: 0/32 = 0.00. Under the conditions of this test, the test sample would not be considered a primary skin irritant since the primary Irritation Index was less than 5.00. Table 6 indicates the irritation scores of animals.

In vivo Draize eye irritation test

The results of the ophthalmic irritation studies were given in Table 7. The possibility of eye irritation due to polymer administration was evaluated in rabbits. The rabbits were observed for ocular lesions and no symptoms of ocular irritation such as redness, tearing, inflammation or swelling were observed after polymer administration. No ophthalmic damage or abnormal clinical signs to the cornea, iris or conjunctivae were visible. Thus, the developed ocular drug delivery systems are apparently free from any ocular irritation potential and can be safely administered to humans. The scores were calculated according to Draize scale.

CONCLUSION

Monomer acrylic acid was subjected to polymerization by using three different crosslinking agent namely divinylbenzene, divinyl glycol and 2,5-dimethyl-1,4-hexadiene. These crosslinking agents were used in different concentrations (0.3,0.6,1 g) with respect to 100 g monomer. The effect of different crosslinking agents on polymer swelling and hydration property was found to be insignificant. Similar results were obtained with respect to different concentrations of crosslinking agent. But the effect of different crosslinking agent on bioadhesion was significant. With decrease in density of crosslinking agent, the mucoadhesive strength was increased. The carboxylic groups present in polymer were responsible for bioadhesion process because the free protons would cause bioadhesion by hydrogen bonding. Thus more the number of free carboylic groups present in the polymer, more is the mucoadhesive strength. The XRD spectra indicated that all the polymers were found to be in semicrystalline in nature. The irritation potential measured by *ex vivo* HET- CAM test indicated that DG polymer were nonirritant and did not show any signs of inflammation and swelling. Thus DG polymer was further tested by *in vivo* draize skin and eye irritation test. The results coincided with the *ex vivo* test proving that the DG polymer was found to be non-irritant polymer. Thus the most appropriate and suitable polymer for ocular drug delivery with optimum swelling properties and good bioadhesive strength, non-irritant in nature is DG polymer viz poly acrylic acid linked by divinyglycol polymer. The DG polymer was further studied and used as bioadhesive polymer in ocular formulation (niosomal *in situ* gel) for retention of drug at the target site.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

ABBREVIATIONS

HET-CAM: Hen's egg test- Chorioallantoin membrane; **MgSO4, 7H2O:** Magnesium sulphate heptahydrate; **FTIR:** Fourier Transform Infra Red; **KBr:** Potassium

bromide; **DG:** Polyacrylic acid with divinyl glycol as crosslinking agent; **DB:** polyacrylic acid with divinyl benzene as crosslinking agent; **HD:** Polyacrylic acid with 2,5-dimethyl 1,5- hexadiene as crosslinking agent; **NaOH:** Sodium hydroxide; **NaHCO3:** Sodium bicarbonate; **K2HPO3:** Potassium phosphite; **NIH:** National Institute of Health; **FHSA:** Federal Hazardous Substance Act; **XRD:** X ray diffraction; **PII:** Primary Irritation Index.

SUMMARY

Poor bioavailability of ocular drug (≤1 % of drug absorbed ocularly) can be attributed to precorneal factors, limited residence time, nasolachrymal drainage, relative permeability through cornea, etc. Thus prescribed dose is higher along with increased frequency. Thus there is need to minimize the dose and dosing frequency which will lead to lesser side effects.

The aim of the present study was to synthesize and evaluate bioadhesive polymer which will not only remain adhered to conjunctiva for a long a period of time but also increase the bioavailability of drug thus reducing its side effects.

The bioadhesive polymer synthesized was evaluated for density, polymer hydration, effect of pH, ion and time on polymer hydration, DSC, FTIR, XRD, mucoadhesive strength *ex vivo* and *in vivo* irritation test.

The polymer demonstrated pH dependent hydration properties thus swelling at neutral and basic pH. There was no significant effect of crosslinking agent either on density or on swelling properties except for mucoadhesive strength.

It was observed that with decrease in density of cross-linking agent, there was increase in bioadhesive strength.

The HET-CAM results coincided with *in vivo* draize skin irritation and eye irritation test indicating that the polymer were found to be nonirritant to slight irritant in nature.

DG polymer was found to most appropriate polymer for ocular delivery with optimum biaodhesive strength and least irritation potential of all the polymers synthesized.

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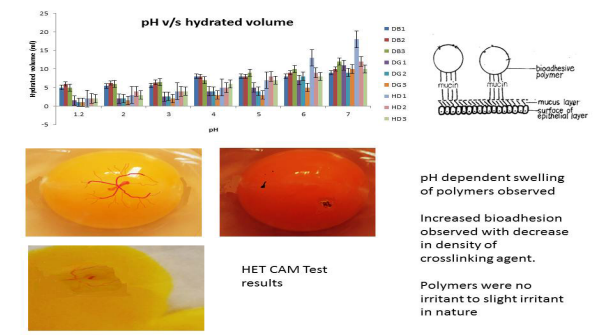
PICTORIAL ABSTRACT



SUMMARY

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Original Article

FORMULATION AND EVALUATION OF NIOSOMAL IN SITU GEL OF PREDNISOLONE SODIUM PHOSPHATE FOR OCULAR DRUG DELIVERY

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ABSTRACT

Objective: The main purpose of the study was to develop niosomal in situ gel of prednisolone sodium phosphate (PSP) with increased bioavailability (enhanced permeation) and sustained action (drug retention at the target site).

Methods: Using different ratios of span 60 and cholesterol (chol), niosomes were prepared by thin film hydration method and optimized by evaluating different parameters like drug content, entrapment efficiency, particle size and *in vitro* drug diffusion study. The niosomal pellets were further incorporated in in situ gel, prepared by the cold method and further optimized by parameters like gelling parameters, mucoadhesive strength and *in vitro*, *in vivo* drug release study.

Results: The optimized niosomal formulation containing span 60 and chol in equal proportion (1:1) showed better drug content (DC) i.e. 86.3±0.39% and entrapment efficiency (EE) i.e. 83.4±0.22 with vesicle size of 465±0.24 nm. The *in vitro* drug diffusion study indicated t90 value of 490 min thus proving sustained action of the formulation. The optimized in situ gel containing poloxamer 407 (P407) and poloxamer 188 (P188) in the ratio of 1:2.7 showed gelation temperature at 37 ^{0}C (physiological temperature of the body) and t90 value of 10 h thus depicting sustained action. The increased area under curve (AUC) value by 1.75 folds proved increased bioavailability of the drug.

Conclusion: Thus sustained drug delivery with increased bioavailability was designed for prednisolone sodium phosphate for the treatment of ocular inflammation.

Keywords: Niosomes, in situ gel, Sustained drug release, Ocular, Prednisolone sodium phosphate

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INTRODUCTION

Poor bioavailability of ocularly administered drug can be attributed to factors which include tear dynamics (blinking reflex and tear turnover) [1], non-productive absorption, transient residence time in the cul-de-sac, relative impermeability through corneal epithelial membrane, rapid precorneal elimination, drainage by gravity, frequent installation, enzymatic metabolism, nasolacrimal drainage, and the absence of controlled release [2-8]. Due to these physiological and anatomical constraints, a very small fraction of the administered drug (approx.1% or even less) of the instilled dose is available for ocular absorption [9-10]. Frequent dosing of drugs thus becomes a necessity to achieve the therapeutic concentration at the targeted site. This often results in the corresponding increase in local and systemic side effects. The high dose and dosing frequency cause unavoidable systemic side effects like stomach upset and disturbed GI motility [11]. The systemic route can overcome this but due to the presence of the blood-aqueous barrier and blood-retinal barrier, it ultimately leads to high loading dose at the target site. Various approaches, like viscosity enhancement, use of mucoadhesive, particulate drug delivery, vesicular drug delivery, prodrugs, and other controlled systems like ocuserts, iontophoresis, bioadhesive gels, ocular insert, contact lenses etc. are being explored [3,12-14].

Vesicular systems (niosomes and liposomes) can act as drug reservoirs. Niosomes offers advantages like no variation in the purity of surfactants, cost-effective, chemically stable, low toxicity because of their non-ionic nature, flexibility in the structure which helps them to form micelles and can improve the performance of the drug via better availability and controlled delivery at a particular site. Niosomes are capable of encapsulating both hydrophilic and lipophilic drugs and can serve as effective drug carriers [15]. However, nonionic surfactant vesicles may promote drug absorption by preferentially modifying the permeability characteristics of the conjunctival and scleral membranes as surfactants in lower concentration are used as penetration enhancers [16, 17].

The in-situ gel is drug delivery system that is in sol form before administration in the body, but undergo gelation with the change in physiological conditions, to form sol to gel (from the lat. *gelu*—freezing, cold, ice or *gelatus*—frozen, immobile). Vesicular systems in combination with mucoadhesive polymers show a controlled as well as a prolonged effect [18, 19]. Prolonging the drug contact time with the surface of the eye can also increase their penetration through the cornea, hence increasing the accessibility of the drug to aqueous humor [20-22]. This can be accomplished by use of bioadhesive polymer which helps the drug to remain adhered to eye surface by forming a noncovalent bond for a long period of time thus preventing the drug from undergoing nasolachrymal drainage. This will reduce the amount of drug (dose of the drug) and the dose frequency necessary for therapeutic effect. A reduction in dose will help to reduce the incidence of systemic side-effects.

PSP is corticosteroid drug effective in the treatment of steroid responsive inflammatory conditions such as allergic conjunctivitis, acne rosacea, superficial punctate keratitis, herpes zoster keratitis, iritis, cyclitis etc. PSP decreases inflammation by suppressing migration of polymorphonuclear leukocytes and reversing increasing capillary permeability for the treatment [23]. The major drawback of steroid treatment is the side effects associated with it if used over a long period of time.

The aim of the present investigation was to achieve the increased permeation of PSP by loading in niosome vesicles and improve its retention time at the particular site of action by incorporating the drug-loaded niosome into in situ gel which significantly reduces dosage frequency hence increase patient compliance.

MATERIALS AND METHODS

Materials

PSP was obtained from Sai Life Sciences, Pune. Span 60 from Loba chemicals, chol from Analab fine chemicals, P407and P188 from

BASF Chemicals were procured. All the chemicals used were of analytical grade.

Preparation of niosomes

Ethanol injection method

Niosomes containing PSP was prepared by modified ethanol injection method. Surfactant and chol in different ratios were dissolved in methanol. The resulting solution was slowly injected using microsyringe at a rate of 0.25 ml/min into 15 ml of Phosphate buffer (PBS) pH 7.4 containing PSP. The solution was stirred continuously on a magnetic stirrer (Remi, 2MLH) and the temperature was maintained above 60 °C. Stirring continued for 1-1.5 h. Vaporization of solvent takes place, resulting in spontaneous vesiculation and formation of unilamellar spherical niosomes [24].

Thin film hydration

Accurately weighed quantity of surfactant and chol in different molar ratios (table 1) were dissolved in chloroform and methanol mixture in a round bottom flask. The solvent mixture was evaporated in a rotary flash evaporator (Trident labotech, Thane) under a vacuum of 20 inches of Hg at a temperature of 25±2 °C and the flask rotated at 100 rpm until a smooth, dry lipid film was obtained. The film was hydrated with 10 ml of PBS pH 7.4 containing 25 mg prednisolone sodium phosphate drug for 45 min at 60 °C with gentle shaking on a water bath. The niosomal suspension was further stored at 2-8 °C for 24 h [24].

Batches of niosomes were prepared to vary in the method of preparation (table 1), the combination of surfactants (table 2), an individual surfactant in different concentrations (table 3), selection of surfactant grade (table 4) and the ratio of chol and span 60 (table 5) respectively. Optimization was carried out on the basis of DC, EE, vesicle size and *in vitro* drug diffusion study.

Preparation of niosomal in situ gel

The "Cold method" was adopted for preparing poloxamer-based gels. The required amounts of P407 and P188 for each formulation were carefully weighed and placed in a flat bottomed vial. After the addition of the required amount of 0.9% NaCl solution for isotonicity, the vial was placed at 4 ^{0}C until P407 and P188 were dissolved completely and a clear solution was obtained. In the study, P407 and P188 concentrations in sols or gels were expressed as the weight percentage (% w/v). The equivalent amount of niosomal pellets obtained from the freeze-drying process was added into the gel formulation along with 100 mg of the synthesized polymer [25] and benzalkonium chloride (0.01%) to form final formulation of niosomal in situ gel. Preliminary blank batches were prepared without niosomes and bioadhesive polymer to find out the ratio of P407 and P188 exhibiting gelation temperature near to 37 ^{0}C [26].

Evaluation of niosomes

Drug content

In 1 ml of niosomal buffer solution, 2 ml methanol was added and further volume was made up by distilled water. Addition of methanol cause breakdown of niosomes and hence drug could freely get dissolve in a solvent. Each of this solution was further diluted according to the requirement by distilled water. Absorbance was measured on UV-visible spectrophotometer (Shimatzo UV visible 1650, Japan) at 247 nm [24]. Drug content was determined by using the formula

$$\text{Percent drug conten} = \frac{\text{Test abs.} \times \text{Standard Conc.}}{\text{Standard abs.} \times \text{weight of drug}} \times \text{Dilution factor} \times 100$$

Table 1: Composition of trial batches for method selection

Batch code	M1	M2	M3	M4	M5	M6	M7
Chol (mg)	25	25	25	25	25	25	-
Span 60 (mg)	25	-	-	25	-	-	-
Tween 20 (mg)	-	25	-	-	25	-	-
Tween 80 (mg)	-	-	25	-	-	25	-
Span 80 (mg)	-	-	-	-	-	-	25
Methanol (ml)	1	1	1	1	1	1	1
$CHCl_3$ (ml)	4	4	4	4	4	4	4
Drug (mg)	25	25	25	25	25	25	25
Buffer (ml)	7.5	7.5	7.5	7.5	7.5	7.5	7.5
Technique	Ethanol injection method			Thin film hydration technique			

Table 2: Composition of trial batches for combination surfactants

Batches	C1	C2	C3	C4	C5	C6
Chol (mg)	25	25	25	-	-	-
Span 60 (mg)	12.5	-	-	12.5	12.5	-
Span 80 (mg)	12.5	12.5	-	-	-	12.5
Tween 20 (mg)	-	12.5	12.5	12.5	-	-
Tween 80 (mg)	-	-	12.5	-	12.5	12.5
Methanol (ml)	1	1	1	1	1	1
$CHCl_3$ (ml)	4	4	4	4	4	4
Drug (mg)	25	25	25	25	25	25
Buffer (ml)	7.5	7.5	7.5	7.5	7.5	7.5

Table 3: Composition of trial batches for shortlisting surfactant

	S1	S2	S3	S4
Chol (mg)	25	25	25	-
Span 60 (mg)	25	-	-	-
Tween 20 (mg)	-	25	-	-
Tween 80 (mg)	-	-	25	-
Span 80 (mg)	-	-	-	25
Methanol (ml)	1	1	1	1
$CHCl_3$ (ml)	4	4	4	4
Drug (mg)	25	25	25	25
Buffer (ml)	7.5	7.5	7.5	7.5

Table 4: Composition of trial batches for selection of surfactant grade

Batches	CS1	CS2	CS3	CS4	CS5	CSS1	CSS2	CSS3	CSS4	CSS5
Chol (mg)	25	25	25	25	25	25	25	25	25	25
Span 60 (mg)	25	50	75	100	125	-	-	-	-	-
Span 80 (mg)	-	-	-	-	-	25	50	75	100	125
Methanol (ml)	1	1	1	1	1	1	1	1	1	1
$CHCl_3$ (ml)	4	4	4	4	4	4	4	4	4	4
Drug (mg)	25	25	25	25	25	25	25	25	25	25
Buffer (ml)	10	10	10	10	10	10	10	10	10	10
Chol: surfactant ratio	1:1	1:2	1:3	1:4	1:5	1:1	1:2	1:3	1:4	1:5

Table 5: Composition of trial batches for selection of ratio between chol and surfactant

Batches	CS6	CS7	CS8	CS9	CS10	CS11	CS12	CS13	CS14	CS15	CS16	CS17
Chol (mg)	25	25	25	25	25	50	62.5	75	87.5	150	200	250
Span 60 (mg)	150	175	200	225	250	25	25	25	25	150	200	250
Methanol (ml)	1	1	1	1	1	1	1	1	1	1	1	1
$CHCl_3$ (ml)	4	4	4	4	4	4	4	4	4	4	4	4
Drug (mg)	25	25	25	25	25	25	25	25	25	25	25	25
Buffer (ml)	10	10	10	10	10	10	10	10	10	10	10	10
Chol: Surfactant ratio	1:6	1:7	1:8	1:9	1:10	2:1	2.5:1	3:1	3.5:1	1:1	1:1	1:1

Entrapment efficiency

Prepared PSP niosomes were separated from the unentrapped drug by centrifugation at 2750 rpm for 60 min using the cooling centrifuge. Cooling centrifuge (Laby instruments, India) was used as niosomes are stable and stored at the refrigerated condition that is at 2-8 ^{0}C. The absorbance of the supernatant was taken after appropriate dilution at 247 nm. Settled pellets were dispersed in distilled water to get a clear solution. After appropriate dilutions, absorbance was recorded [27]. The entrapment efficiency was calculated through the following relationship,

$$\% \text{ EE} = \frac{\text{Entrapped drug}}{\text{Entrapped drug + Drug in supernatant}} \times 100$$

Vesicle size distribution

The average vesicle size of niosomes was measured by the method of laser light diffraction using Nanophox NX0088. Prior to measurements, about 50 mg of each sample was dispersed with 100 ml of hexane and signal to noise ratio was measured in order to eliminate error if any. The vesicle size distributions were estimated by setting the intensity of the scattered light at a wavelength of 750 nm and the scattering angle (θ) of 90 [26].

***In vitro* release study**

Drug release from niosomes was studied using a dialysis method. Dialysis bags were soaked before use in distilled water at room temperature for 12 h to remove the preservative, followed by rinsing thoroughly in distilled water. *In vitro* release of PSP from niosomes was conducted by dialysis in a dialysis sac made up of a cellophane membrane (Sigma-Aldrich) with 100 ml of PBS (pH 7.4) at 37 °C. Two ends of the dialysis sac were tightly bound with threads. The sac was hung inside a beaker with the help of a glass rod so that the portion of the dialysis sac with the formulation dipped into the buffer solution. The beaker was kept on a magnetic stirrer (Remi, 2MLH) and stirring was maintained at 100 rpm at 37 °C with thermostatic control. Samples were collected every at 15 min, 30 min, 45 min, 1 h, 2 h, 3 h and further 1hr interval over a period of 6 h and assayed spectrophotometrically for drug released and the sampled volume of buffer maintained at the same temperature. An equal volume of fresh release medium was replaced at the same time intervals. The diffusion data were analyzed for calculating the amount of drug released and percentage drug released at different time intervals [25].

Release kinetics of drug

The kinetics of the drug release was evaluated by the model fitting method using PCP Disso v3 software and the model with the highest correlation coefficient amongst them was considered to be the best model for the particular formulation

Fourier transform infrared spectroscopy (FTIR)

The infrared spectrum of prednisolone sodium phosphate was determined on Fourier Transform Infrared Spectrophotometer (FT/IR 4100, Jasco) using potassium bromide (KBr) dispersion method. The baseline correction was done using dried KBr. The samples to be analyzed and KBr were previously dried in the oven for 30 min and mixed thoroughly in 1:300 (sample: KBr) ratio in a glass mortar. These samples were then placed in a sample holder and scans were obtained at a resolution of 2 cm^{-1} from 4000 to 400 cm^{-1}[28].

Differential scanning calorimetry (DSC)

DSC measurements were performed on a differential scanning calorimeter containing an intra-cooler (DSC Mettler STAR SW 9.20, Switzerland). Nitrogen gas was purged (50 ml/min) to maintain an inert atmosphere. All accurately weighed samples (about 5-10 mg of samples) were placed in a sealed aluminum pan, and the samples were heated under nitrogen gas flow (20 ml/min) at a scanning rate of 10 °C per min from 40 to 340 °C. An empty aluminum pan was used as reference [29].

Optical microscopy

Optical microscopy of the drug sample was carried out by using a Digital Microscope (Motic). A very slight quantity of the niosomal sample solution was spread on the glass slide. This slide was focused under various magnification lenses and the images were captured [30].

Zeta potential determination

Niosomal dispersion (0.5 ml) was diluted to 50 ml with distilled water in a glass beaker with constant stirring. Zeta-potential of the resulting suspension was determined using the Zetasizer (model: Nano ZS, Malvern Instruments, Westborough, MA, USA) Electrophoretic mobility (μm/s) was measured using small volume disposable zeta cell and converted to zeta potential by inbuilt software using Helmholtz–Smoluchowski equation [26].

Polydispersity index (PDI)

The PDI determination was using photon correlation spectroscopy with in-built Zetasizer (model: Nano ZS, Malvern Instruments, Westborough, MA, USA) at 633 nm [26]. The polydispersity index was calculated by

$$\text{PDI} = \frac{\text{X90} - \text{X10}}{\text{X50}}$$

Transmission electron microscopy (TEM) analysis

TEM (Philips CM 200 super twin stem microscope) was used to determine the morphology of the niosomal vesicles. Few drops of the optimized niosomal formulation (CS17) were deposited on a carbon-coated copper grid and examined under a transmission electron microscope [31].

Evaluation parameters in situ gel

Appearance

The appearance of the gels was examined for clarity. The clarity of various formulations was evaluated by visual inspection under the black and white background.

pH

The pH of each formulation was examined using a digital pH meter (Equip tronics, EQ610). The pH meter was first calibrated using buffer solutions of pH 4 and pH 7. Then gel was taken in a beaker and the pH was measured [24].

Drug content of in situ gel

In this study, each formulation (1 ml) was taken in a 100 ml volumetric flasks diluted with distilled water up to the mark. After suitable dilutions, the amount of drug was measured in the formulation by using ultraviolet spectroscopy at 246 nm [24].

Gelation time

The Tsol–gel of the formulation was determined by test tube inversion method. Niosomal in situ gel (2 ml) was transferred to a test tube and sealed with paraffin. This test tube was placed in the constant temperature water bath at 35±1 ^{o}C. The sample was examined for gelation [24].

Gelation temperature and gel melting temperature

The Tsol–gel of the formulation was determined by test tube inversion method. Niosomal in situ gel (2 ml) was transferred to a test tube and sealed with paraffin. This test tube was placed in the constant temperature water bath (Equitron). The temperature of the water bath was increased in increments of 2 ^{0}C and left to equilibrate at each new temperature. However, in the region of Tsol–gel temperature was raised slowly in the increments of 0.5 ^{0}C. The formulation was examined for gelation which was said to have occurred when the meniscus would no longer move upon tilting through 90^{0}. Measurements were done in triplicate [32].

The obtained temperature is said to be T1. After attaining the temperature T1, further heating of gel causes liquefaction of gel and form the viscous liquid and it starts flowing, this temperature is noted as T2 i.e. gel melting temperature.

Gelling capacity

Determination of *in vitro* gelling capacity was done by a visual method. Colored solutions (1% Congo red solution in water) of *in-situ* gel were prepared. The *in vitro* gelling capacity of prepared formulations was measured by placing 5 ml of the gelation solution (pH 7.4 PBS) in a glass test tube and maintained at 37±1 °C temperature. One ml of colored formulation solution was added with the help of pipette. As the colored solution comes in contact with gelation solution, it was immediately converted into the stiff gel-like structure. The gelling capacity of the solution was evaluated on the basis of the stiffness of the formed gel and time period for which the formed gel remains as such. The *in vitro* gelling capacity was graded in two categories on the basis of gelation time and time period for which the formed gel remains as such [33].

Texture analysis

Texture analysis of the prepared gel formulation was done by using Brookefield texture analyzer CT3. The formulations were evaluated for the following parameters.

Gel strength

The gel strength, which is an indication of the viscosity of the gel at physiological temperature, was measured by measuring the force required for depression of gel at 37 ^{0}C temperature [32].

Mucoadhesive strength

The mucoadhesive potential of each formulation was determined by measuring a force required to detach the formulation from the conjunctival membrane. The mucoadhesive force expressed as the detachment stress in dynes/cm^{2} was determined from the minimum weight that detached the mucosal tissue from the surface of each formulation [34, 35].

$$\text{Mucoadhesive strength} = \frac{\text{mg}}{\text{A}}$$

Where,

m = Weight required for detachment in g

g = acceleration due to gravity (980 cm/s^{2})

A = area of mucosa exposed (cm^{2})

Spreadability

The spreadability was evaluated by measuring the distance to which the 10 ml formulation would spread under the influence of specified force applied on gel [24].

Viscosity measurement

The viscosity of prepared gel formulation was measured by using Brookefield DV-II pro-plus viscometer (Brookefield engineering Labs Inc. Middleboro, USA) equipped with a helipath stand and T bar spindles. Viscosity measurements were made at variable temperature and variable shear rate. For temperature dependent study, the formulation was subjected to constant shear rate at different temperatures from 25 to 40 ^{0}C. During this testing, the temperature was raised gradually by 1 ^{0}C and the viscosity of the sample was measured after attaining the set temperature. Measurements were done in triplicate. Steady shear sweep test was carried out by measuring the viscosity at the constant temperature of 25 ^{0}C and 37 ^{0}C but varying the rotation speed of the spindle from 10 to 100 rpm [32].

***In vitro* drug diffusion study**

In vitro release studies were carried out using Franz diffusion cell and the temperature was adjusted to 37±0.5 ^{0}C. The prehydrated dialysis membrane (cellophane membrane) was used for the study. Samples were withdrawn at periodic intervals of 0.5,1,2,3,5 and 6 h and replaced with fresh simulated tear fluid to maintain sink conditions. The drug content was analyzed using UV-Visible Spectrophotometer at 247 nm using simulated tear fluid as blank [25]. The apparent diffusion coefficient was calculated by using formula

$$\text{Dapp} = \frac{\Delta Q}{\Delta t} \times \frac{1}{\text{ACo60}}$$

Where ΔQ/Δt (µg/min) is the flux across the corneal tissue. A is the area of diffusion (cm^{2}), Co (µg/cm^{3}) is the initial concentration of drug in the donor compartment, and 60 is taken as the factor to convert min into second. The flux across the cornea was obtained from the slope of the regression line obtained from the linear part of the curve between the amount permeated (Q) vs time (t) plot.

Ex vivo diffusion study

Ex vivo drug diffused study was performed for the optimized formulation and marketed formulation by using 5 ml of Franz diffusion cells containing simulated tear fluid. The goat conjunctival epithelium was used for the study. 1 ml of sample was placed in the donor compartment and diffusion study was conducted for 6 h at 37±1 ^{0}C. Sample (0.5 ml) was withdrawn at 1/2 h for an hour and then every 1 h and the same quantity of simulated tear fluid was added [26].

Pharmacodynamic study

Primary skin irritation test

Two healthy albino rabbits were used for the experiment. Animal husbandry was conducted in accordance with the "Guide for the Care and use of Laboratory Animals," National Institute of Health (NIH) publication No.85-23.

Methods: The backs of the animals were cleaned free of fur with a razor at least 4 h before application of the sample. One ml sample of the least irritant polymer obtained from ex vivo test was then applied to the particular site to an area of skin approximately 1" × 1" (2.54 × 2.54 cm) square. The sample applied site was covered with a nonreactive tape. Animals were returned to their cages. After a 24 h exposure, the tape was removed and the test sites were wiped with tap water to remove the test sample. At 24 and 72 h after the test sample application, the test sites were examined for dermal reactions in accordance with the Federal Hazardous Substance Act (FHSA)-recommended Draize scoring criteria (Appendix 1). Primary Irritation Index (P. I. I.) of the test sample was calculated following test completion [36].

***In vivo* draize eye irritation test**

The Draize test was performed on white albino rabbits. In this test 100 µg test sample was placed into the lower cul-de-sac of rabbit's right eye (1.5-2 kg, 13 w of age). The left eye was treated as a control. Rabbits' eyes were observed periodically for redness, swelling and watering of the eye at 1 h, 4 h and every 24 h for 7 d. Three rabbits were used for the test substance. These parameters were calculated from the weighted score for each part of the rabbit eye such as (cornea, iris, and conjunctiva) and also from the sum of these scores. The maximal average Draize total scores (MAS) are classified into non-irritants (0<MAS<0.5), slight irritants (0.5<MAS<15), mild irritants (15<MAS<25), moderate irritants (25<MAS<50) and severe irritants (50<MAS) [37]. Approval of the institutional animal ethics committee (Approval No. MCP/IAEC/01/2016) was obtained prior to the commencing of the study from Modern College of Pharmacy, Nigdi, Pune.

Pharmacokinetic study

The drug pharmacokinetics in the aqueous humor on ocular instillation of the optimized formulation (B) and the drug solution was measured on male New Zealand albino rabbits. Rabbits (2–2.5 kg) were kept in cages kept in a light-controlled (alternate night and day cycles, 12 h each) air-conditioned chamber under controlled humidity (45±5%). All the experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) of Modern College of Pharmacy, Nigdi, Pune constituted under the guidelines of Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA, India) with protocol approval number MCP/IAEC/01/2016.

HPLC analysis of prednisolone sodium phosphate

The quantitative determination of PSP pharmacokinetics in the rabbit aqueous humor was based on a previously validated HPLC method [31]. A reversed-phase HPLC-UV method was used to measure PSP in aqueous humor. The HPLC (Water 600) apparatus consisted of quaternary (gradient system), HPLC pump (isocratic) equipped with 30 w high resolution UV/Vis detector DATA ACE Chromatography Software (version 1.50) integrator software and a Grace smart RP C18 column (4.6 mm × 250 mm and 10 mm particle size).

The mobile phase was prepared by mixing 2.5 ml of isopropanol with 0.2 ml of phosphoric acid and diluting with deionized water to 90 ml. The pH was adjusted to 3.0 with 1 M NaOH and then diluted further to 100 ml. The mobile phase was degassed and filtered through 0.45-micron nylon filters. The flow rate of the mobile phase was set at 1 ml/min. The detector was set at 246 nm (absorption maxima of PSP). The injection volume was 20 µl; run time was 10 min. The calibration concentration ranges for the standards were 0.050 to1.0 µg/ml. An internal standard 6-alpha-methyl-prednisolone was added to every standard and sample [38].

Study design

A two treatment, non-blind, randomized, parallel design was adopted to compare the drug pharmacokinetics in the rabbit aqueous humor following ocular instillation of the optimized niosomal in situ gel (B). Twelve rabbits were randomly divided into two groups. The niosomal in situ gel (B) was instilled (50 µl) in the lower conjunctival sac of the right eye of each rabbit belonging to the first group (Treatment A) while the same volume of the drug solution was applied to those of the second group (Treatment B). The rabbits were systemically anaesthetized with ketamine hydrochloride (intramuscular injection, 50 mg/kg). At 5 min intervals, the formulation was instilled into the eye using a micropipette. At 0.5, 1, 2, 4, and 8 h post-instillation, the rabbits were locally anaesthetized (benoxinate HCl, 0.4% w/v) and aqueous humor samples (0.15 ml) were withdrawn by anterior chamber paracentesis [39].

Aqueous humor samples were stored at −20 °C until HPLC analysis. Prior to HPLC analysis, the thawed aqueous humor samples were spiked with 6 alpha methylprednisolone. In order to precipitate the associated proteins, the spiked samples (0.1 ml) were vortex mixed (30 s) with acetonitrile (0.2 ml). Following centrifugation (15 min, 12000 rpm), the drug content in the organic phase layer was determined by HPLC.

Pharmacokinetic analysis

The graph of concentration of prednisolone sodium phosphate (mean±SD) in the rabbit aqueous humor was plotted against time. The maximum drug concentration (Cmax, in µg/ml) and the time to reach Cmax (Tmax, in h) were directly obtained from the individual subject curves. The mean residence time (MRT, in h) was estimated using graph prism® software. The area under the aqueous humor concentration-time curve (AUC, in µg/h/ml) was calculated using the trapezoidal rule method. The results were statistically evaluated, using one-way ANOVA, at $P<0.05$. The increase in the ocular drug bioavailability (folds) was estimated by dividing the AUC of the niosomal in situ gel (B) over that of the drug solution.

Sterility test

The formulation was sterilized by membrane filtration method and was incubated with different culture media like soybean casein digest medium and fluid thioglycate medium for a period of 14 d and observed for the presence of microbial growth if any [33].

Stability study

On the ICH, Harmonized Tripartite Guidelines on stability testing of new drug substance and product, fundamental recommendations are summarized.

For the drug substances intended for storage in a refrigerator are given in table 6.

Table 6: ICH guidelines for the drug substances intended for storage in a refrigerator.

Study	Storage condition	Time period
Long term	5 °C±3 °C	12 mo
Accelerated	25 °C±2 °C/60% RH±5% RH	6 mo

In order to determine the stability of gels, the samples were kept in airtight glass vials packed by aluminum foil. The niosomal in situ gels were stored at 25 ^{0}C±2 ^{0}C/60%±5% RH for 6 mo [40]. These samples were evaluated for drug content, gelation temperature and

physical characteristics. Samples were also stored at 5 °C±3 °C for 3 mo. These samples were also evaluated for drug content, gelation temperature, and physical characteristics.

Statistical analysis

The obtained data were analyzed using the one-way analysis of variance (ANOVA) test (P<0.05) was considered as an estimate of significance while evaluating the degree of difference between various formulations.

RESULT AND DISCUSSION

Optimization of niosomes on the basis of DC, EE and vesicle size

Method selection

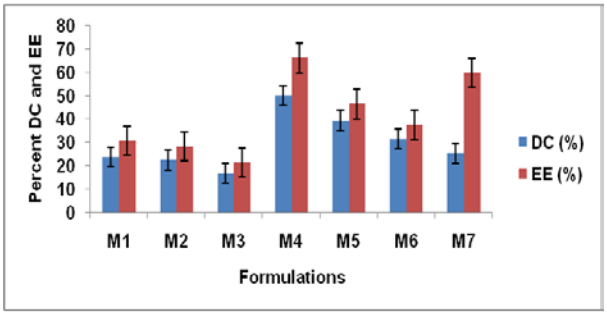

Fig. 1: Graph of DC and EE of preliminary batches for method selection data expressed mean±SD (n=3)

The niosomes were prepared by two methods namely thin film hydration and ethanol injection method. It was observed that the DC and EE of niosomes prepared by thin film hydration method were significantly (p<0.05) higher than of niosomes prepared by the ethanol injection method (fig. 1). In addition to this, niosomes were also evaluated on the basis of their morphology and appearance by motic digital microscopy. Niosomes prepared by thin film hydration technique were spherical and uniform in size. Hence thin film hydration technique was selected for niosomes preparation**.** Similar results were obtained in salbutamol sulphate liposomes preparation by Honmane SM *et al.* 2014, where entrapment efficiency was higher for liposomes prepared by thin film hydration technique.

Combination of surfactants

Niosomes were evaluated for the combined effect of surfactants. By preparing niosomes using a single surfactant and in combination, DC and EE were calculated (fig. 2). It was observed that the use of surfactants in combination did not show a significant effect on DC and EE. The drug content and encapsulation efficiency depended on the HLB value of the mixture of surfactants used. The higher HLB of the mixture of surfactants with respect to individual surfactant reduced its potential in solubilizing and thus entrapping the PSP molecule. The results coincide with the previous study by Taymouri S *et al.* 2016where effect of different surfactants on physical properties of carvedilol nanoniosomes was studied. Hence single surfactant niosomes were prepared instead of a combination to prevent interaction.

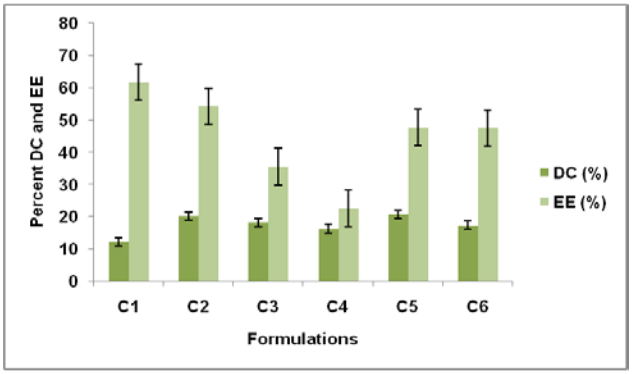

Fig. 2: Graph of DC and EE of trial batches for the combination of surfactants data expressed mean±SD (n=3)

Selection of surfactants

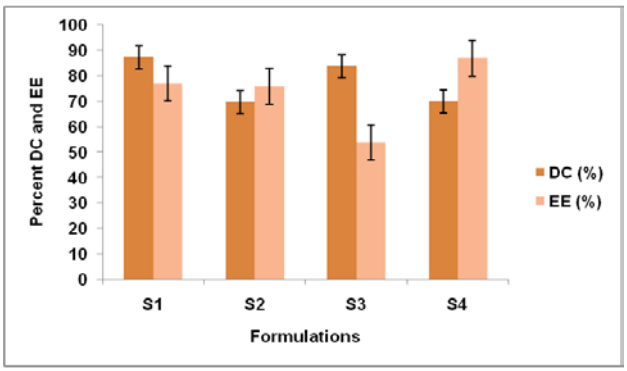

Fig. 3: Graph of DC and EE of trial batches for selection of surfactants data expressed mean±SD (n=3)

Four surfactants used during formulation were span 60, span 80, tween 20 and tween 80 to prepare niosomes. It was observed that use of spans showed significantly (p<0.05) higher DC and entrapment efficiency over tweens (fig. 3). The probable reason behind this is the Hydrophilic-Lipophilic Balance (HLB) value. HLB is a dimensionless parameter, which is the indication of the solubility of the surfactant molecule. Surfactants with HLB between 4 and 8 can be used for the preparation of vesicle [41]. Hydrophilic surfactants with an HLB value ranging from 14 to 17 are not suitable to form a bilayer membrane due to their high aqueous solubility. However, with the addition of an optimum level of chol, niosomes are indeed formed from Tween 20 (HLB value = 16.7). Spans possess lower HLB which makes the drug entrapment of steroid drug moiety more efficient. The result was found to be in agreement with previous data reported by Shaji J *et al.* 2016 and Taymouri S *et al.* 2016 which indicated that lower the HLB of the surfactant; the higher will be the entrapment efficiency.

Selection of surfactant grade

Table 7: DC, EE, and vesicle size for the selection of surfactant grade

Batch code	DC (%)*	EE (%)*	Vesicle size (nm)*
CS1	60.16±0.12	72.4±0.23	423±0.03
CS2	51.59±0.16	86.95±0.36	401±0.43
CS3	57.98±0.47	86.06±0.41	494±0.52
CS4	41.96±0.14	90±0.33	485±0.16
CS5	59.33±0.05	91.54±0.16	469±0.38
CSS1	54.4±0.27	87.19±0.36	356±0.26
CSS2	61.29±0.62	91.31±0.22	407±0.14
CSS3	42.12±0.29	80±0.43	396±0.49
CSS4	43.47±0.32	62.33±0.62	404±0.04
CSS5	55.57±0.38	81.61±0.04	375±0.55

*(mean±SD, n=3)

As discussed in the selection of surfactant section, HLB value plays an important role in the formation of stable niosomes. The hydrophilic-lipophilic balance (HLB) system, which is a measure of the relative contributions of the hydrophilic and lipophilic regions of the surfactant molecules, is more commonly used as an indicator on potential niosomes formation. The HLB value of span 60 is 4.7 and HLB value of span 80 is 4.3 respectively. So considering the HLB value, Span 80 having lower HLB value compared to span 60 must be able to incorporate steroid moiety more efficiently than span 60. But there is an exception. Sorbitan monooleate (Span 80, HLB of 4.3) cannot assemble into niosomes (on their own) due to their inadequate geometry, hence packing properties. The oleate moiety of this surfactant molecule has a double bond (with relatively high electron density) at the C9 which repels adjacent hydrocarbon chains resulting in the characteristic "kink" in the structure [42]. Vesicle size of niosomal dispersion containing span 80 was less as compared to that of span 60 (table 7). This might be due to the increase in the hydrophobicity of the surfactant from Span 60 to Span 80. The decrease in surface free energy with increasing the hydrophobicity of surfactants may be the major attribute of reduction in the vesicle size of niosomes. Since the DC of niosomes obtained by using span 80 was significantly ($p<0.05$) less due to kink in the structure as compared to span 60, the span 60 was selected as surfactant. The results coincide with the previous study by Essa 2010 where effect of formulation and processing variables on sorbitan monopalmitate niosomes was studied.

Selection of chol: surfactant ratio

Table 8: DC, EE and vesicle size for a selection of chol: surfactant ratio

Batch code	DC (%)*	EE (%)*	Vesicle size (nm)*
CS6	64.6±0.12	63.9±0.16	324±0.05
CS7	55.8±0.35	69.2±0.26	357±0.15
CS8	66.5±0.41	70.9±0.38	373±0.22
CS9	57±0.55	79.4±0.41	410±0.29
CS10	56.3±0.26	73.2±0.64	428±0.45
CS11	42.1±0.09	66.9±0.28	385±0.62
CS12	68±0.034	70.1±0.36	448±0.54
CS13	53.5±0.49	53.8±0.05	424±0.32
CS14	68.5±0.54	61.4±0.19	401±0.41
CS15	72.4±0.06	80±0.32	436±0.38
CS16	78.1±0.42	80.7±0.46	417±0.61
CS17	86.3±0.39	83.4±0.22	465±0.24

*(mean±SD, n=3)

In order to find the optimum concentration ratio of chol: surfactant, different batches of niosomes were prepared (table 8) ratio value (chol: span 60) ranged from 1 to 10. Reverse order of ratio (chol: span 60) from 2 to 3.5 was also used to prepare niosomes. The amount of chol to be added depends on the HLB value of the surfactants. As the HLB value increases above 10, it is necessary to increase the minimum amount of chol to be added in order to compensate for the larger head groups [43]. EE decreases as the HLB value decreases from 8.6 to 1.7. It was seen that the addition of chol enables more hydrophobic surfactants to form vesicles, suppresses the tendency of the surfactant to form aggregates, and provides greater stability to the lipid bilayer by promoting the gel-liquid transition temperature of the vesicle [44]. The EE is affected by the phase transition temperature (Tc) of the surfactant. Thus Span 60 with a high Tc exhibits the highest EE. The encapsulation efficiency is improved when the chol content was increased to 50% molar ratio due to the reduction of drug permeability. A similar result was reported by Mokhtar *et al.* 2008, who studied the effect of some formulation parameters such as the chol content of niosomes on flurbiprofen encapsulation and release rates of niosomes prepared from proniosomes. The optimization of the niosomal batch was not possible only on the basis of DC and EE as prolong the effect of PSP was expected (fig. 4). Hence the batch was selected on the basis of *in vitro* drug release.

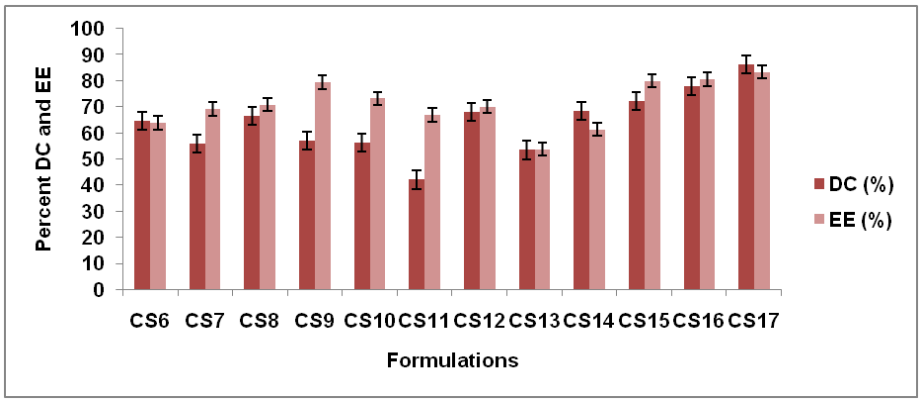

Fig. 4: Graph of DC and EE of trial batches for selection of chol: surfactant ratio data expressed mean±SD (n=3)

***In vitro* drug release**

In vitro dissolution of niosomal batches was carried out by dialysis bag method. The value of t90 played an important role in determining the optimized niosomal batch. Our main purpose of this study was to sustain the release of drug and hence t90 was expected to be higher for the optimized batch. The batch CS17 (chol: span 60 ratio was 1) exhibited t90 of 490 min that is the release was

sustained upto 8 h of the drug by this formulation (fig. 5 and 6). The change in chol: span60 ratio did not showed linear correlation either with DC, EE or *in vitro* drug release. The values differed randomly without showing any correlation. The niosomal batches CS1, CS15,CS16, CS17 all contained chol: span 60 ratio 1 i.e. both the ingredients were in equal quantities but still there t90 values were variable viz 112 min, 370 min, 435 min and 490 min respectively (table 9). The difference in these batches was the change in concentration with respect to the whole composition. The amount of surfactant used in CS1 was ten times lesser than that used in CS17. Hence the value of t90 was shifted from 112 to 490 min. Surfactant concentrations were within the limits in accordance with safety guidelines where the concentration of surfactant does not exceed by 1-2.5 % w/w. In batch CS17 the amount of span is 1% w/v. The primary function of surfactant is to improve the solubility of substance but PSP being water soluble, this function need not has to be achieved. The surfactants in higher concentrations act as sustained release polymers which cause the drug to release at the controlled rate. This was in agreement with findings of other studies like Tabbakhian M et. al 2006;Das k et. al 2011;Azeem A et. al 2008 indicating a more sustained drug permeation and possibly a greater drug deposition and increased drug release where drug containing vesicular systems used, as compared to a pure drug solution.

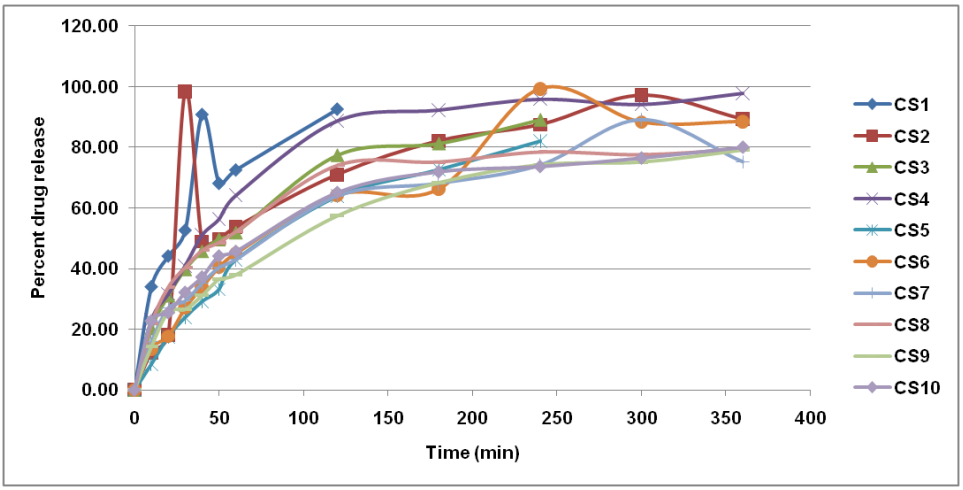

Fig. 5: Dissolution drug profile of batches CS1 to CS10data expressed mean±SD (n=3)

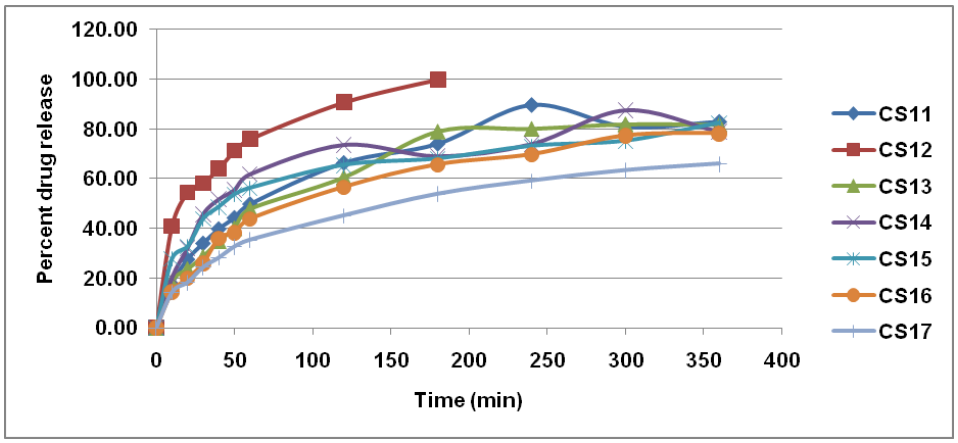

Fig. 6: Dissolution drug profile of batches CS11 to CS17data expressed mean±SD (n=3)

Release kinetics of drug

The kinetics of the drug release was evaluated by model fitting method using PCP Disso v3 software and the model with the highest correlation coefficient amongst them was considered to be the best model for a particular formulation.

Different t90 values were observed for different batches. The batch CS17 showed (chol: span 60–1:1) maximum t90 value of 491.9 min (table 9). This indicates that 90% drug release occurred in 490 min thus sustaining the release to upto 8 h. The kinetics of the drug release was evaluated by a model fitting method using PCP Disso v3 software and the model with the highest correlation coefficient amongst them was considered to be the best model. The release kinetics indicated that the optimized batch (CS17) followed Korsmeyer Peppas kinetics where R is 0.9935, n is 0.4352 and k is 5.846. The Korsemeyer Peppas release model equation is, F = (M t/M) = k m t n Where F = fraction of drug release at time t; Mt = amount of drug release at time t; M = total amount of drug in dosage form; K = constant. 'n' is estimated from linear regression of log (Mt/M) vs log t. If n = 0.45, it indicates Fickian diffusion; n<0.45≤0.89 indicates non fickian diffusion. Nonfickian diffusion involves a combination of both diffusion and erosion controlled release rate [45].

Fourier transform infrared spectroscopy (FTIR)

FTIR spectra of pure drug, the physical mixture of niosomes, niososme pellets and niosomal in situ gel were evaluated (fig. 7-10). The FTIR study for the pure drug was carried out and the observed peaks were noted. From fig. 10, it was found that following were the peaks of the PSP present in the IR graph of the standard and this

confirms the purity of the drug. The peaks were observed at 715.5, 892.8, 984.5, 1113.7, 1245.8, 1441.5, 1656.5, 1715.4, 2200.4, 2870.5, 2937.06 and 3327.6 cm^{-1} which corresponded to =C-H out of plane bending, C-H aromatic out of plane bending, C-H stretching of alkyl substituted alkenes, C-H in-plane bending, C-O stretching, C=C aromatic stretching, C=C aromatic stretching, C=O ester stretching, Weak combination and overtone of–C-H indicating mono-substitution on aromatic ring,-CH_2-symmetrical stretching, C-H aromatic stretching and O-H (free) alcoholic stretching respectively [46]. An overlay of all the spectra confirmed that all the peaks of the drug were retained in physical mixture and formulation as in pure drug which indicates drug and excipients were found to be compatible with each other.

Table 9: Release kinetics of niosomal batches

Batch Code	Zero order model	First order model	Higuchi/Matrix model	HixsonCrowell model	Korsmeyer–Peppas model			Best fit kinetic model	t_{90}
	R	R	R	R	R	n	K		Min
CS1	0.6148	0.9860	0.9877	0.9286	0.9987	0.4034	13.37	KP	112.8
CS2	0.6615	0.9251	0.9646	0.8872	0.9526	0.5329	4.8165	M	271.9
CS3	0.6229	0.9635	0.9817	0.9027	0.9820	0.4598	7.8542	KP	201.2
CS4	0.5982	0.9818	0.9726	0.9250	0.9832	0.4718	8.3280	KP	155.2
CS5	0.8932	0.9926	0.9849	0.9742	0.9866	0.6975	2.0973	FO	307.9
CS6	0.8892	0.8520	0.9785	0.9333	0.9867	0.6135	3.3073	KP	218.1
CS7	0.6320	0.8788	0.9653	0.8419	0.9836	0.4461	6.6215	KP	347.1
CS8	0.6321	0.7365	0.8837	0.5983	0.9683	0.3460	11.909	KP	345.8
CS9	0.7173	0.9369	0.9842	0.8888	0.9891	0.4683	5.5747	KP	379.7
CS10	0.4564	0.8718	0.9504	0.7877	0.9860	0.3827	9.1718	KP	390.7
CS11	0.5505	0.8696	0.9561	0.8192	0.9815	0.4330	7.6207	KP	299.5
CS12	0.2242	0.9414	0.9506	0.8244	0.9946	0.3199	19.98	KP	110.4
CS13	0.6615	0.9251	0.9646	0.8872	0.9526	0.5329	4.8165	KP	271.9
CS14	0.1567	0.7936	0.8777	0.6439	0.9339	0.3713	11.318	KP	266
CS15	0.4219	0.7193	0.8344	0.5055	0.9733	0.2875	15.69	KP	370.8
CS16	0.6864	0.9312	0.9800	0.8766	0.9834	0.4759	5.3902	KP	435.2
CS17	0.6671	0.8889	0.9826	0.8347	0.9935	0.4352	5.846	KP	491.9

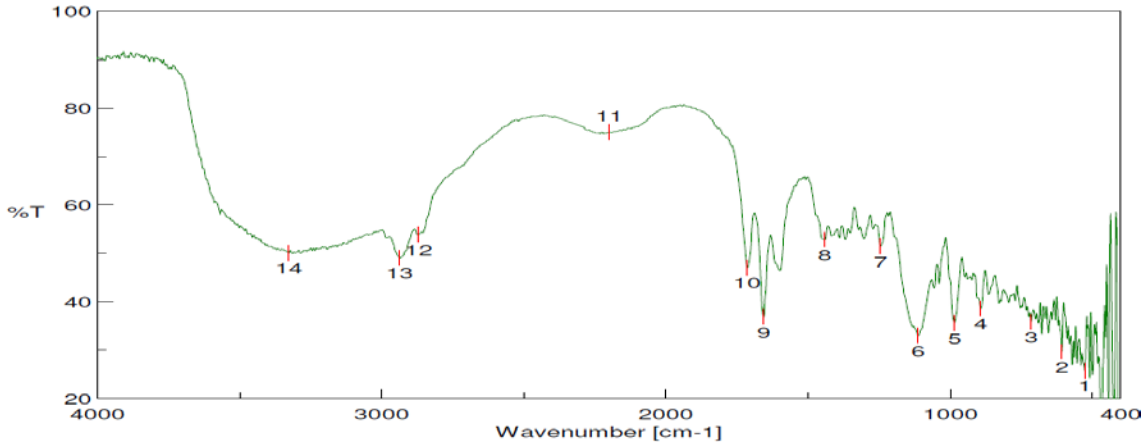

Fig. 7: FTIR spectra of PSP

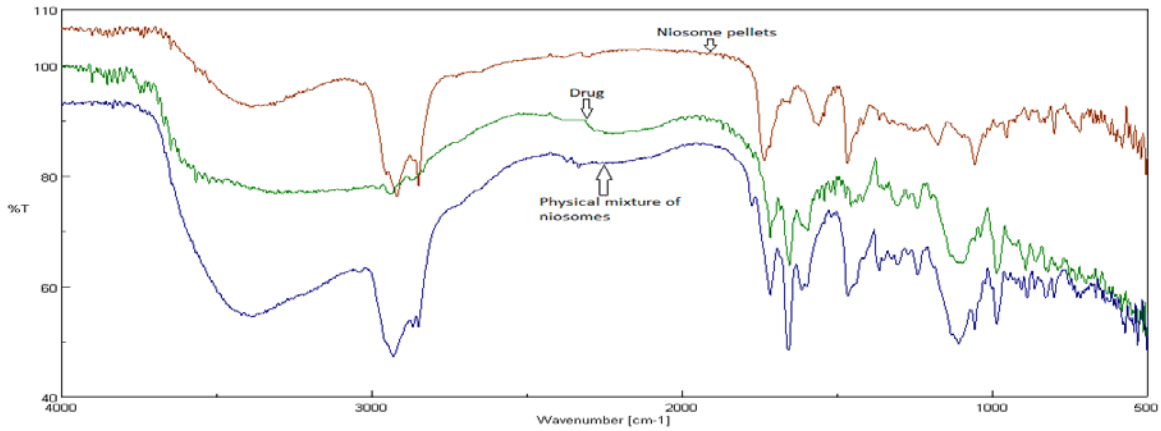

Fig. 8: Overlay of drug, physical mixture of niosomes and noisome pellets

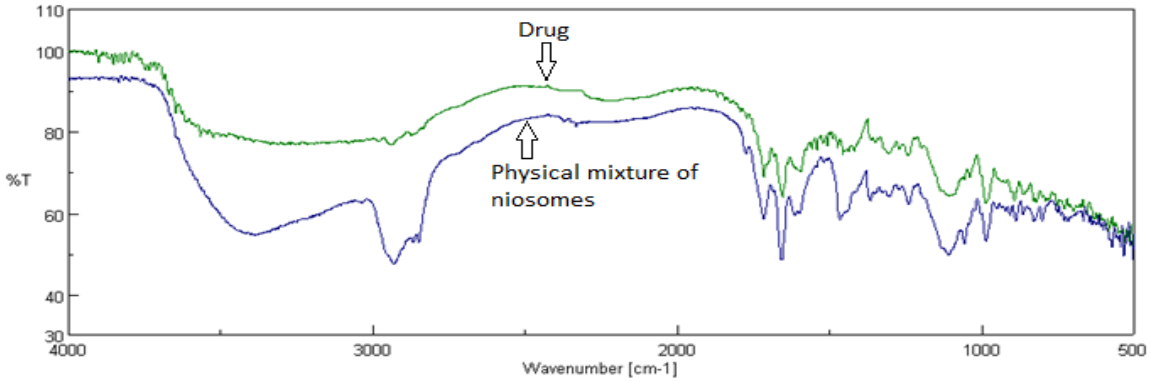

Fig. 9: Overlay of drug and physical mixture

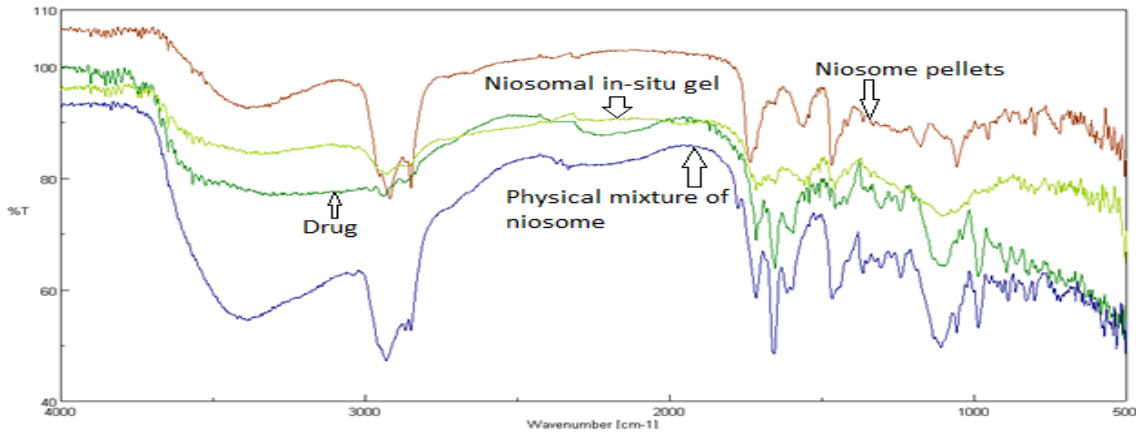

Fig. 10: Overlay of drug, physical mixture of niosomes, niosome pellets and niosomal in-situ gel

DSC

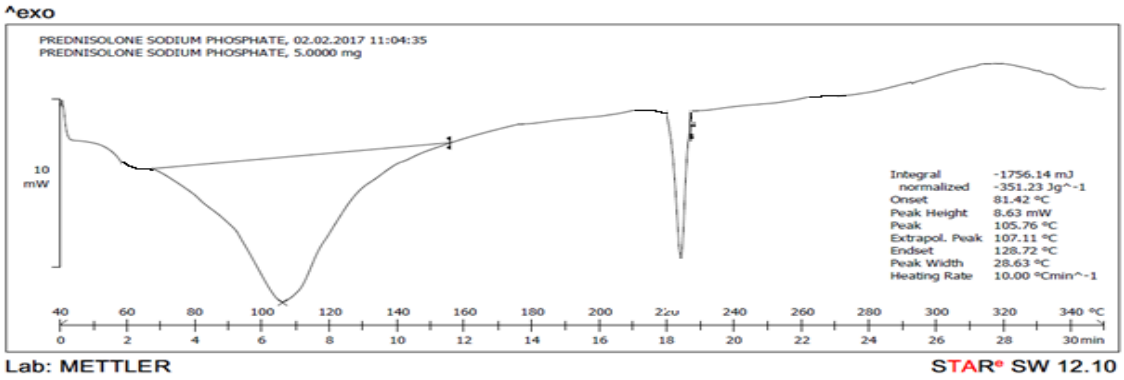

Fig. 11: DSC spectra of PSP

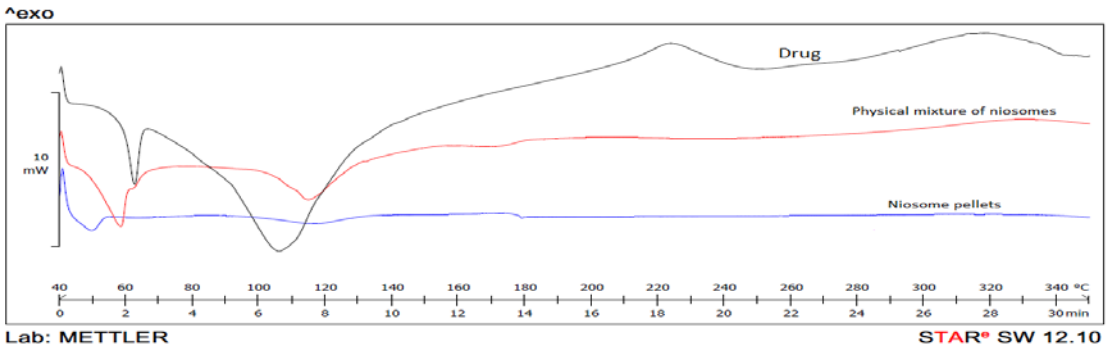

Fig. 12: Overlay of drug, the physical mixture of niosomes, and noisome pellets

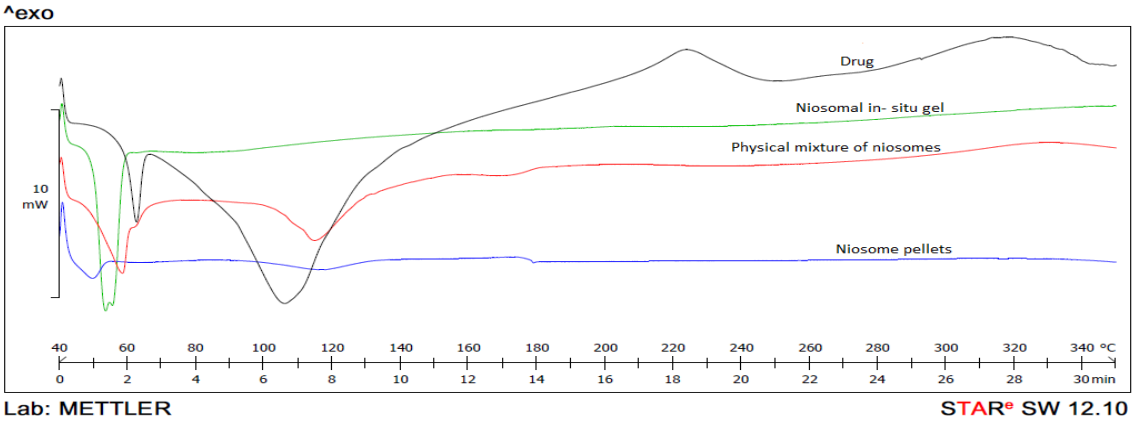

Fig. 13: Overlay of drug, physical mixture of niosomes, niosome pellets and niosomal in-situ gel

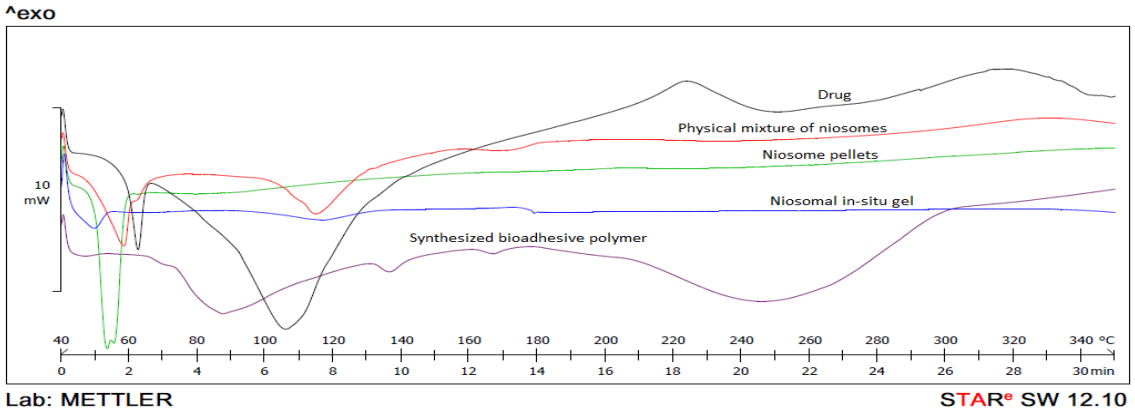

Fig. 14: Overlay of drug, physical mixture of niosomes, niosome pellets, niosomal in-situ gel and synthesized bioadhesive polymer

DSC of the drug was performed to evaluate the thermal profile of the drug (fig. 11). The onset of the peak observed at 80 ^{0}C represented the peak of evaporation of water molecules due to heat. A sharp endothermic peak was observed at 220 ^{0}C indicating the melting point of PSP (reported value 216 ^{0}C). PSP crystals exhibit an endothermic peak at 320 °C due to decomposition of PSP. The DSC thermograms of pure drug, physical mixture of niosomes, niososme pellets and niosomal in situ gel along with synthesized polymer were obtained (fig. 12, 13 and 14). The peaks observed between 50-60 ^{0}C indicated melting points of maximum excipients used in the formulation like span 60, P407 and P188 [47,48]. The cholesterol has the melting point of 148 ^{0}C which was shifted to 120 ^{0}C due to evaporation of water absorbed by the poloxamer during formulation of in situ gel. The broad peak observed at 240 ^{0}C indicates the melting point of synthesized bioadhesive polymer and drug. The absence of PSP peak on this thermogram has been taken to represent the only evidence of PSP amorphization. The physical state of the drug inside the carrier system is important because it can affect the *in vitro* and *in vivo* drug release [49]. The PSP peak was absent in the thermogram of niosomes. This situation shows that PSP was in amorphous structure and molecularly dispersed in niosomes as emphasized by Dong Y *et al.*2005. The amorphous PSP may be favorable due to the enhanced solubility of active agent [50].

It was stated by Nasr *et al.* 2008 that absence of drug's crystalline melting peak after niosomal encapsulation shows the high interaction between drug and surfactant bilayers of niosomes. This also explains the high entrapment of PSP into niosomes. In accordance with this, the entrapment of an active agent in niosomes was high in prepared formulations.

Optical microscopy

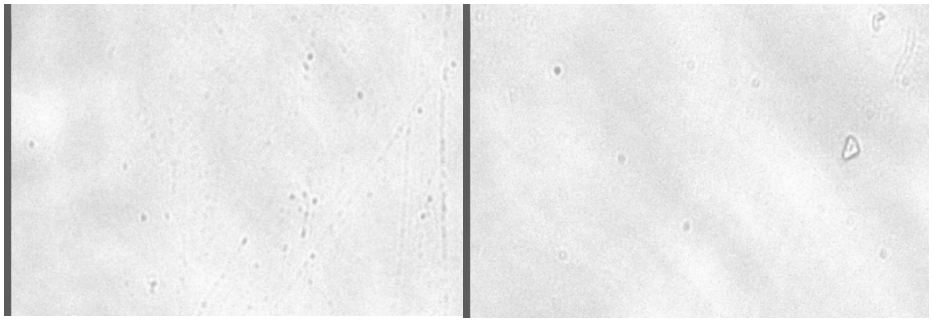

Fig. 15: Optical microscopic image of niosomes

The niosomes were observed under the digital motic microscope (fig. 15). Vesicle size of niosomes was found to be in 0.2-0.5 µm range. The vesicles were circular in shape with uniform vesicle size distribution.

Vesicle size distribution

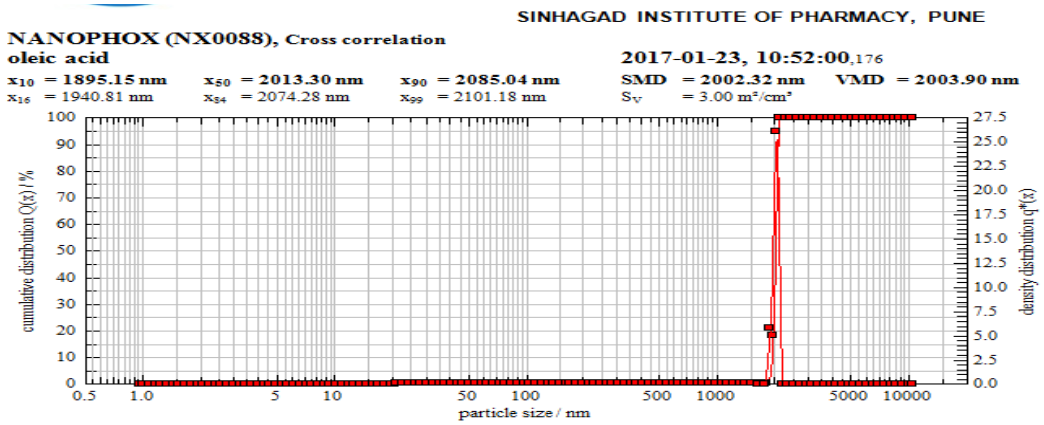

Fig. 16: Vesicle size distribution of pure drug

Vesicle size of pure drug and vesicle size of niosomal dispersion was measured by Nanophox NX0088 (fig. 16 and 17). Vesicle size of the pure drug was found to be 2002 nm which was reduced to 465 nm for niosomal dispersion. Thus the particle size was reduced by 4 times making the formulation feasible for ocular use to enhance their penetration through different biological barriers of the eye. According to previous studies of ophthalmological applications, the size of complex drug particles should be less than 10 µm to avoid a foreign body sensation after administration [51]. Especially for ocular drug delivery, larger sized particles (>1 µm) may potentially cause ocular irritation [52]. In the ophthalmic field, particles of size range 10 to 1000 nm allow for the improved topical passage of large molecules through the barriers of the ocular system [53]. Based on these results, delivery of ocular therapeutics via niosomes can be used to reduce the sensation and irritation of the eye.

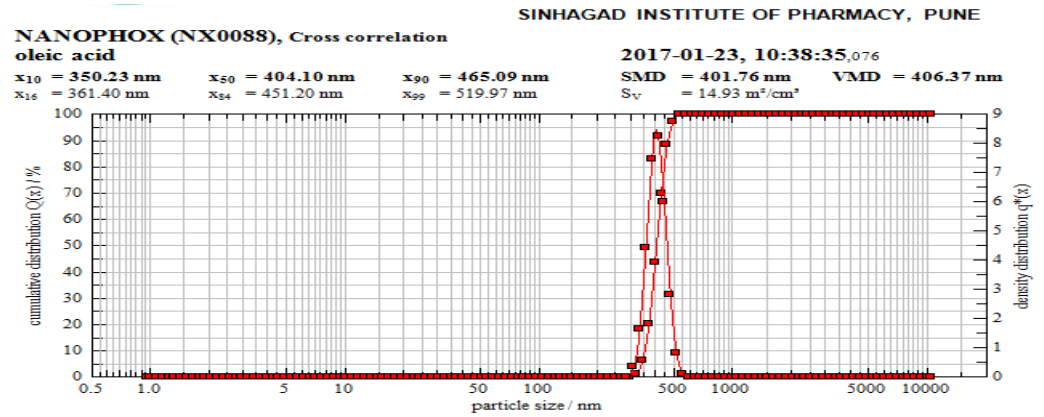

Fig. 17: Vesicle size distribution of niosomal dispersion

Zeta potential determination

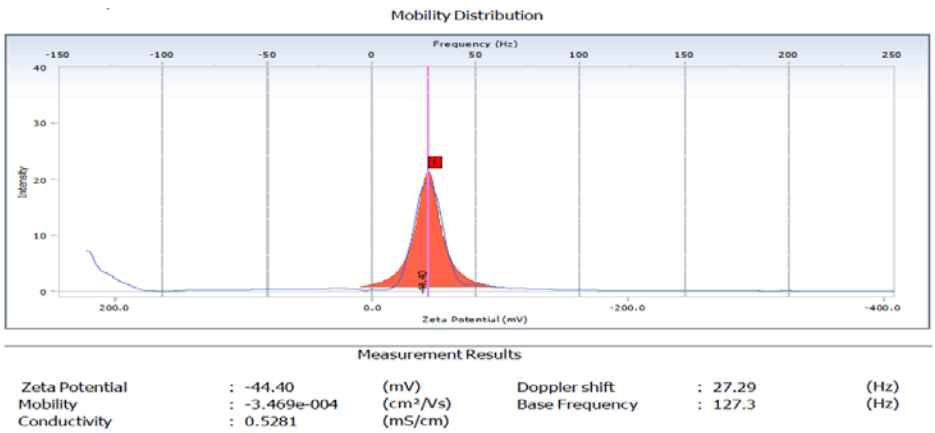

Fig. 18: Zeta potential of niosomal formulation

The zeta potential for niosomal formulation was measured by zetasizer. It has long been recognized that the zeta potential is a very good index of the magnitude of the interaction between colloidal particles. Dissociation of acidic groups on the surface of a particle will give rise to a negatively charged surface. The magnitude of the surface charge depends on the acidic or basic strength of the surface groups and on the pH of the solution. The zeta potential of the noisome under study was found to be-44 mV (fig. 18). The negative charge is contributed by the negatively charged sulfonate groups present in span 60. The presence of a net charge, whether negative or positive, can increase water uptake within the double layer. It implies that equal molarity of nonionic surfactant and chol can make the membrane compact and well organized [54]. The magnitude of the zeta potential gives an indication of the potential stability of the colloidal system. If all the particles in suspension have a large negative or positive zeta potential, then they will tend to repel each other and there will be no tendency for the particles to come together. However, if the particles have low zeta potential values then there will be no force to prevent the particles from coming together and flocculating. The general dividing line between stable and unstable dispersions is generally taken at either+30 or-30 mV. Particles with zeta potentials more positive than+30 mV or more negative than-30 mV are normally considered stable. Thus it indicates that the formulation is stable and particle size will not increase due to aggregation or coagulation even after the formulation is kept for a long period of time**.**

PDI

Table 10: PDI of pure drug and niosomal formulation

S. No.	Sample name	PDI*
1.	Pure drug	0.095±0.003
2.	Niosomal Formulation	0.284±0.026

*(mean±SD, n=3)

PDI values were calculated from vesicle size distribution data. The PDI value of formulation was found to be in the 0.284 which was found to be in the standard range i.e. less than 1 indicating that the formulation is monodisperse (table 10). The quality and uniformity of the dispersed systems are expressed with the PDI values. The values less than 0.7 are considered as suitable measurements. The low PDI values demonstrated the narrow size distribution and uniformity of the niosomal suspension [55]. Homogeneity of niosomal dispersions was indicated by the PDI values.

TEM analysis

Morphological characteristics of niosomal formulations were further confirmed by TEM analysis. TEM photomicrograph of (CS17) niosomal formulation at 40,000x and 45,000x (fig. 19) magnification revealed the spherical shape and morphology of the niosomes. Further, it was observed from the TEM images that niosomes are with hollow vesicular structure**.** The vesicle size (432 nm) observed in TEM was found to coincide with the value obtained by vesicle size determination thus confirming the size of vesicles to be in the colloidal range.

In situ gel preparation

Preliminary batches were prepared by varying the concentrations of P407 and P188 indifferent ratios and evaluated for gelation temperature (table 11). The concentration of P407 was varied from 10 to23% w/v whereas the concentration of P188 was varied from 10-30% w/v. While defining the ratios, care was taken that the total poloxamer concentration should not exceed 40%w/v. The formulations containing only P407 showed higher sol-gel transition temperatures than the gel bases containing P188 in combination with it. The sol-gel transition temperature increased when the P407 concentration was decreased. This observation was in accordance with the data available in the literature [56]. When the mixtures were compared according to the ratio of P407/P188, it was observed that the w/w percent ratio of P407/P188 was important to reach the desirable gelation temperature and the result was compatible with the literature [57, 58].

It was found that with the increase in the concentration of P188, the gelation temperature decreased significantly ($p<0.05$) and found to be equivalent to the physiological temperature that is 36-37^{0}C. Gelation temperatures for P188 and P407 gels were observed for the different concentration range of polymer, and it was found that the gelation temperature of formulation decreased with increasing concentration of polymer. As the concentration of polymer increases, the gel structure becomes more closely packed with the arrangement in the lattice pattern [59].

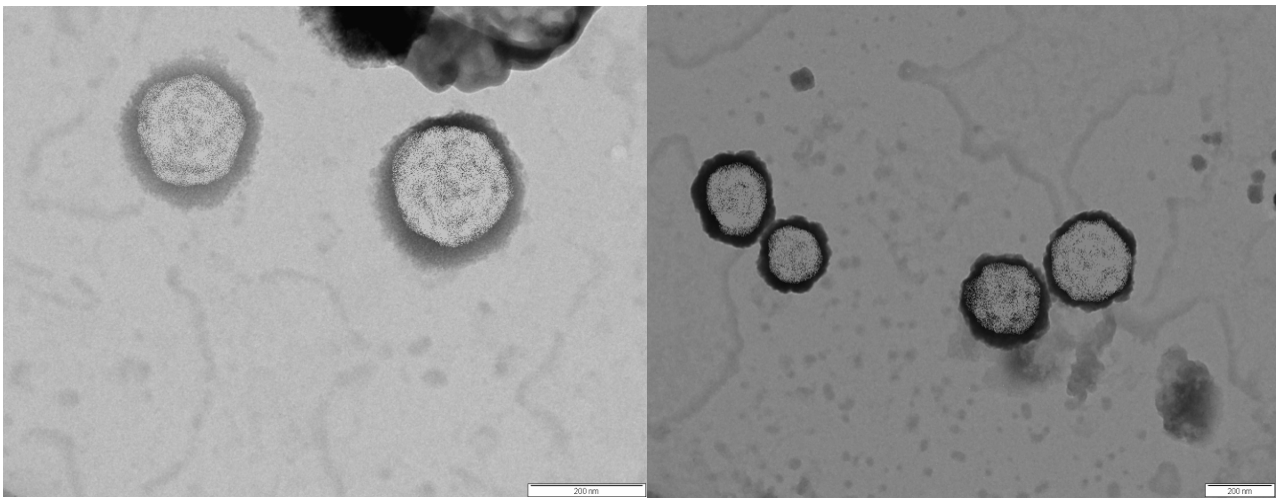

Fig. 19: TEM images of optimized niosomal batch (CS17)

Evaluation of in situ gel

Preliminary batches of in situ gel

Table 11: Formulation batches of in situ gel along with gelation time, gel capacity and gel temperature

Batch code	P407 (%w/v)	P188 (%w/v)	Gelation temperature (°C)	Gel capacity*	Gelation time (min)
G1	20	-	>60	-	-
G2	18	-	>60	-	-
G3	16	-	>60	-	-
G4	21	-	>60	-	-
G5	22	-	>60	-	-
G6	23	-	>60	-	-
G7	20	14	>60	-	-
G8	20	15	>60	-	-
G9	20	16	>60	-	-
G10	23	14	>60	-	-
G11	22	15	>60	-	-
G12	21	16	>60	-	-
G13	15	15	>60	-	-
G14	20	10	>56	-	-
G15	17	17	>60	-	-
G16	10	11	>57	+	-
G17	11	10	>57	+	-
G18	11	11	>57	+	-
G19	10	12	>52	-	-
G20	12	20	>52	-	-
G21	12	12	>52	-	-
G22	20	13	48-52	-	-
G23	13	20	48-52	-	-
G24	13	13	48-52	-	-
G25	10	15	43	+	-
G26	15	20	43	-	-
G27	15	15	42	-	<1
G28	10	16	43	+	<1
G29	17	10	42	+	<1
G30	17	17	43	++	<1
G31	10	18	42	+	<1
G32	18	20	42	++	<1
G33	20	18	40	++	<1
G34	10	25	37-38	+++	<1
G35	10	27	37-38	+++	<1
G36	10	30	36-37	+++	<1

*(–): The solutions which did not undergo a phase transition at all. (+): The solutions which exhibited a phase transition only after 60 s. and the formed gels which collapsed within 1-2 h. (++): The solutions which formed the gels after 60 s. however, the gels formed did not remain stable for more than 3 h. (+++): The solutions which exhibited phase transition within 60 s and the gels so formed remained stable for more than 7-8 h.

Evaluation parameters of in situ gel

Table 12: Evaluation results of shortlisted formulations

Formulations	G34 (A)	G35 (B)	G36 (C)
Appearance	Translucent	Translucent	Translucent
pH*	6.8±0.1	6.5±0.1	6.7±0.1
Drug content (%)*	88.45±0.37	91.37±0.26	86.13±0.15
Gelation temperature (°C)*	37.7±0.5	37.2±0.5	36.9±0.5
Gel strength (s)*	29±0.36	32±0.42	35±0.18
Gelation time (min)	<1	<1	<1
Mucoadhesive strength (dynes/cm^2)*	1837±0.35	2043±0.26	2465±0.14
Spreadability (cm)*	2.3±0.33	2.1±0.52	1.8±0.08

*(mean±SD, n=3)

The appearance of the formulation was translucent since niosomal pellets equivalent to drug dose were introduced into the gel formulation (table 12). The pH of the solution was found to be in the range of 6.5 to 6.8 since the pH of the formulation was maintained by addition of 0.1 M NaOH. The effect of polymer concentration on drug content was negligible because the drug was incorporated into niosomes and the drug was not directly dissolved into the gel formulation. Thus, DC was affected by niosomal formulation parameters and not by gel formulation parameters. With the increase in the concentration of P188, the gelation temperature was decreased and thus the optimum temperature of 37.2±0.2 ^{0}C was obtained. Gel strength is the indication of the viscosity of the gel formulation. It was observed that with the increase in polymer concentration, gel strength was also increased. The mucoadhesive

strength limits the total clearance of drug from the ocular surface. Defining the mucoadhesive characteristics is of great importance when prolonged residence time and a decreased leakage of the formulation with the mucosal secretion are required [56]. The bioadhesive strength was not significantly affected by polymer concentration because the amount of addition of synthesized bioadhesive polymer was constant in all the three formulations. The bioadhesive strength of formulation was increased with increase in the concentration of polymer forming in situ gel formulation. In the literature, it was shown that P407 increased the mucoadhesive force of the formulation. It was also presented that the formulation prepared with the mixture of P407/P188 increased the bioavailability by preventing the migration of the formulation from the mucosal tissue [58]. This was in accordance with our data and the studies on the mucoadhesive property of poloxamers. The spreadability of the gel formulation was measured by texture analyzer. It was found that with the increase in polymer concentrations, the spreadability decreased significantly ($p<0.05$) due to the increase in viscosity of the formulation. It has been observed that increasing the concentration of any of the gelling agents was always associated with a decrease in the spreadability [60]. One of the criteria for a gel to meet the ideal quality is that it should possess good spreadability. It is the term expressed to denote the extent of area, to which gel readily spreads on application site. Lesser the time is taken for separation of two slides, better the spreadability. The gelling capacity of the formulation was measured on the basis of gelation time and time for which the gel remains to hold its solid state. It was observed that with significant ($p<0.05$) increase in P188 concentration (above 20% w/v), the gelling capacity improved thus forming within 60 s and holding its state for more than 7-8 h.

Viscosity measurements

The viscosity of the optimized formulation was measured at a different temperature from 25 ^{0}C to 40 ^{0}C (fig. 20). It was observed that the viscosity of gel was suddenly increased between temperature 35-37 ^{0}C which indicated sol to gel transformation. The viscosity of gel formulation was found to be in the range of 800-900 cps below 35 ^{0}C which increased to about 4000-5000 cps above 35 ^{0}C indicating gelation temperatures. The increase in the concentration of P188 caused the decrease in the gelation temperature.

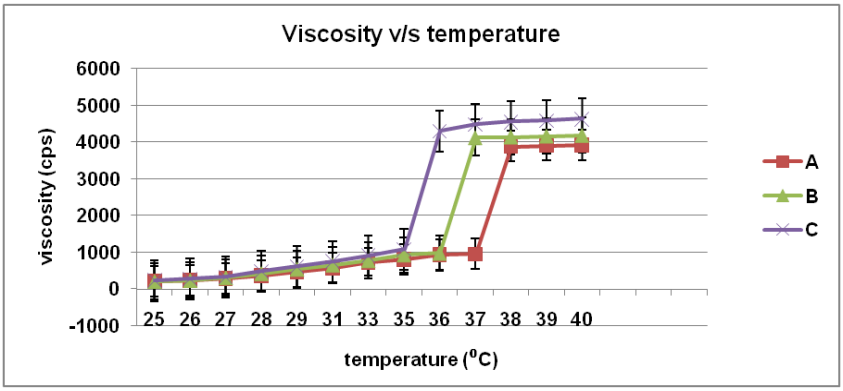

Fig. 20: Effect of temperature on viscosity of in situ gel data expressed mean±SD (n=3)

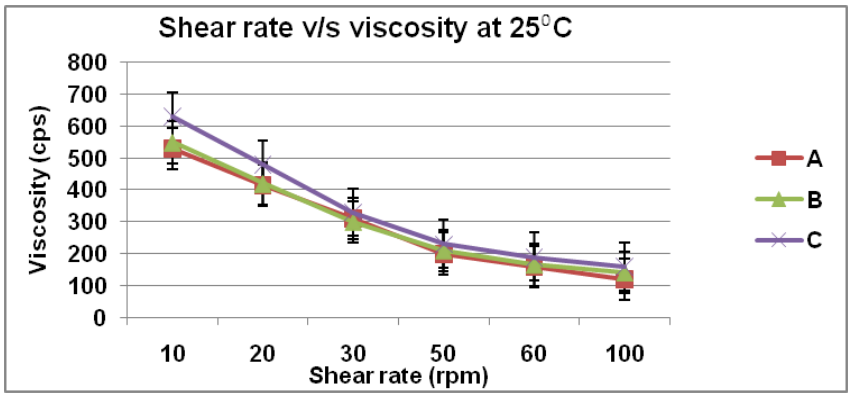

Fig. 21: Comparison of viscosity change with change in shear rate at 25^{0}C for different formulations data expressed mean±SD (n=3)

All the formulations showed pseudoplastic rheological flow after studying at various temperatures, as evidenced by shear thinning and increase in shear stress with increased angular velocity. It was found that the rheological parameter was directly dependent on polymer concentration of formulation. At 25 ^{0}C, all formulations were having low viscosity (fig. 21) and at 37 ^{0}C, the formulations showed high viscosity (fig. 22). This indicates conversion of these formulations from sol to gel. It was also observed that viscosity of all the formulations was decreasing with the increase in shear rate. The non-newtonian formulations with pseudoplastic properties can acquire a viscosity decrease with increasing shear rate, creating blinking and ocular movement. Pseudoplasticity is thus interesting because it offers significantly less resistance to blinking and shows much greater acceptance than viscous newtonian formulations [61].

***In vitro* drug diffusion study**

The *in vitro* drug release profile of gel formulations was calculated by PCP disso software where the percent drug release along with dissolution kinetics and best fit model was found out. T90 value was found out using this PCP disso V3 software and comparison was done on its basis (fig. 23). The apparent diffusion coefficient values are given in table 12. It was observed that the diffusion coefficient for optimized formulation (1.998×10^{-5} cms^{-1}) was higher as compared to the pure drug (0.710×10^{-5} cms^{-1}) and marketed

formulation (0.865 x 10^{-5} cms^{-1}) thus proving increased permeability of drug through the membrane (table 13). These results can be attributed to the presence of bioadhesive polymer which retains the formulation in contact with the eye for a long period of time.

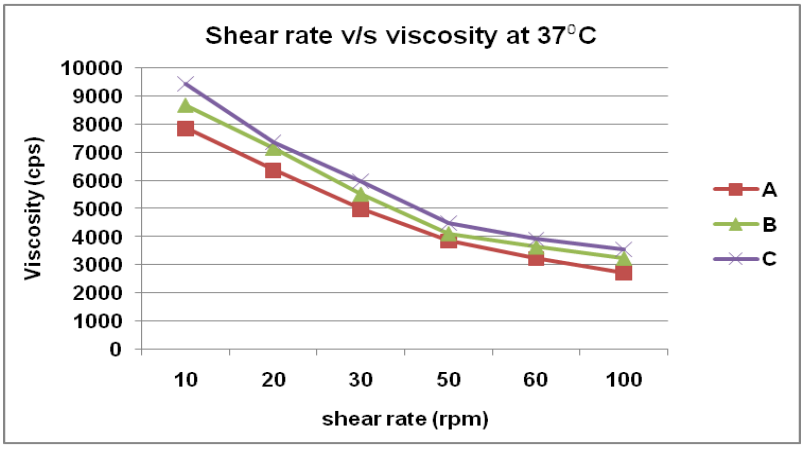

Fig. 22: Comparison of viscosity change with change in shear rate at 37^{0}C for different formulations data expressed mean±SD (n=3)

The presence of polymers in in situ gel (P188 and P407) in lower concentrations acts as penetration enhancers and thus helps in penetration of PSP through the membrane. T90 value is the time at which 90% of the drug is released (table 14). A, B and C batch exhibited 90 value of 523 min (8 h and 43 min), 596 min(9 h 56 min) and 555 min (9 h 15 min) respectively. The release kinetics indicated that it followed zero order which means drug release does not depend on initial concentration.

The B batch (P407:P188-1:2.7) was found to be the optimized batch with maximum t90 value thus sustaining the action for a long period of time. The marketed formulation and the pure drug showed 20-23% drug diffusion in 6 h whereas the drug released by niosomal formulation was found to be in the range of 45-50 % in 6 h. Thus the amount of drug diffused was increased by niosomal in situ gel formulation. O. Inal *et al.* 2013 observed that the presence of P188 polymer increased the release of meloxicam due to the change in the ratio of Poly Propylene oxide/Poly Ethylene Oxide (PPO/PEO) units in the polymer. Comparably shorter chain and low PPO/PEO molar ratio of hydrophilic P188 tend to disrupt the hydration shells around the hydrophobic portion of P407 molecules, which resulted as the high degree of water molecules around the PPO units. During gelation, those ordered water molecules had to be squeezed out into the bulk solution. Therefore, an increase in temperature required to promote the hydroscopic interaction between poloxamer micelles [62].

Thus, the gel prepared with P188 has more tendencies to erode. As seen in fig. 23, formulations including P407:P188 combination (A, B, C) showed higher erosion profiles than the others probably due to the decrease in PPO/PEO molar ratio of polymer in the gel.

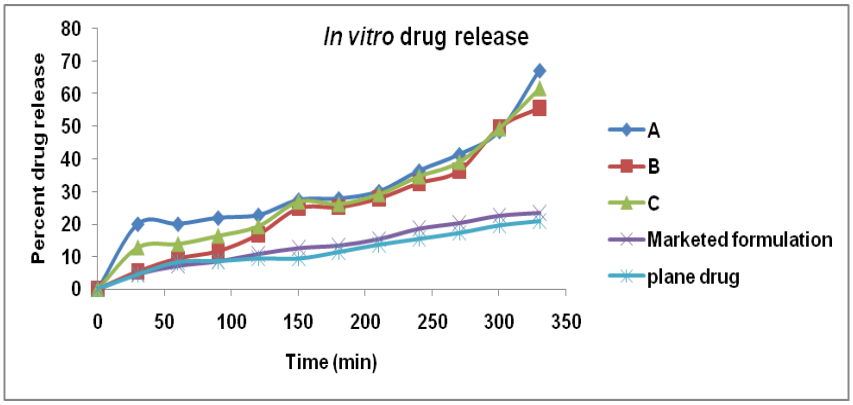

Fig. 23: *In vitro* drug release profile of in situ niosomal batches data expressed mean±SD (n=3)

Table 13: Amount of drug released in 6 h and apparent diffusion coefficient of different batches

S. No.	Batch	Amount of drug diffused in 6 h*	Apparent diffusion coefficient (cm s^{-1})*
1.	A	48.55±0.12	1.853* 10^{-5}
2.	B	49.72±0.37	1.998* 10^{-5}
3.	C	49.04±0.24	1.892*10^{-5}
4.	Pure drug	19.52±0.48	0.710*10^{-5}
5.	Marketed formulation	22.64±0.59	0.865*10^{-5}

*(mean±SD, n=3)

Table 14: Release kinetics of in situ niosomal batches

Batch Code	Zero-order model	First order model	Higuchi/Matrix model	Hixson-Crowell model	Korsmeyer–Peppas model			Best fit kinetic model	$t_{90\%}$
	R	R	R	R	R	N	K		Min
A	0.9146	0.883	0.892	0.902	0.8526	0.4819	2.7082	ZO	523.4
B	0.9777	0.946	0.888	0.959	0.9728	0.9384	0.2028	ZO	596.1
C	0.9631	0.928	0.904	0.944	0.9353	0.6425	1.0740	ZO	555.6

Ex vivo drug release studies

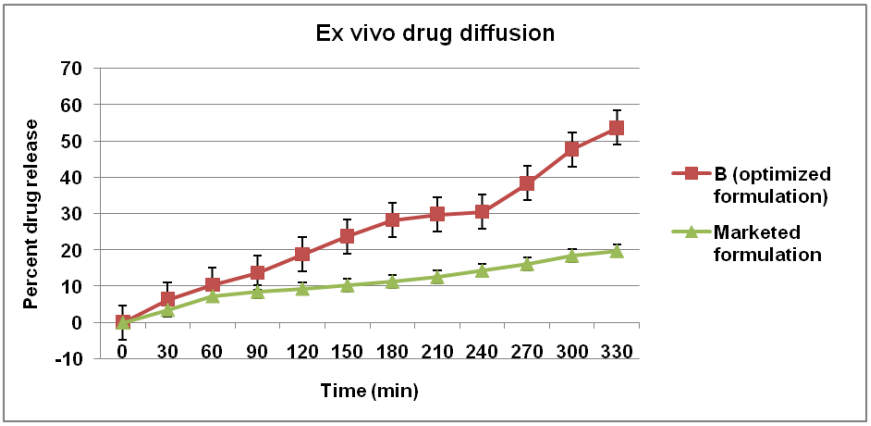

Fig. 24: Ex vivo drug diffusion of optimized formulation data expressed mean±SD (n=3)

The ex vivo drug diffusion study of optimized formulation showed that 90% of drug release was obtained in 10 h providing sustained effect (fig. 24). It is clear that a significantly higher amount ($p < 0.05$) of PSP permeated from the optimized formulation as compared to the marketed formulation. It is clear that niosomes prepared using Span 60 showed the more amount of PSP permeated, this might be attributed to the transition temperature of the used surfactant, where the high transition temperature of Span 60 made the niosomes in a more packed ordered gel state at the specified permeation temperature (37 °C) as studied by Vora *et al.*, 1998. The marketed formulation showed just 20% drug release after 6 h which may be due to its larger molecule weight and thus large molecule size which indicates there may be a problem in the conjunctival absorption of the drug. Thus this problem was overcome by entrapment of PSP in niosomal in situ gel formulation. The size of vesicle was reduced due to its incorporation into niosomes and the release was sustained by in situ gel formulation.

Pharmacodynamic study

Primary skin irritation test

The PII of the test sample was calculated to be 0.00;no irritation was observed on the skin of the rabbits/rats. The scores for erythema and edema were summed for intact and abraded skin for rabbits at 24 and 72 h, PII was calculated. Based on the sum of the scored reactions divided by 32 (two scoring intervals multiplied by two test parameters multiplied by 8 animals). PII: 0/32 = 0.00. Under the conditions of this test, the test sample would not be considered a primary skin irritant since the PII was less than 5.00.

***In vivo* draize eye irritation test**

The possibility of eye irritation due to niosomal in situ gel administration was evaluated in rabbits. The rabbits were observed for ocular lesions, and no symptoms of ocular irritation such as redness, tearing, inflammation, or swelling were observed after niosomal in situ gel administration. No ophthalmic damage or abnormal clinical signs to the cornea, iris or conjunctivae were visible. Thus, the developed ocular drug delivery systems are apparently free from any ocular irritation potential and can be safely administered to humans. The scores were calculated according to Draize scale.

Pharmacokinetic study

The aqueous humor concentration (mean±SD) time profiles of prednisolone sodium phosphate following ocular instillation of optimized niosomal in situ gel (B) and the drug solution in rabbits are depicted in fig. 25. The differences between the estimated drug pharmacokinetic parameters (Cmax, Tmax, and MRT (0–8 h) of the two treatments are illustrated in table 15. The mean (±SD) Cmax of the drug solution and that of the optimized formulation (B) were found to be equivalent. The delay in the median Tmax (from 1 to 2 h), as well as the prolongation in the MRT (0–8 h) from 3.583±0.526 to 5.349±0.035 h for the drug solution and the optimized formulation respectively, could indicate the sustained-release characteristics of the latter. Based on the calculated $AUC_{(0-8)}$ value, the increase in the ocular bioavailability was found to be 1.754-fold. The drug penetration enhancement following the instillation of the optimized formulation could be attributed to the presence of surfactant (span 60) in niosomal formulation which also acts as penetration enhancer. In addition to this, PSP being hydrophilic in nature can easily pass the epithelial barrier and thus contribute to increased penetration.

The bioadhesive synthesized polymer macromolecular hydrocolloids have numerous hydrophilic functional groups (carboxylic acid). The cornea and conjunctiva have a negative charge where these mucoadhesive polymers may interact intimately with these extraocular structures [25], would increase the concentration and residence time of the associated drug. The elevated PSP levels in the cornea and aqueous humor following the administration of PSP-Gel might be due to the increase in the amount of PSP dissolved in the precorneal area leading to the high concentration gradient, favoring good permeation, together with higher contact time with the corneal area [63].

Sterility test

In order to ensure the sterility of the finished product, the final formulation (B) was subjected to the sterility test. The formulation sterilized by membrane filtration method and incubated with different culture media like soybean casein digest medium; fluid thioglycate medium etc., for a period of 14 d of incubation did not show growth of the organism on the culture medium. This indicated that the formulation was sterile.

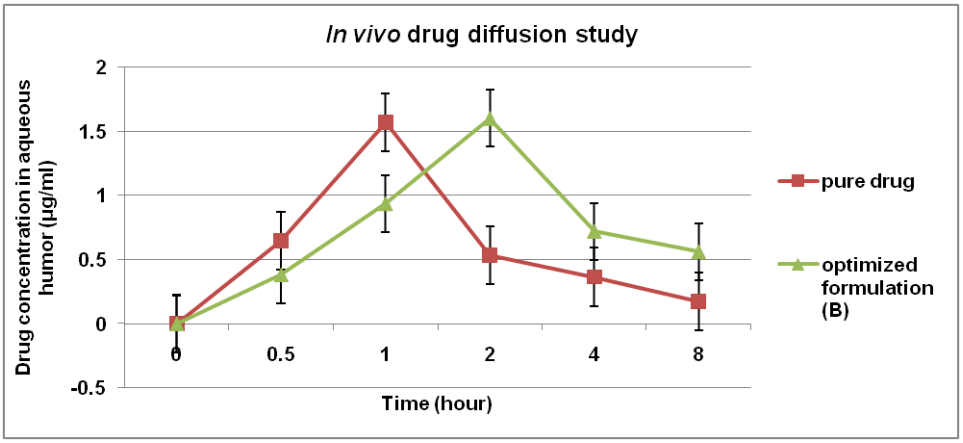

Fig. 25: Aqueous humor concentration-time profiles of PSP following ocular instillation of the drug solution and optimized B formulation to rabbits data expressed mean±SD (n=3)

Table 15: The pharmacokinetic parameters of PSP following ocular instillation of the drug solution and optimized formulation B to rabbits

Batch	C_{max} (µg/ml)*	T_{max} (h)	$AUC_{(0-8)}$ (µg h^{-1} ml^{-1})*	$MRT_{(0-8)}$ (h)*	Increase in bioavailability (folds)
Pure drug solution	1.573±0.345	1	3.75225±0.254	3.583±0.526	-
Optimised formulation (B)	1.602±0.427	2	6.584±0.127	5.349±0.035	1.754

*(mean±SD, n=3)

Stability study

Accelerated stability study

Table 16: Stability testing by appearance, percent drug content determination and gelation temperature

Time period	Appearance	Percent drug content of optimized formulation*	Gelation temperature (^{0}C)*
Initial	Translucent	91.36±0.36	37.2±0.23
After 3 mo	Translucent, little gel formation at the base which can be	90.53±0.27	37.0±0.41
After 6 mo	converted into sol after shaking	88.45±0.47	37.0±0.05

*(mean±SD, n=3)

The results obtained show that all the formulations were found to contain almost same amount of drug after 6 mo. There was a little decrease in percent drug content but it was less than 5% for each formulation after 6 mo. The results obtained showed little decrease in the gelation temperature of the formulation with the increase in time but that was so less that it can be neglected (table 16).

Long term stability study

Table 17: Stability testing by appearance, percent drug content determination and gelation temperature

Time period	Appearance	Percent drug content of optimized formulation*	Gelation temperature (^{0}C)*
Initial	Translucent	91.36±0.36	37.2±0.23
After 3 mo	Translucent and stable	89.53±0.27	37.1±0.41

*(mean±SD, n=3)

The results obtained showed that all the formulations were found to contain almost same amount of the drug after 3 mo. So it can be concluded that there wasn't any drug loss from formulation during storage. The results obtained showed there wasn't any significant change in the gelation temperature of the formulations after 3 mo. So, it can be predicted that the formulation would be stable during storage (table 17).

CONCLUSION

Different approaches have been used till now in order to overcome the drawbacks related to conventional ocular delivery systems and to improve patient compliance without losing the therapeutic activity of the drug. Niosomal in situ gel proved to be one of the successful approaches to accomplish the goals successfully. Niosomes optimal batch (CS17) was able to entrap PSP where entrapment efficiency was found to be 83.4±0.22%. As a result, the optimal formula was further incorporated into on situ gel with desirable mucoadhesive behavior essential to achieve increased retention at the target site. The formula exhibited significant permeation with almost 2.5 fold increased flux and sustained for longer periods (t90= 10 h) compared to the pure drug and marketed formulation at the same dose level. The pharmacokinetic study in

rabbits proved that the total concentration of PSP in aqueous humor was higher as compared to that of pure drug solution. The mean residence time and the shift in tmax value indicated sustained release of the drug. Thus niosomal in situ system proved to be a very useful system for ocular drug delivery with promising results.

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AUTHORS CONTRIBUTIONS

There was an equal contribution of both the authors in carrying out the research work

CONFLICT OF INTERESTS

There is no conflict of interest

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