ORIGINAL ARTICLE

Investigation of hydrogel membranes containing a combination of timolol maleate and brimonidine tartrate for ocular delivery

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Hydrogels are comprised of a cross-linked network of polymers. Water penetrates these networks, resulting in swelling and giving the hydrogel a soft and rubbery consistency, thereby maintaining the integrity of the membrane. Because of the drawback of conventional therapy for ocular delivery, a hydrogel membrane containing a combination of timolol maleate and brimonidine tartrate were formulated for the treatment of glaucoma. In the present investigation, hydrogel membranes were prepared using polymers like gelatin, PVA and chitosan, which were cross-linked using physical and/or chemical methods. The cross-linking of the membranes was confirmed by Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD) and Differential scanning calorimetry (DSC) studies. From the scanning electron microscopy (SEM) of the membranes, it appeared homogenous and showed no separation. The pH of the membranes ranged from 7.21-7.4. The hydrogels showed a considerably good swelling ratio ranging from 91.66-372.72%. The drug content ranged from 82.78-95.62%. The *in vitro* drug release study indicated that there was a slow and sustained release of the drug from the membranes that were sufficiently cross-linked and followed zero order release. The Intraocular pressure (IOP) lowering activity of the prepared formulation was compared with the marketed formulation, and it was found that the IOP lowering action was sustained for a long period of time. Stability studies proved that the formulations could be stable when stored at room temperature. Results of the study indicate that it is possible to develop a safe and physiologically effective hydrogel that is patient compliant.

Key words: *Cross-linking, differential scanning calorimetry, fourier transform infrared spectroscopy, glaucoma, hydrogels, scanning electron microscopy, X-ray diffraction*

INTRODUCTION

Controlled drug deliveries to the eye remain a challenging task due to the normal ocular protective mechanisms such as blinking and tear drainage that promote rapid clearance and reduced bioavailability, resulting in a short duration of pharmacological response. Current research efforts are focused toward the design and evaluation of ocular delivery systems that are easy to administer, require decreased administration frequency and provide controlled and possibly sustained drug release in order to increase therapeutic efficacy and patient compliance.[1,2] The conventional ocular delivery systems like solutions, suspensions and ointments show drawbacks such as increased pre-corneal elimination, high variability in efficiency and blurred vision, respectively. The major problem encountered with solution is the rapid and extensive elimination of drugs from the pre-corneal lachrymal fluid by solution drainage, lachrymation and non-productive absorption by the conjunctiva, which may lead to undesirable side-effects. It must be noted that this high drainage rate is due to the tendency of the eye to maintain its residence volume at 7-10 μL permanently, whereas volumes topically instilled range from 20-50 μL. Ointments increase the contact time,

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minimize the dilution by tears and resist nasolachrymal drainage; however, these are responsible for blurring of vision. Suspensions show high variability due to inadequate dosing, mainly due to lack of patient compliance.[3]

Glaucoma is a disease of the major nerve of vision, called the optic nerve. The optic nerve receives light from the retina and transmits impulses to the brain that we perceive as vision. Glaucoma is characterized by a particular pattern of progressive damage to the optic nerve that generally begins with a subtle loss of side vision (peripheral vision). If glaucoma is not diagnosed and treated, it can progress to loss of central vision and blindness.[4]

Hydrogels are three-dimensional, cross-linked networks of water-soluble polymers. They can be made from virtually any water-soluble polymer, encompassing a wide range of chemical compositions and bulk physical properties. They can be formulated in a different variety of physical forms, including slabs, microparticles, nanoparticles, coatings and films.[5] They are endowed with the ability to swell in water or aqueous solvents, and their highly porous structure can easily be tuned by controlling the density of cross-links in the gel matrix and the affinity of the hydrogels for the aqueous environment in which they are swollen. Their porosity also permits loading of drugs into the gel matrix and subsequent drug release at a rate dependent on the diffusion coefficient of the small molecule or macromolecule through the gel network.[5,6]

Timolol maleate is a beta blocker that acts by reducing the synthesis of aqueous humour production through blockade of β receptors on the ciliary epithelium, and has a half-life of 2.5-5 h. Brimonidine tartrate is an α 2 agonist, which acts by decreasing the synthesis of aqueous humour and increasing the amount that drains from the eyes through uveoscleral outflow, and it has a half-life of 3 h. The above combination is marketed in the form of eye drops; however, due to the drawbacks associated with any other eye drops such as rapid tear turnover, lachrymal drainage rate and drug dilution by tears, it has been demonstrated that 90% of the administered dose was cleared off within 2 min for an instilled volume of 50 μL. The ocular residence time of conventional solution is limited to few minutes and the overall absorption is limited from 1-10%. Consequently, most drugs get absorbed systematically via the nose or gut after drainage from the eye. This excessive systemic absorption not only reduces the ocular bioavailability but may also lead to unwanted side-effects and toxicity.[7] The two main strategies for improving ocular absorption are increasing the corneal permeability and prolonging the contact time on the ocular surface.

With all the above aspects in mind, the present work was aimed at investigating the potential of hydrogel membranes containing a combination of timolol maleate and brimonidine tartrate as ocular drug delivery systems for the treatment of glaucoma so as to increase the contact time of the drug with the eye, reduce systemic side-effects, reduce the number of applications and better patient compliance.[8] The device is non-biodegradable to prevent disintegration of the membrane and leakage of the drug. This is based on drug loaded in hydrogels, the ocular device is placed under the eyelid, where the hydrogel takes up fluid, swells and releases the drug.

MATERIALS AND METHODS

Materials

Brimonidine tartrate was obtained from Indoco Remedies Ltd, Goa, India, as a gift sample. Timolol maleate was obtained from FDC Aurangabad, India, as a gift sample. Chitosan was obtained from CIDF, Cochin, India. Gelatin was obtained from Thomas Baker, Mumbai, India. Propylene glycol was obtained from Loba Chemie Pvt.Ltd., Mumbai, India. Benzalkonium chloride was obtained from Merck India Ltd., Mumbai India. Fluid thioglycolate medium and soyabean casein digest were provided by Hi Media Ltd., Mumbai, India. All other solvents and reagents used for the study were of analytical grade.

Methods

Preparation of hydrogel membranes

The hydrogel membranes were prepared by the solvent casting method after cross-linking the polymers [Table 1].

Cross-linking of polymers

Preparation of PVA- gelatin cross-linked membranes

Gelatin was dissolved in a solution of PVA, which was prepared using phosphate-buffered saline of pH 7.4, by heating PVA to 80°C for 30 to 40 min, adding one drop of 0.1 M HCl and stirring the resulting dispersion stirred at 70°C for half an hour to carry out the esterification between PVA and gelatin.[9]

Preparation of cross-linked PVA membranes

Aqueous solutions of PVA were prepared by dissolving PVA in phosphate-buffered saline (pH 7.4) by heating for 30 to 45 min at 80°C. It was then frozen at 0°C (for 14 h) and thawed at 30°C (for 6 h) for one to three cycles.[10]

Preparation of cross-linked chitosan- PVA membranes

A clear solution of chitosan was prepared by dissolving chitosan in 0.1 M HCl and similarly the PVA solution was also prepared by heating in phosphate-buffered saline of pH 7.4 for 30-40 min at 80°C. Both the solutions were then mixed and autoclaved for cross-linking.[11]

The pH of all the above cross-linked polymer solutions was adjusted in the range of 7 -7.5 using 0.1 M NaOH. A stock solution of the mixture of drug and preservative was prepared and 1mL of the drug solution was pipetted out and added to each of the polymeric solutions. The solutions

Table 1: Formulation of hydrogel membranes

Formulation code	TM (mg)	BT (mg)	GL (%w/v)	PVA (%w/v)	CHT (%w/v)	PG (%w/v)	BZK (%w/v)
GP							
F1	0.5	0.25	2.0	3.0	-	0.1	0.02
F2	0.5	0.25	1.5	2.0	-	0.1	0.02
F3	0.5	0.25	1.0	3.0	-	0.1	0.02
PP							
F4	0.5	0.25	-	1.5	-	0.1	0.02
F5	0.5	0.25	-	3.0	-	0.1	0.02
F6	0.5	0.25	-	1.5	-	0.1	0.02
F7	0.5	0.25	-	3.0	-	0.1	0.02
CP							
F8	0.5	0.25	-	3.0	2	0.1	0.02
F9	0.5	0.25	-	3.0	3	0.1	0.02
F10	0.5	0.25	-	1.0	4	0.1	0.02

GP: Gelatin-PVA cross-linked membrane, PP: PVA-PVA cross-linked membrane, CP: Chitosen-PVA cross-linked membrane, TM: Timolol maleate, BT: Brimonidine tartrate, GL: Gelatin, PVA: Polyvinyl alcohol, CHT: Chitosan, PG: Polyglycolate, BZK: Bezalkonium chloride

were then poured into a sterilized mould (5 cm × 5 cm or 25 cm^2) under aseptic conditions and dried in oven at 40°C for 12 h. Membranes (0.4 cm × 0.5 cm or 0.2 cm^2 for *in vivo* study and 2 cm × 2 cm or 4 cm^2 for *in vitro* drug release study) were then cut, packed and stored for further evaluation.[12] The entire procedure was carried out under aseptic conditions using sterilized glassware and moulds.

Characterization of hydrogel membranes

Determination of the dimensions and weight of the membrane

The thickness of the membranes was measured using a micrometer screw gauge at three different points on each of the membranes. The length and breadth of the membranes were determined using a Vernier caliper scale. For each formulation, five randomly selected membranes were tested for their thickness, length and breadth. For the determination of weight, five membranes from each formulation were selected and weighed individually using a digital balance. The mean weight of the membranes was noted.[12]

Determination of pH

The membranes were allowed to swell in a closed Petri dish at room temperature for 1h in phosphate-buffered saline of pH 7.4. The pH was noted after bringing the electrode of the pH meter in contact with the surface of the formulation and allowing them to equilibrate for 1 min. The average of five determinations for each of the formulation was taken.[13]

Determination of folding endurance

The folding endurance is expressed as the number of folds (number of times the membrane is folded) at the same place either to break the specimen or to develop visible cracks as the test is important to check the ability of the sample to withstand folding. This also gives an indication of brittleness. The specimen was folded in the center, between the fingers and the thumb, and then opened. This was termed as one folding. The process was repeated till the insert showed breakage or cracks in the center of the insert. The total folding operations were termed as folding endurance value.[14]

Determination of tensile strength

This mechanical property was evaluated using an Instron universal testing instrument (Model 1121, Instron Ltd., Japan) with a 5 kg load cell. Hydrogel membranes in special dimension and free from air bubbles or physical imperfections were held between two clamps positioned at a distance of 3cm. During measurement, the strips were pulled by the top clamp at a rate of 100 mm/min; the force and elongation were measured when the film broke. Results from film samples, which broke at and not between clamps, were not included in the calculations. Measurements were run in triplicate for each membrane. Two mechanical properties, namely tensile strength and % elongation, were computed for the evaluation of the membrane. Tensile strength is the maximum stress applied to a point at which the film specimen breaks and can be computed from the applied load at rupture as a mean of three measurements and cross-sectional area of fractured membrane as described from the following equation:[15]

Tensile strength = Force at break (N)/Initial cross-sectional area of the sample (mm^2)

Percentage elongation can be obtained by the following equation:

% Elongation at break = (Increase in length/Original length) ×10

Determination of the swelling index

After measuring the initial weight of the membrane, the membrane was directly immersed in 20 mL isotonic phosphate buffer pH 7.4 at room temperature. The excess

surface water was removed with the aid of a filter paper and the weight of the swollen samples was measured at various time intervals.[16] The procedure was repeated thrice. The swelling index was determined by the following formula:

Swelling index = (We-Wd)/Wd × 100
We = weight of membrane after hydration
Wd = weight of dry membrane.

Determination of degree of cross-linking
The degree of cross-linking of a polymer is the ratio of the mass of the cross-linked state to the whole mass of the individual monomer. In order to fabricate a device, an aluminum cylinder of height of 30-50 mm was chosen. Five to six holes were drilled into the base of the metallic cylinder. The cross-linked polymer was weighed and placed inside the container. The mouth of the container was closed with an aluminum foil and holes were drilled similarly. The container, after weighing, was then immersed into a solvent responsible for solubilization of the monomer under suitable conditions. After 1 h, the container was dried at 40°C in oven for 4 h to allow the material to dry and then the container was reweighed.[17] The procedure was repeated three times and the degree of cross-linking was determined by the following formula:

C= (mP - mC)*100/(mS - mC) ×100
C = degree of cross-linking of hydrogel
mP = mass of the container after the whole process
mC = mass of the dry container
mS = mass of the container with cross-linked polymer.

Surface morphology by scanning electron microscopy
To study the surface topography of the hydrogel membrane before and after hydration, SEM photographs were taken with a JEOL, JSM5610-LV scanning microscope, Japan. Samples were coated with gold for 60s under argon atmosphere using sputter coater in a high-vacuum evaporator. Images were taken at an acceleration voltage of 15 kV and magnification of 33 to 200.

FTIR studies
To investigate and predict any physicochemical interactions between components in the formulation and to confirm the cross-linking of polymers, an FTIR study was conducted.

Differential scanning calorimetry
The DSC study was used to study the melting and crystalline behaviors of the polymeric membrane. The temperature and energy scales were calibrated with standard procedures. The study was performed in the temperature range of 30 to 350°C at a heating range of 10°C/min in an N_2 atmosphere.[15]

X-ray diffraction studies
XRD patterns were obtained with an X-ray diffractometer using Cu α-radiation generated at 40 kV and 35 mA; the range of diffraction angle was 3.00 to 80.00° 2θ.[16]

Mucoadhesion studies
The working of a double-beam physical balance formed the basis of the bioadhesion test assembly. The right pan was removed and hung with a stainless steel chain. A Teflon block with 1.5 inches height × 1.5 inches diameter was hung with the stainless steel chain to balance the weight of the other pan. The height of the total set up was adjusted to accommodate a glass container or beaker below it, leaving a headspace of about 0.5 cm in between. Another Teflon block of 2 inches height and 1.5 inches diameter was kept inside the glass container, which was then placed below the top hung Teflon block. Suitable weights were added (15.0 g) on the left pan to balance the beam of the balance. The conjunctival membrane of a goat was attached with the mucosal side upward over the lower Teflon block, which was then placed into the glass container, which was then filled with simulated tear fluid, such that the tear fluid just touches the surface of the mucosal membrane to keep it moist. This was then kept below the upper Teflon block. The hydrogel membrane under test was fixed to the surface of the upper block with glue. The 15.0 g weight on the right pan was removed and this lowered the upper Teflon block along with the membrane, so that it was in contact with the mucosal surface. A load of 20.0 g was placed as initial pressure on the upper block for 3 min and then slowly weights were added on the left pan starting from 100 mg till the patch separated from the mucosal surface. The excess weight on the pan (i.e. the total weights minus 15.0 g) required to separate the hydrogel from the mucosa was noted and the bioadhesion force was calculated per unit area of the membrane as follows:

F = (Ww × g)/A

Where F is the bioadhesion force (kg/m/s^2), Ww is the mass applied (g), g is the acceleration due to gravity (cm/s^2) and A is the surface area of the patch (cm^2).[17]

Drug loading
The drug content and uniformity of drug content were determined by assaying individual membranes of size 2 cm × 2 cm^2 or 4 cm^2. Each membrane was grounded in a glass mortar and pestle after cutting it into small pieces, stirred in 5 mL of phosphate-buffered saline of pH 7.4 and kept for 5 h to extract the entire drug present. The solution was the filtered through a Whatmann filter paper No. 1 and 1 mL of solution was transferred into a 10 mL volumetric flask, and the volume was made up with isotonic phosphate buffer of pH 7.4 and analyzed using a UV spectrophotometer.[18]

***In vitro* drug release**
As dissolution apparatus, vials in a modified oscillating water bath were employed to evaluate the release of drug from the hydrogel membranes. A hydrogel membrane (2 cm × 2 cm^2, containing 0.08 mg of Timolol maleate (TM)

and 0.04 mg of Brimonidine tartrate (BT) was transferred into a vial containing 5 mL of phosphate-buffered saline of pH 7.4. To avoid evaporation, the vials were covered with rubber caps and placed on a mechanical shaker that was attached to a water bath, which was maintained at a temperature of 32 ± 1°C. Aliquots of 3 mL were withdrawn throughout the experiment at 30, 45, 60, 90, 120, 150,180, 240, 300 and 360 min and replaced by an equal volume of fresh buffer solution. It was filtered and diluted if necessary and analyzed using a UV spectrophotometer.[19]

Release kinetics

The release kinetics was evaluated considering four different models including zero order, first order, Higuchi's equation and Korsmeyer's equation, and the selection was based on the comparisons of the relevant correlation coefficients and linearity test.[20-22]

Test for sterility

The test for sterility was conducted on formulations as per the Indian Pharmacopoeia (IP) by following the direct inoculation method. At intervals during the incubation period and at its conclusion, the media were examined for macroscopic evidence of microbial growth. If no evidence of growth was found, the preparation passed the test for sterility.[19,23]

Ocular irritation studies

An ocular irritation study was performed on 12 New Zealand white Albino rabbits weighing 2-3 kg. Animals were housed in standard cages in a number of two per cage. They were fed with suitable diet and water as much as required. A dark and light cycle of 12 h was maintained. The temperature and humidity were maintained at 28 ± 2°C and 60 ± 15°C, respectively. Of 10 formulations, the best ones were chosen for the study. The formulation was applied into the cul-de-sac region once a day for a period of 7 days and the rabbits were monitored periodically for irritation, inflammation, etc., by naked eye or by means of a pen torch. The test may be considered positive if there are one or more positive reactions at any observation period. One eye was used as the test and the other eye was used as the control. Rabbits were grouped into three (4 + 4 + 4) groups. For the first group containing four rabbits, formulation F1 were applied to one eye and the other eye was kept as the control (to which nothing was applied). For the second group containing four rabbits, formulation F8 was applied to one eye and the other eye was kept as the control. For the third group containing four rabbits, marketed hydrogel was instilled to one eye and the other eye was kept as the control. During the time of the examination period, each rabbit was scored for ocular reaction.[19,23]

***In vivo* IOP-lowering activity**

Glaucoma was induced in rabbits by instilling prednisolone eye drops (1% w/v) up to 3-4 weeks. The study was performed on 16 New Zealand white Albino rabbits weighing 2-3 kg divided into four groups. The animals were procured from K.S. Hegde Medical Academy. The IAEC Certificate number is KSHEMA/AEC/25/2010. The first group received TL, the second group received CB, the third group received F1and the fourth group received F8 in the right eye and the other eye was untreated. IOP was measured using a Schiötz tonometer after instilling a drop of procaine hydrochloride local anesthetic (1% w/v). The left eye was used as the control and treatment was carried out on the right eye. All the formulations were instilled into the lower conjunctival sac. At regular intervals, the IOP was measured.[24] Change in IOP was expressed as follows:

Δ IOP = IOP untreated eye- IOP treated eye

Results are reported as mean (±S.E.). ANOVA - One-way statistical test was used to identify statistical significance at $P < 0.05$.

Stability studies

The membranes were wrapped in aluminum foil and placed in Petri dishes. These Petri dishes were stored at ambient humidity conditions at refrigerated temperature (2-8°C), room temperature (27 ± 2°C) and oven temperature (45 ± 2°C) for a period of 60 days. The formulations were evaluated for changes in drug content, pH and maximum *in vitro* drug release.[23,24]

RESULTS AND DISCUSSION

The present investigation on hydrogel membrane as an ocular delivery system is largely based on the delivery of drugs through the cross-linked polymers for the purpose of sustained release of drugs; thereby, the frequent administration and efficiency of drugs can be improved.

Hydrogel membranes are polymeric materials. At physiological conditions, they do not dissolve in water. However, they swell considerably in aqueous medium and exhibit extraordinary capacity (>20%) to imbibe water into the network structure.[25] Gels that exhibit a phase transition as a response to change in external conditions like pH, ionic strength, temperature and electric currents are known as "stimuli-responsive" or "smart" gels.[26] Being insoluble, these three-dimensional hydrophilic networks can retain a large amount of water that contributes to their good blood compatibility and maintains a good degree of structural integrity and elasticity.[27] This phenomenon may be attributed to the presence of hydrophilic functional groups in their structure, like -OH, -COOH, $-CONH_2$ and $-SO_3H$, capable of absorbing water without undergoing dissolution. Suitability of the present study was an attempt to investigate the hydrogel membranes in ocular delivery as they offer a better delivery system than the conventional methods.

They can be prepared from natural and synthetic polymer materials and classified using various criteria depending on their preparation method and physicochemical properties.[28] Natural polymers, such as proteins,[29] polysaccharides[30] and deoxyribonucleic acids (DNAs), are cross-linked by either physical or chemical bonds and synthetic hydrogels can be easily prepared by cross-linking polymerization of synthetic monomers.[31] To obtain different properties in the same hydrogel, natural polymers can be combined with synthetic polymers.[32] A great variety of chemical and physical methods can be followed for cross-linking of the polymers and thereby hydrogels.[33] Covalent bonds are present between different polymer chains in chemically cross-linked gels, whereas in physically cross-linked gels, dissolution is prevented by physical interactions that exist between different polymer chains. The network structure of a hydrogel will determine its properties as a drug delivery device. Hence, in the present study, a synthetic polymer like PVA was used along with two other natural polymers like chitosan and gelatin. PVA has excellent film-forming and adhesive properties, apart from excellent transparency, and is biocompatible. Gelatin is a natural protein and is biodegradable, whereas chitosan (200-800 cp with 190-300 kDa) is a natural polysaccharide obtained from chitin and is biocompatible.[34] It has been used extensively for ocular delivery.[35,36]

The physicochemical properties of the hydrogel membranes were investigated before being put into its *in vitro* and *in vivo* studies. The thickness of the membranes ranged from 0.4 to 0.59 mm or 400 to 590 nm, which is ideal for the membranes intended for ocular delivery (US Patent), and the pH was found to be in the range of 7.21-7.42, which indicated the compatibility of the membranes with the ocular system. All the membranes had good folding endurance and weighed around 3-4 mg.

Drug - polymer interaction by FTIR

The presence of any drug: Excipient interactions in the formulation were studied by performing the FTIR of the mixture of drug and other excipients. The FTIR peaks of the drug: Polymer mixtures were compared with the principal peaks of the drug in the literature to observe any changes. The FTIR peaks of TM, BT and the mixture of the two drugs with chitosan, PVA and gelatin were investigated. The principal peaks of TM and BT obtained from the literature were matched with the pure drug and the drug: Polymer mixture in order to rule out any interaction of the drug with the polymer mixture. The principal peaks for TM as per the literature were at 1497 cm^{-1}, 1527 cm^{-1}, 1120 cm^{-1}, 1230 cm^{-1}, 1590 $cm^{-1}$and 1620 cm^{-1}. The principal peaks for BT as per the literature were 3473 cm^{-1}, 3438 cm^{-1}, 1300 cm^{-1}, 2362 cm^{-1}, 2341 $cm^{-1}$and 1718 cm^{-1}. The characteristic peaks of TM and BT were approximately matched with the drug: Polymer mixture and hence it was concluded that there was no interaction between the drug and the polymers used in the formulation of the hydrogel membranes [Figure 1a-c].

SEM

The blend membrane was clear to the eye and neither showed separation into two layers nor any precipitation. The drug-loaded films were yellowish in color due to BT. The swollen hydrogel membranes showed the presence of pores. These pores neither fixed in size nor localized in any definite location. As a result of water uptake, the macromolecular segments exhibit enhanced mobility so that the size, shape and location of the pores continuously change. From the

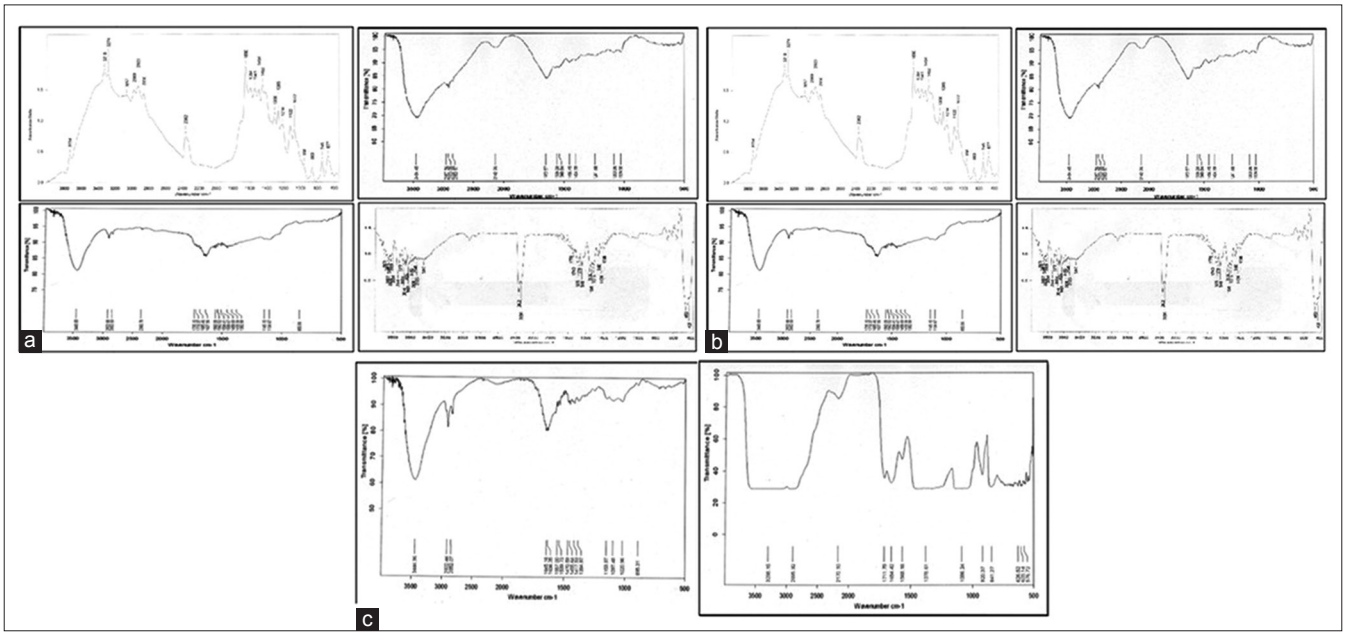

Figure 1: (a) FTIR spectra of TM, BT, chitosan with TM and BT and PVA with TM and BT. (b) FTIR spectra of gelatin, PVA with TM and BT, gelatin PVA and cross-linked gelatin-PVA hydrogel membrane. (c) FTIR spectra of chitosan and cross-linked PVA membrane

SEM images of cross-linked PVA hydrogel membranes, it can be interpreted that the membranes were homogenous and uniform. They developed pores on hydration. These pores were responsible for the rapid uptake of water and swelling of the hydrogel membranes. The hydrogel membranes showed fine crystals on the surface, which may be due to excess amount of gelatin that was unable to form cross-links. On hydration, the membranes showed interconnection between the swollen polymeric chains; hence, it can be interpreted that there exists cross-linking between the two polymeric chains [Figure 2].

Polymer - polymer interaction (cross-linking) by XRD and DSC studies

The XRD of gelatin showed no peak, indicating that the sample lacks crystallinity. The XRD pattern of PVA [Figure 3b] indicated three peaks at 2θ=19.238, 2θ=22.57 and 2θ=40.35 having intensities of 4700, 1300 and 600, respectively. The hydrogel membranes of cross-linked gelatin with PVA [Figure 3c] showed peaks at 2θ=19.16, 2θ=26.89 and 2θ=53.7, indicating that the crystallinity of the membrane was mainly due to interaction between gelatin and PVA. The diffraction for chitosan sample showed peaks at 2θ=5.74, 2θ=10.1, 2θ=19.65 and 2θ=21.81 having intensities 600, 2100 and1100, respectively. The hydrogel membranes showed peaks at 2θ=5.574, as was present in the XRD of chitosan, and at 2θ =26.905, having intensities of 190 and 300, respectively. However, the non-appearance of peaks of PVA at 2θ=19.23 and 2θ=22.5 indicated an interaction between chitosan and PVA [Figure 3d]. The XRD pattern of the cross-linked PVA membranes indicated peaks at 2θ=19.16 and 2θ=26.8, having intensities of 300. The decrease in the intensities indicated intermolecular hydrogen bond.

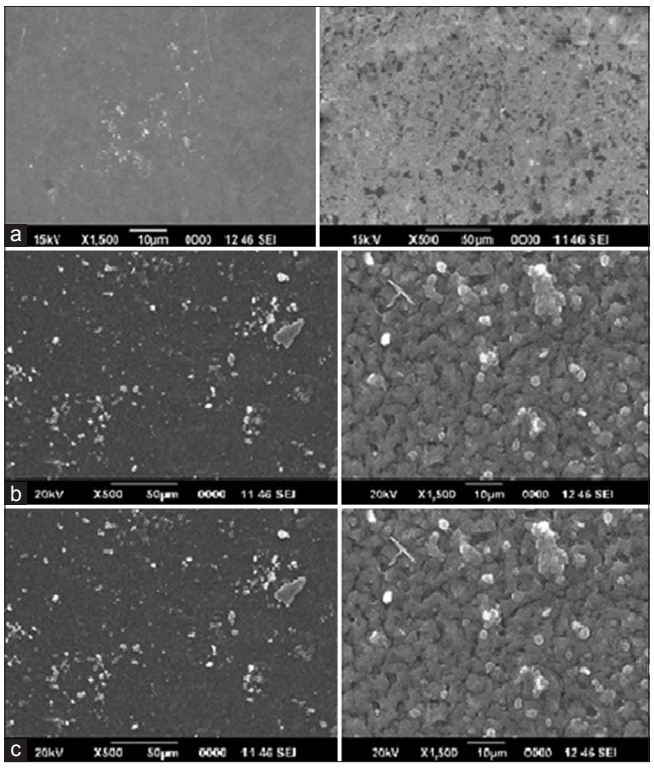

Figure 2: Scanning electron microscopy: (a) CP before hydration and CP after hydration, (b) PP before hydration and PP after hydration and (c) GP before hydration and GP after hydration

DSC studies carried out on the cross-linked hydrogel membranes of Chitosan-PVA (CP) [Figure 5] indicated a shift in the peaks and also formation of new peaks due to the interaction between polymers. The peaks obtained indicated glass transition temperature at 48.58°C and melting endotherms at 134.93°C, 203°C and 243°C. Gelatin showed a glass transition temperature at 104.39°C and a small peak at 228.67°C. It showed a melting endotherm at 289.75°C. The cross-linked hydrogel membrane showed glass transition temperature at 142°C and melting endotherms at 215°C and 284°C. These peaks were not seen in the DSC thermogram of pure gelatin and pure PVA, which indicated existence of cross-linking among the two polymers. The DSC of PVA showed a glass transition temperature at 49.83°C and a melting endotherm at 221.3°C. The DSC thermogram of cross-linked PVA showed the presence of endotherm peaks at 219°C and 320 -- 340°C, indicating the existence of some interaction between the polymeric chains after freeze-thawing [Figures 3 and 5].

Tensile strength and percentage elongation

The tensile strength gives an indication of the strength and elasticity of the film reflected by the parameters tensile strength (TS) and elongation at break (E/B). A weak and soft polymer is characterized by a low TS and E/B; a hard and brittle polymer shows a moderate TS and low E/B; a soft and tough polymer shows a high TS and E/B. The PVA membranes showed high percentage elongation but a very poor tensile strength. Among the Gelatin- PVA (GP) formulations, F1 showed maximum tensile strength and hence was least elongated. Among the PVA-PVA (PP) formulations, when comparing the cross-linked membranes with the PVA membranes of the same ratio, it can be inferred that cross-linking causes an increase in the tensile strength, which may be due to the crystalline regions formed on cross-linking. F7 showed the maximum tensile strength. Among the CP formulations, as the concentration of chitosan increased, the tensile strength decreased, except in the case of F9, which may be due to the presence of equal quantity of PVA, which may cause considerable increase in percentage elongation as well.

Degree of cross-linking

Among the formulations of GP, F3 showed minimum cross-linking and hence maximum swelling capacity was observed. The degree of cross-linking was found to be inversely proportional to the swelling ratio. This may be due to the high PVA content in F3. Among the formulations of PP, we saw that as the freeze-thaw cycle increased, the degree of cross-linking increased, which may be due to the fact that initially only few PVA chains participated in the crystalline formation process and increasing the freeze-thaw cycles

led to further crystal formation and therefore increased physical cross-linking between the PVA chains. Among the formulations of CP, F8 showed the maximum degree of cross-linking. As the content of PVA increased, the degree of cross-linking was found to be decreased and the swelling ratio increased [Table 2].

Swelling studies

Formulation F3 showed maximum swelling compared with formulations F1 and F2 due to the high percentage of both PVA and gelatin. As the amount of PVA in the gel decreased, the swelling ratio was also found to be decreased. Among formulations of PP, F4 and F5 showed maximum swelling in the first 1 h and then reached equilibrium. The decrease in the swelling ratio at the end of 5 h may be likely due to chain dissolution and changes in the crystalline structure that inhibit the gel from maintaining a gel structure. F6 and F7 showed less swelling ratio when compared with F4 and F5 because of higher degree of cross-linking among the polymeric chains. Both F6 and F7 attained equilibrium swelling at the end of 2 h. Among the formulations of CP, the swelling ratio of F9 was found to be more than F8 and F10 and all three membranes were found to be stable after 24 h.

Table 2: Degree of cross-linking

Formulation code	Degree of cross-linking %
GP	
F1	68.22±0.355
F2	50.69±1.031
F3	40.84±0.3554
PP	
F4	45.69±0.827
F5	41.11±0.593
F6	69.21±0.394
CP	
F7	68.88±1.236
F8	62.73±0.570
F9	51.78±0.634
F10	58.99±0.935

Average of three readings, Data are presented as mean±SD (n=2). GP: Gelatin-PVA cross-linked membrane, PP: PVA-PVA cross-linked membrane, CP: Chitosen-PVA cross-linked membrane

As chitosan is insoluble in alkali and PVA is a water-soluble polymer, due to the hydrogen bonding that occurs between the functional groups of chitosan and PVA, the physically cross-linked composite material is insoluble in a non-acidic aqueous solution. But, PVA being more hydrophilic, the swelling ratio increased with an increase in the weight percentage of PVA.

It may be observed that as the degree of cross-linking of polymers increases, swelling proposition of the membranes decreases. Highly cross-linked hydrogels have a tighter structure and will swell less compared with the same hydrogels with lower cross-linking ratios. Cross-linking hinders the mobility of the polymer chain hence lowering the swelling ratio[25] [Figure 4].

Bioadhesive force

Bioadhesive force (kg/m/s^2) for different formulations was shown to be in the normal range. It was found that among the formulations of GP, F3 showed the maximum bioadhesive force because of a high percentage of both gelatin and PVA. Among the PP formulations, it was found that cross-linked PVA subjected to one freeze-thaw cycle has greater bioadhesive force than the samples subjected to two cycles. In the CP series, formulation F9 showed maximum bioadhesion as it contains chitosan and PVA in a higher percentage. As the amount of chitosan decreased, the bioadhesive force also decreased [Figure 6].

Drug content

The percentage drug content of all the formulations was found to be in the range of 82.78-95.62%. Because the drugs were hydrophilic in nature and all the polymeric solutions were aqueous in nature, the drug was uniformly distributed in the membrane.

***In vitro* release studies**

The release of drug from the hydrogel membrane depends on the type of polymer used, its degree of cross-linking and the percentage swelling ratio. Among the GP formulations, F1 was found to better sustain the release of the drugs

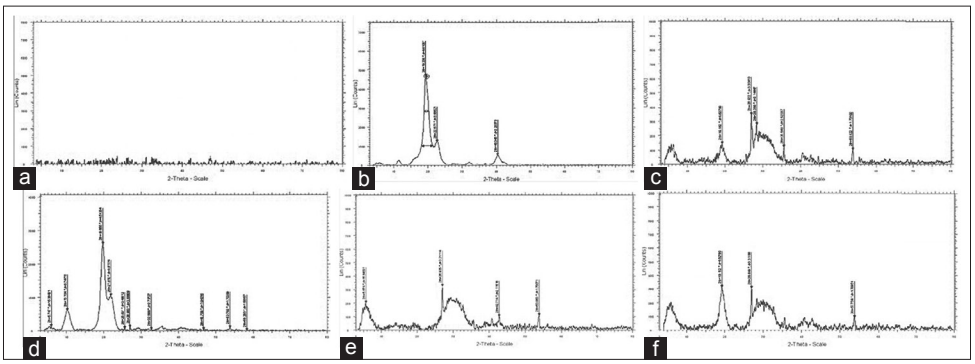

Figure 3: X-ray diffraction (XRD) studies: (a) XRD spectra of gelatin, (b) XRD spectra of PVA, (c) XRD spectra of cross-linked gelatin-PVA hydrogel membrane, (d) XRD spectra of chitosan, (e) XRD spectra of CP and (f) XRD spectra of PP

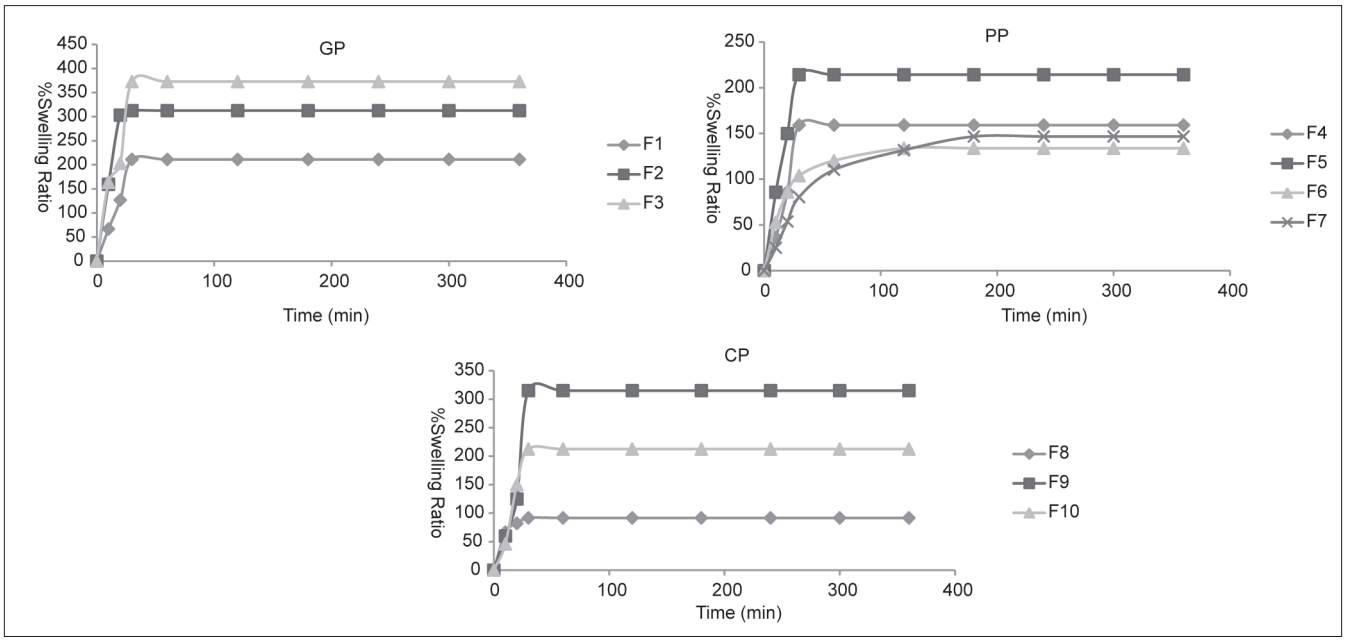

Figure 4: Percentage swelling ratio of GP, PP and CP

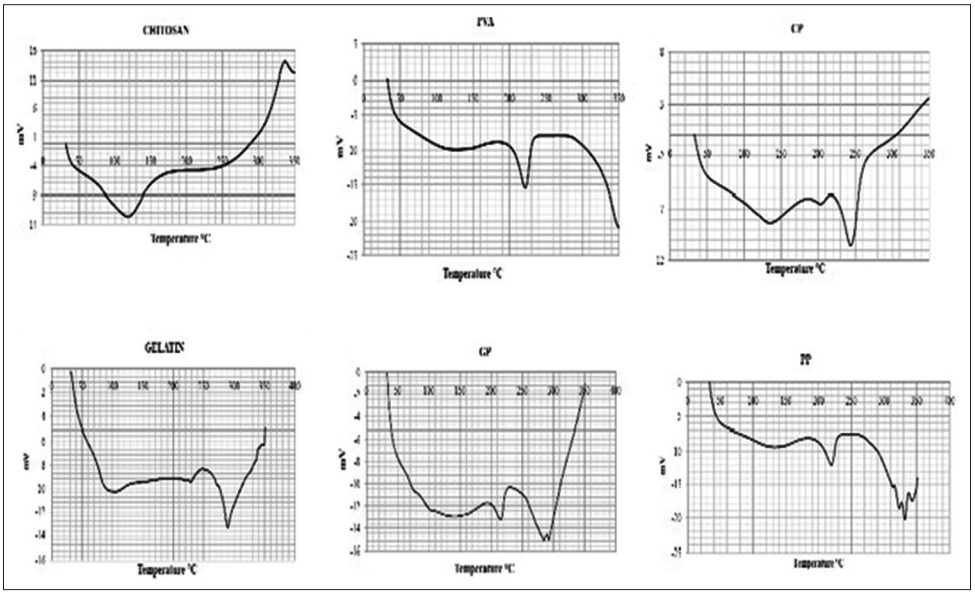

Figure 5: Differential scanning calorimetry interpretation

due to its non-porous nature and a mesh-like structure as confirmed by SEM and hence the drug was released by diffusion through the mesh network. The swelling index was found to be around 211.1% at the end of 6 h and the degree of cross-linking was around 68.22%. Percentage cumulative drug release was found to be 32.20% and 37.35% for TM and BT, respectively, at the end 6 h. F3 showed a cross-linking of 40.84% and a swelling ratio of 372.72%. The drug release was found to contain 52.66% and 58.01% of TM and BT, respectively. This may be attributed to the hydrophilic nature of the drug. Among the PP formulations, there was not much of a difference in the degree of cross-linking of F6 and F7 -- 69.21 and 68.88, respectively. The percentage swelling ratio of F6 and F7 was 133.84 and 146.66%; hence, the release of the drug did not vary much among F6 and F7. Among CP, F8 showed the maximum degree of cross-linking and a percentage swelling ratio of 91.66%. F9 showed a high % swelling ratio of 315% and low cross-linking of 51.78%; hence, the % drug release was found to be 58.91% and 62.17%, respectively. There was not much variation in the % drug release of F8 and F7; however, F8 was chosen over F7 due to its low % swelling capacity and hence may be better patient compliant. F1 and F8 are considered to be the best formulations based on the above

factors. From the above, it was concluded that as the degree of cross-linking increases, the % swelling ratio decreases; however, as the % swelling ratio increases, the % release of the drug also increases. This may be attributed to the hydrophilic nature of the drug. F1 showed percentage cumulative drug release of 32.20% and 37.35% of both TM and BT, respectively, whereas F8 released 39.6% and 31.54% of TM and BT, respectively.

Release kinetics

In the present study, four different mathematical equations were employed to model the dissolution profile, i.e. (a) First order equation, (b) zero order (c) Higuchi square root of time equation and (d) Korsmeyer-Peppas diffusion models to determine the mechanism of release. Depending on the strength of the gel layer network (external phase) formed, drug release can be controlled by different mechanisms with different kinetics. Erosion of the swollen polymer represents the release mechanism and generally leads to a zero order release kinetics as indicated in the results with a correlation coefficient of 0.923-0.992 for all the formulations. Release of the drug from a matrix system containing hydrophilic polymers generally involves factors of diffusion. Diffusion is related to transport of drug from the dosage form into the *in vitro* study fluid depending on the concentration. As gradient varies, the drug release varies because the distance for diffusion increases. This could explain why the drug diffuses at a comparatively slower rate as the distance for diffusion increases, which is referred to as square-root kinetics or Higuchi's kinetics. In our experiments, the *in vitro* release profiles of drug from all the formulations could be best expressed by Higuchi's equation as the plots showed linearity in the range of 0.887-0.995. Drug is released from the swollen polymeric network principally through a diffusion-controlled mechanism, described by the well-known Fick's law. Often, both diffusion and erosion contribute to the release of the drug. This transition, between the two limit mechanisms, results in kinetics between square root of time dependence and zero order, generally described as "anomalous transport" used when contribution of both diffusion and relaxation happens. For all the analyzed hydrogel membranes, the values of diffusional exponent "*n*" obtained from the slopes of the fitted Korsemeyer-Peppas model, with "*n*" value found between 0.463 and1.032, suggest that the combination of passive diffusion (Fickian diffusion) and erosion was the drug release controlling mechanism. This means that the release rates of TM and BT are not connected to polymer chain relaxation but the drug is released by diffusion through the polymer chains that form the firm gel structure. This appears to indicate a coupling of diffusion and erosion mechanisms, the so-called anomalous diffusion. The relative complexity of this formulation and its components may indicate that the drug release is controlled by more than one process. Hence, diffusion coupled with erosion may be the mechanism for the drug release from the hydrogel formulation[20-22] [Figures 7 and 8].

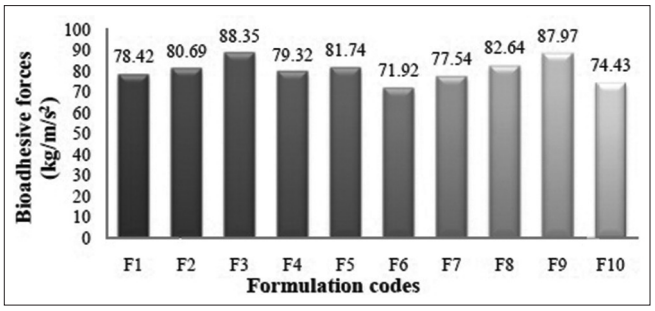

Figure 6: Mucoadhesion studies

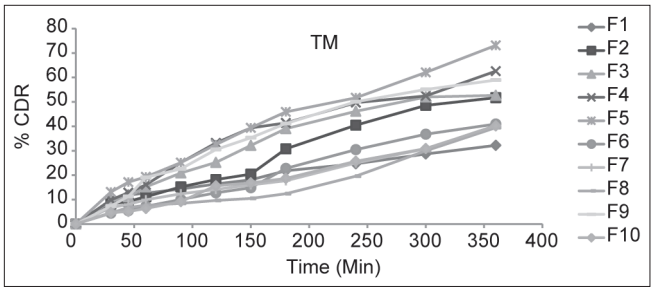

Figure 7: Drug release profile of timolol maleate

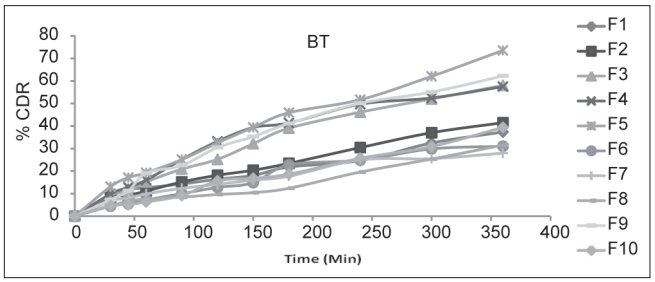

Figure 8: Drug release profile of brimonidine tartarate

Test for sterility

The results of the sterility when compared with the positive and negative controls showed that the medium used was sterile and provided necessary nutrients for the microorganism. Further, it could also be interpreted that the presence of drugs did not show any antimicrobial or antifungal activity in the given test. After the examination of tests for sterility, there was no macroscopic evidence of microbial growth. Hence, the formulations were found to be sterile.[37]

Ocular irritation study

The results of the ocular irritation studies indicated that all formulations are non-irritant to the eye. Excellent ocular tolerance was noted. No ocular damage or abnormal signs to the cornea, iris and conjunctiva were visible.

***IOP*-lowering activity**

In the present study, 0.5 cm × 0.4 cm or 0.2 cm^2 membranes were used so that it contains 0.004 mg of TM and 0.002 mg of BT, which is equivalent to the dose of the marketed eye drops when instilled into the eye. The marketed product

contains 0.5% of TM and 0.25% of BT, which is equivalent to 0.05 mg of TM in 1mL and 0.025 mg of BT in 1mL. When one drop (0.06mL) is instilled into the eye, the dose of the drugs becomes equivalent to 0.003 mg of TM and 0.0016 mg of BT. Hence, the present study requires a membrane of the size 0.2 cm^2 so that the dose of the drugs becomes equivalent to the marketed preparation during one-time instillation into the eye.

The physiological effectiveness of the formulations was determined in terms of their IOP-lowering effect in glaucoma-induced rabbits. There was a drop in IOP in rabbits as a function of time after administration of formulations containing TM as the single drug entity and also in combination with the eye drop. No change in the IOP was observed in the untreated eye during the course of measurement in any of the formulations. This clearly indicated that all the formulations exerted a local action within the eye and that the activity shown is not due to any systemic absorption followed by subsequent redistribution. The marketed formulation of Combigan containing TM (0.5%) and BT (0.25%) and Timolet GFS containing TM 0.5% were used as control. The formulations F1 and F8 showed significant differences when compared with the marketed formulations. CB decreased IOP by 5 mmHg at the end of 30 min whereas the TL decreased the IOP by3 mmHg at the end of 30 min. F1 and F8 decreased IOP by 2 mmHg, and the values were not statistically significant. This may be due to the time lag before the swelling of the membrane takes place. The change in IOP between F1 and F8 becomes statistically significant at the end of 2 h. This may be due to the swelling of the hydrogel. CB showed a decrease in IOP up to 13 mmHg at the end of 4 h, but then there was an increase in the IOP, which may be due to the elimination of the drug from the site of action. Hence, it was unable to sustain the activity for a long period of time, which calls for frequent administration of the formulation. TL decreased the IOP by 10 mmHg at the end of 6 h. However, F1 and F8 decreased IOP by 13 mmHg and 12 mmHg, respectively. The decrease in IOP was greater in the hydrogel when compared with TL because of the presence of two drug candidates. Hence, the IOP-lowering activity of the hydrogel formulation was better compared with the marketed formulations. All values are negative, indicating that IOP returns to normal. The baseline IOP did not show any significant change during the course of the study, indicating the absence of systemic side-effects. All values for all formulations are statistically significant ($P < 0.05$) except F1and F8 [Figure 9].

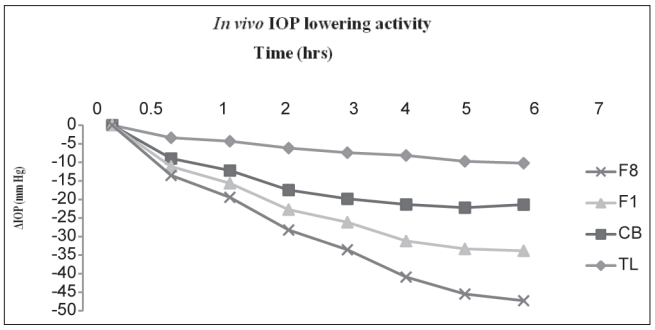

Figure 9: IOP measurement and effect of formulation on IOP

Stability study

The prepared formulations were subjected to stability studies as described under "methodology" and were checked for any change in the physical appearance, pH, drug content and *in vitro* release studies. All the formulations showed good stability at 25-30°C/60% RH. There was no significant change in the physical appearance, pH, drug content and *in vitro* release. The drug content did not deviate by more than 3%, indicating that the drug is stable in the hydrogel formulations and also that there was no significant change in the *in vitro* release profile at the end of 60 days.

CONCLUSION

Hydrogel membranes offer a promising avenue to fulfill the need for an ophthalmic drug delivery system that can localize and maintain drug activity at the site of action for a longer period of time thus allowing a sustained action, minimizing frequency of drug administration with patient compliance. The hydrogel membranes containing a combination of TM and BT were found to be promising ocular delivery systems for the treatment of glaucoma. These findings with further extensive research and application of a certain concept of a novel drug delivery system may help the industry to scale up for commercial production.

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