Original Research Article

Investigation of hydrogel membranes containing combination of gentamicin and dexamethasone for ocular delivery

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Abstract

Background: Hydrogel is a cross-linked network of polymers. Water penetrates these network causing swelling and giving the hydrogel a soft and rubbery consistency and there by maintaining the integrity of the membrane. Due to the drawback of conventional therapy for ocular delivery, hydrogel membranes containing the combination of gentamicin (GT) sulfate and dexamethasone (DX) were formulated for the treatment of conjunctivitis. The objective of this study was to formulate and evaluate the hydrogel membranes containing the combination of GT and DX for the treatment of conjunctivitis. **Materials and Methods:** In the present investigation, hydrogel membranes were prepared by using polymers such as gelatin, polyvinyl alcohol, and chitosan, which were cross-linked using physical/chemical methods. **Results:** The cross-linking of the membranes was confirmed by Fourier transform infra-red studies. The pH of the membranes ranged from 7.19 to 7.45 and drug content ranged from 69.82% to 89.19%. The hydrogels showed a considerably good swelling ratio ranging from 22.5% to 365.56%. The *in vitro* drug release study showed that there was a slow and sustained release of the drug from the membranes which were sufficiently cross-linked and followed zero order release. *In vivo* studies showed that the severity of conjunctivitis was remarkably lowered at day 3 with hydrogel membrane compared to marketed eye drops. Results of unpaired *t*-test of significance between two groups indicated that the hydrogel membrane showed a better response in the treatment of conjunctivitis compared to the marketed products. Stability studies proved that the formulations could be stable when stored at room temperature. **Conclusion:** Results of the study indicated that it is possible to develop a safe and physiologically effective hydrogels which are patient compliant.

Key words: Conjunctivitis, cross-linking, Fourier transform infra-red, hydrogels, polyvinyl alcohol

INTRODUCTION

Ocular drug delivery has been a major challenge to pharmacologists and drug delivery scientists due to its unique anatomy and physiology. Static barriers (different layers of cornea, sclera, and retina including blood-aqueous and blood-retinal barriers), dynamic barriers (choroidal and conjunctival blood flow, lymphatic clearance, and tear dilution) and efflux pumps in conjunction pose a significant challenge for delivery of a drug alone or in a dosage form, especially to the posterior segment. Identification of influx transporters in various ocular tissues and designing a transporter-targeted delivery of a parent drug has gathered momentum in recent years. Novel drug delivery strategies such as bio adhesive gels and fibrin sealant-based approaches were developed to sustain drug levels at the target site. Designing noninvasive sustained drug delivery systems and exploring the feasibility of topical application to deliver drugs to the posterior segment may drastically improve drug delivery in the years to come.[1]

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A number of ocular inserts were prepared to utilize different techniques to make soluble, erodible, nonerodible, and hydrogel inserts. Ocular inserts can be the valuable technique for the treatment of glaucoma.[2]

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. They are endowed with the ability to swell in water or aqueous solvents, their highly porous structure can easily be tuned by controlling the density of crosslinks in the gel matrix and the affinity of the hydrogels for the aqueous environment in which they are swollen. Their porosity also permits the loading of drugs into the gel matrix and the subsequent drug release at a rate dependent on the diffusion coefficient of the small molecule or macromolecule through the gel network.[3-5]

Ocuserts containing fluconazole β-cyclodextrin complex has been evaluated to treat eye infections and found that the drug delivery system retained the drug concentration at the intended site of action for sufficient period of time and elicited desired pharmacological activity.[6]

In situ forming gel of pefloxacin mesylate was investigated against *Pseudomonas* induced conjunctivitis in rabbits and compared its efficacy with marketed eye drops. It was found that *in situ* forming gel of pefloxacin mesylate remain at the site of absorption over an extended period of time and resulted in a large increase in bioavailability.[7]

Gentamicin (GT) inhibits bacterial protein synthesis mainly through binding with the 30S ribosomal subunit and acts through two different mechanisms. In one mechanism, GT can interfere with the correct amino acid polymerization and elongation. This mechanism takes place at high concentrations. Another mechanism predominates at low concentrations in which the amino acid codons are misread by tRNA and proof reading is impaired. This leads to incorrect amino acid sequencing and nonsense proteins. Dexamethasone (DX) is a glucocorticoid class of steroid drug. It acts by binding with the high affinity to specific cytoplasmic glucocorticoid receptors. This complex binds to DNA elements which results in a modification of transcription and hence protein synthesis in order to achieve the inhibition of leukocyte infiltration at the site of inflammation, interference in the function of mediators of inflammatory response, suppression of humoral immune responses, and reduction in inflammation. The combination of GT and DX is available in the market as eye drops and ointment (GENTOP-D and GENTAGEN-D).[8,9]

With all the above aspects in mind, the present work was aimed at investigating the potential of hydrogel membranes containing combination of GT and DX as ocular drug delivery systems for the treatment of conjunctivitis so as to increase the contact time of the drug with the eye, reduce systemic side effects, reduce the number of application, and better patient compliance. The device is non-biodegradable to prevent the disintegration of the membrane and leakage of the drug. This is based on a 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

GT was obtained from Mediwin Ltd., Ahmadabad, India, as gift sample. DX was obtained from Matish healthcare Ltd., Indore, India, as gift sample. Chitosan was obtained from CIDF, Cochin. Gelatin was obtained from Thomas baker, Mumbai. Propylene glycol was obtained from Loba Chemie Pvt Ltd., Mumbai. Benzalkonium chloride was obtained from Merck India Ltd. Fluid thioglycolate medium and Soybean casein digest were provided by Hi Media Ltd., Mumbai. All other solvents and reagents used in the study were of analytical grade.

Preparation of hydrogel membranes

The hydrogel membranes were prepared by a solvent casting method, after cross linking the polymers.

Crosslinking of polymers

Preparation of cross-linked polyvinyl alcohol membranes

An aqueous solution of polyvinyl alcohol (PVA) was prepared by dissolving PVA in phosphate buffer saline pH 7.4 (5 ml) by heating on a water bath for 30-45 min at 80°C, and then was frozen at 0°C (for 14 h) and followed by thawed at 30°C (for 6 h) for 1-3 cycles.[10]

Preparation of cross-linked chitosan-polyvinyl alcohol membranes

A Clear solution of chitosan was prepared by dissolving chitosan in 0.1 M HCl, mixed with PVA solution (as prepared by above said a method) and autoclaved for cross-linking.[11]

Preparation of polyvinyl alcohol-gelatin cross-linked membranes

Aqueous solution of PVA was prepared as above, in which gelatin was added to the resulting solution, and a drop of 0.1 M HCl was added and the resulting dispersion was stirred at 70°C for half an hour to carry out the esterification between PVA and gelatin.[12]

The pH of the solutions was adjusted in the range of 7-7.5 using 0.1 M NaOH. A sterile stock solution of the drug DX (20 mg), GT (3 ml of marketed product equivalent to 120 mg) and preservative polyglycolate (0.1% w/v) and benzalkonium chloride (0.02% w/v) were added in such a way that the final concentrations in the formulation remains as specified. All the solutions (drugs and preservatives) were mixed with each of the polymeric solutions. The solutions were then poured into a sterilized petri dish (70 cm^2) under aseptic condition and dried in oven at 40°C for 12 h. Required sizes of the membranes (2 cm × 2 cm) were

then cut, packed, and stored for further evaluation of physical parameters and drug release profile. However, for *in vivo* study 0.4 cm × 0.5 cm or 0.20 cm^2 size membranes were used. The entire procedure was carried out under aseptic conditions using sterilized glassware and molds [Table 1].

Characterization of hydrogel membranes

Determination of the dimensions and weight of the membrane

The thickness of the membranes was measured using micrometer screw gauge at three different points of each the membrane. The length and breadth of the membranes were determined by using vernier caliper scale. For each formulation, five randomly selected membranes were tested for thickness, length, and breadth. For the determination of weight, five membranes from each formulation were selected and weighed individually using digital balance. The mean weight of the membranes was noted.[13]

Determination of pH

The membranes were allowed to swell in closed petridish at room temperature for an hour in phosphate buffer saline pH 7.4. The pH was noted after bringing the electrode of a pH meter in contact with the surface of the formulation and allowing to equilibrate for 1 min. The average of five determinations for each of the formulation was taken.[14]

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.[13]

Determination of tensile strength

This mechanical property was evaluated using 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 3 cm. 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 (TS) and % elongation were computed for the evaluation of the membrane. TS 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 by the following equation.[15]

TS = 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 (E/B) = (Increase in length/original length) × 10

Drug loading

The drug content and uniformity of drug content was determined by assaying individual membranes. Each membrane was grounded in a glass mortar and pestle after cutting it into small pieces stirred in 5 ml of phosphate buffer saline pH 7.4 and kept for 5 h to extract the entire drug present. The solution was then filtered through Whatman filter paper No. 1, and 1 ml of solution was transferred to a 10 ml volumetric flask and the volume was made up with isotonic phosphate buffer pH 7.4 and analyzed by ultraviolet (UV) spectrophotometer at 235 nm and 561 nm for DX and GT, respectively.[14]

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.[14]

Table 1: Formulation of hydrogel membranes

Formulation code	GT (mg)	DX (mg)	PVA (% w/v)	GEL (% w/v)	CHT (% w/v)	PG (% w/v)	BZK (% w/v)
PP							
F1	120	20	3.0	—	—	0.1	0.02
F2	120	20	1.5	—	—	0.1	0.02
F3	120	20	2.0	—	—	0.1	0.02
CP							
F4	120	20	3.0	—	—	0.1	0.02
F5	120	20	3.0	—	1.0	0.1	0.02
F6	120	20	1.5	—	1.0	0.1	0.02
F7	120	20	2.5	—	1.0	0.1	0.02
GP							
F8	120	20	1.5	—	—	0.1	0.02
F9	120	20	2.5	—	—	0.1	0.02
F10	120	20	1.5	—	—	0.1	0.02

GT: Gentamycin, DX: Dexamethasone, PVA: Polyvinyl alcohol, GEL: Gelatin, CHT: Chitosan, PG: Polyglycolate, BZK: Benzalkonium chloride

The procedure was repeated for 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 the dry membrane.

Determination of degree of crosslinking

The degree of crosslinking of a polymer is the ratio of the mass of cross-linked state to the whole mass of the individual monomer. In order to fabricate a device, the aluminium cylinder of the 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 aluminium foil and holes were drilled similarly. The container after weighing was then immersed into a solvent responsible for the solubilization of the monomer under suitable conditions. After an hour, the container was dried at 40°C in an oven for 4 h to allow the material to dry and then the container was reweighed.[16] The procedure was repeated 3 times and the degree of crosslinking was determined by the following formula:

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

Surface morphology by scanning electron microscopy

Scanning electron microscopy (SEM) photographs were taken with JEOL, a JSM5610-LV scanning microscope, Japan. Samples were coated with gold for 60 s under an argon atmosphere using sputter coater in a high vacuum evaporator. Images were taken at an acceleration voltage of 15 kV and magnification of 33-200. SEM study was conducted to study the topography of the hydrogel membrane before and after hydration.[17]

Fourier transforms infra-red spectroscopy studies

Fourier transform infra-red (FTIR) study was conducted to investigate and predict any physicochemical interactions between components in the formulation and to confirm the crosslinking of polymers.[16,18]

Differential scanning calorimetry

Differential scanning calorimetry (DSC) study was conducted to study the melting and crystalline behavior of the polymeric membrane. The temperature and energy scales were calibrated with standard procedures. The study was performed in the temperature range of 30-350°C at a heating range of 10°C/min in an N_2 atmosphere.[19]

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 equivalent to 2.28 mg of GT and 1 mg of DX) was transferred into a vial containing 5 ml of phosphate buffer saline pH 7.4. To avoid evaporation of the medium, the vials were covered with rubber caps and placed on a mechanical shaker which was attached to a water bath, which was maintained at a temperature of 37°C ± 1°C. Aliquots of 3 ml were withdrawn throughout the experiment at 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, and 360 min interval and replaced by an equal volume of fresh buffer solution. It was filtered and diluted if necessary and analyzed at 235 nm and 561 nm using Shimazdu Double beam UV-visible spectrophotometer for DX and GT, respectively.[20] Analysis of variance (ANOVA) studies were conducted to support the results.

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.[21,22]

Test for sterility

The test for sterility was conducted on formulations as per Indian Pharmacopoeia 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 passes the test for sterility.[23,24]

Ocular irritation studies

Ocular irritation study was performed on 12 New Zealand white albino rabbits weighing 2-3 kg, grouped into 3 for each formulation group (GP, PP, and CP). 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°C ± 2°C and 60°C ± 15°C, respectively. The guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India, were followed and prior permission was sought from the Institutional Animal Ethics Committee for conducting the study (SDCP/IAEC-22/2012-13). Out of 10 formulations, the best ones were chosen for the study. The formulation was applied to 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 the naked eye or by means of a pen torch. The test may be considered positive if there are one or more positive reactions during any observation period. One eye was used as a test and other as a control. Rabbits were grouped into three (4 + 4 + 4). For 1st group containing four rabbits, formulation F3 was applied to one eye and the other eye was kept as control (to which nothing applied). For 2nd group containing four rabbits formulation, F5 was applied to one eye and the other eye was kept as control. For 3rd group containing four rabbits, F9 was applied to one eye and

the other eye was kept as control. During the time of examination period, each rabbit was scored for ocular reaction.[25]

In vivo studies

Pseudomonas aeruginosa causes severe and rapid ocular infection and is one of the most common causes of bacterial conjunctivitis. In this study, 8 rabbits were induced conjunctivitis by swapping the sterile cotton which was dipped in the culture of the microorganism of *P. aeruginosa*. Both the eyes were induced conjunctivitis initially, followed by formulation application; to assess the grade of infection occurred in the eyes. Parameters which were considered for this study are redness, lacrimal secretion, mucoid discharge, response to ocular stimuli, and swelling of the eyelid.[7]

Parameters of conjunctivitis were graded as follows: Redness of the mucous membrane of the eye was observed visually and the grades were given from 0 to 4 that is, 0 = absent; 1 = mild; 2 = moderate; 3 = severe; 4 = extensive; Lacrimal secretion: It was graded from 0 to 3 as 0 = normal; 1 = slightly more than normal and 2 = more than normal; 3 = severe; mucoidal discharge: Whitish to yellowish white semi-solid discharge if any was noted and recorded as a grade of 0 to 3 in which 0 = absent; 1 = little; 2 = more and 3 = extensive; Response to ocular stimulus: It was assessed by throwing torch light on the eye from a particular distance and noticing the response to this stimulus. It was graded from 0 to 2 as 0 = normal; 1 = fast; 2 = very fast; Swelling of eye lid: It was graded from 0 to 2 as 0 = absent; 1 = slight; 2 = prominent. Rabbits developed conjunctivitis symptoms 48 h after the inoculation of bacteria into eyes. The formulation (F5 for group II, marketed product for group I) was placed into the eyes 24 h after the development of infection (complete development of infection, grade 4), and observed for the recovery of infected eye day by day by following the above said grading system till the full recovery of the eye (grade 0). Treatment effects were compared with those of the marketed formulations, and significance was determined using the unpaired t-test. Significance levels were determined for $P < 0.05$, for two-tailed test. The theoretical 't' value is 2.306 (i.e., the table 't' value) at this level of degrees of freedom. Treatment was given significance (S) if 't' value exceeded the table 't' value, and if it did not exceed then, treatment was considered as nonsignificant.

This study was conducted in accordance with CPCSEA guidelines, and the experimental protocol was approved by the Institutional Animal Ethics Committee (SDCP/IAEC-22/ 2012-13).

Stability studies

The membranes were wrapped in aluminum foil and placed in petridishes. These petridishes were stored at ambient humidity conditions at refrigerated temperature (2-8°C), room temperature (27°C ± 2°C), and oven temperature (45°C ± 2°C) for a period of 60 days. The formulations were evaluated for changes in drug content.[26]

RESULTS AND DISCUSSION

The present investigation on hydrogel membrane as 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.

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 0.40 to 0.26 mm, which is ideal for the membranes intended for ocular delivery (US Patent) and the pH was found to be in the range of 7.21-7.45, which indicated the compatibility of the membranes with the ocular system. All the membranes had good folding endurance.

Drug polymer interaction by Fourier transforms infra-red

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 mixture was compared with the principal peaks of the drug in the literature to observe any changes. The principle peaks of the drug GT were observed at 2347 cm^{-1} (C=C stretching), 1631 cm^{-1} (C=O stretching), 1390 cm^{-1} (C-H stretching), and 630 cm^{-1} (N-H stretching). The principle peaks of the drug DX were observed at 3421 cm^{-1} (O-H stretching), 1330 cm^{-1} (C-H stretching), and 1014 cm^{-1} (C-F stretching). The characteristic peaks of GT and DX were approximately matched with the drug: Polymer mixture in the formulation and hence, it was concluded that there was no interaction between the drug and the polymers used in the formulation of the hydrogel membranes [Figures 1-3].

Differential scanning calorimetry

The DSC of chitosan showed a glass transition temperature at 40.6°C and a melting endotherm at 118°C. The DSC of PVA shows a glass transition temperature at 49.83°C and a melting endotherm at 221.3°C. Gelatin shows the glass transition temperature at 104.39°C and a small peak at 228.67°C. It shows a melting endotherm at 289.75°C. 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 shows the presence of endotherm peaks at 219°C and 320-340°C indicated that existence of some interaction between the polymeric chains after freeze thawing. DSC studies carried out on the cross-linked hydrogel membrane of CP indicated a shift in the peaks and also the formation of new peaks due to the interaction between polymers. The peaks obtained indicated glass transition temperature at 48.58°C and a melting endotherm at 134.93°C, 203°C, and 243°C. The cross-linked hydrogel membrane shows glass transition temperature at 142°C and a melting endotherm at 215°C and 284°C. These peaks were not seen in the DSC thermogram of pure gelatin and pure PVA, which indicated the existence of crosslinking among the two polymers [Figures 4 and 5].

Scanning electron microscopy

The blend membrane was clear to the eye and neither showed the separation into two layers nor any precipitation. The swollen hydrogel membranes showed the presence of pores. These pores neither fixed in size nor they localized in any definite location. As a result of water uptake of the macromolecular segments exhibit enhanced mobility so that the size, shape and location of the pores continuously change. From the SEM images of cross-linked PVA

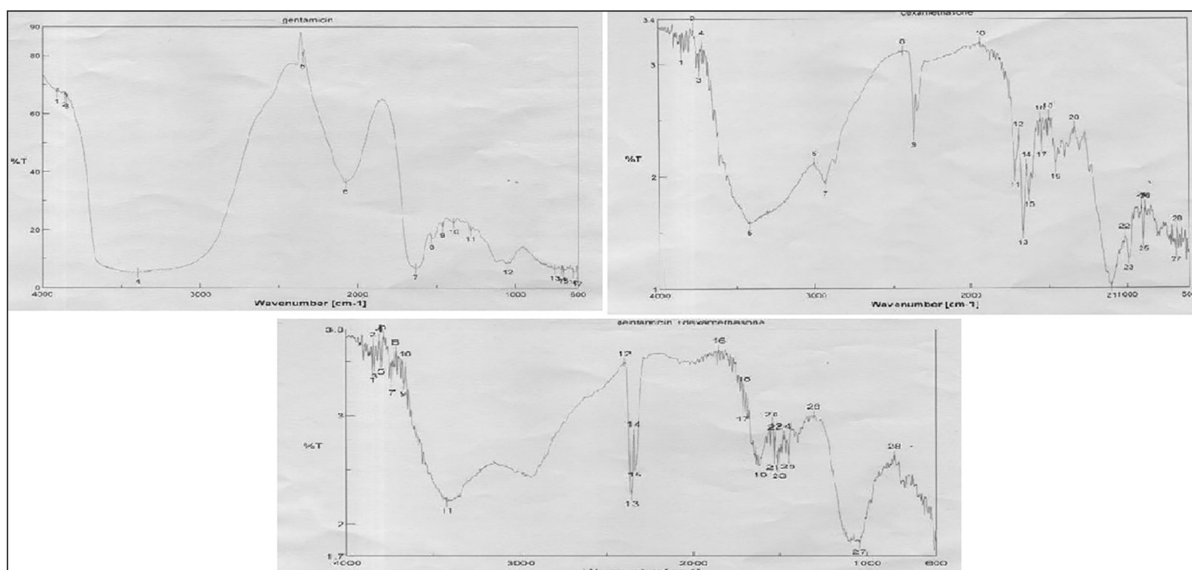

Figure 1: Fourier transforms infrared spectra of gentamicin, dexamethasone and both

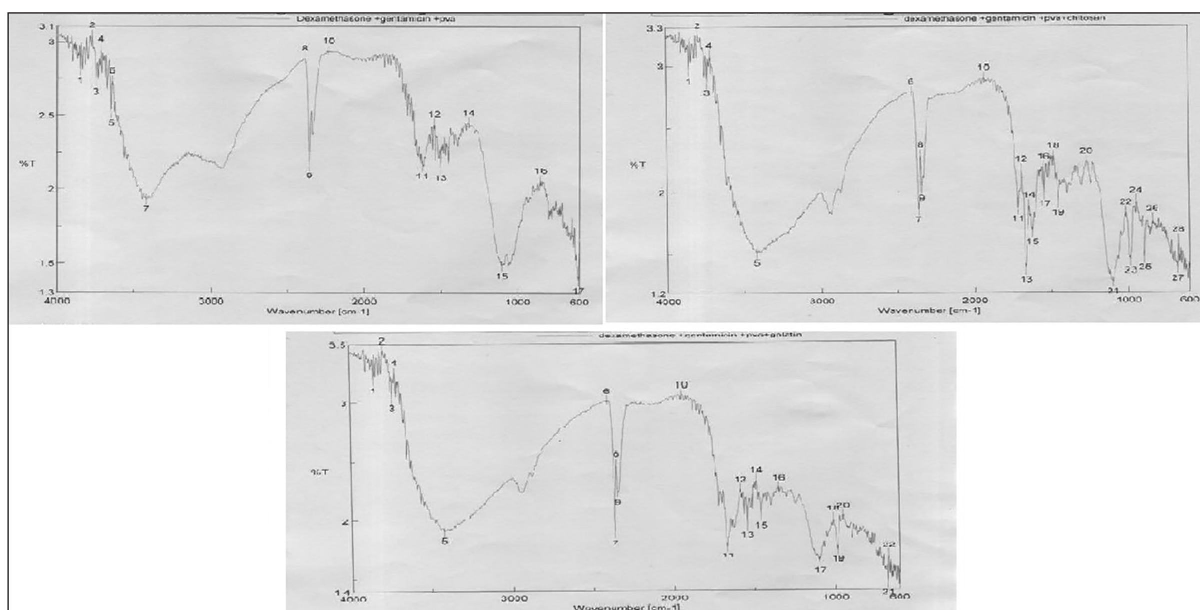

Figure 2: Fourier transforms infrared spectra of dexamethasone and gentamicin with polyvinyl alcohol, dexamethasone and gentamicin with chitosan, dexamethasone and gentamicin with polyvinyl alcohol with gelatin

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 which was unable to form crosslinks. On hydration, the membranes showed the interconnection between the swollen polymeric chains hence, it can be interpreted that there exists crosslinking between the two polymeric chains [Figure 6a-c].

Tensile strength and percentage elongation

The TS gives an indication of the strength and elasticity of the film reflected by the parameters, TS, and 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. Among the PP formulation, F4 showed maximum TS and hence was least elongated. Among the CP formulations, as the concentration of chitosan increased the TS decreased F8 showed the maximum TS. Among the GP formulation, F6 showed maximum TS.

Degree of crosslinking

Among the PP formulations, we see that as the freeze thaw cycle increased the degree of crosslinking increased, due to the fact that initially only a few PVA chains participated in the crystalline formation process and increasing the freeze thaw cycles leads to further crystal

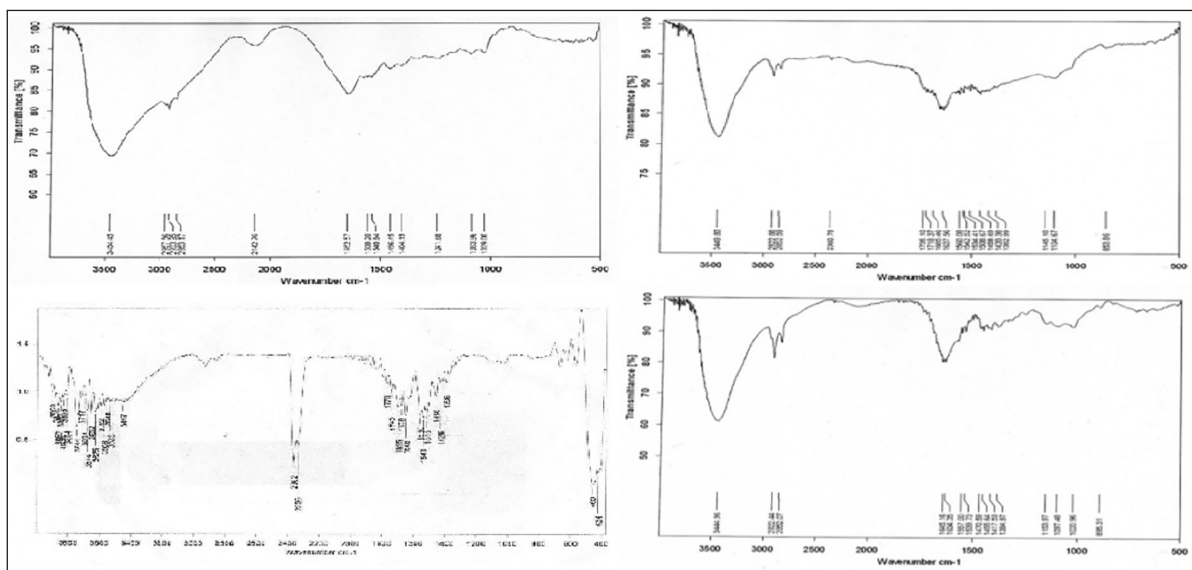

Figure 3: Fourier transforms infrared spectra of gelatin, polyvinyl alcohol, hydrogel polyvinyl alcohol, chitosan, cross-linked polyvinyl alcohol

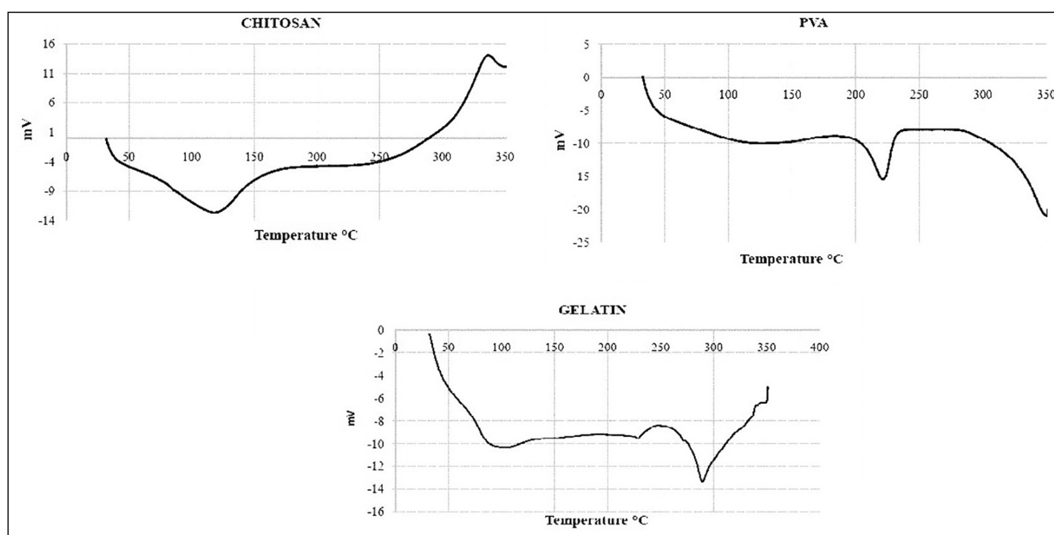

Figure 4: Differential scanning calorimetry thermogram of gelatin, chitosan and cross-linked polyvinyl alcohol

formation and, therefore, increased physical crosslinking between the PVA chains. In the formulation CP, F5 showed a maximum degree of crosslinking. As the content of PVA increased the degree of crosslinking was found to be decreased, and the swelling ratio increased. In the formulation GP, F9 shows minimum crosslinking and hence maximum swelling capacity was observed. The degree of crosslinking is found to be inversely proportional to the swelling ratio. This may be due to the high PVA content in F9 [Table 2].

Swelling studies

Among formulation PP, F2 and F4 showed maximum swelling in the first 30 min and then reached equilibrium by the end of 2 h. The decrease in the swelling ratios at the end of 2 h may be likely due to chain dissolution and changes in the crystalline structure which inhibits the gel from maintaining gel structure. F1 and F3 showed less swelling ratio when compared to F2 and F4 because of a higher degree of crosslinking among the polymeric chains. Both F2 and F4 attained equilibrium swelling at the end of 2 h.

Table 2: Degree of crosslinking

Formulation code	Percentage degree of crosslinking*
PP	
F1	45.73±0.825
F2	41.11±0.597
F3	69.24±0.395
F4	68.90±1.234
CP	
F5	62.76±0.573
F6	51.80±0.638
F7	58.51±0.941
GP	
F8	68.26±0.359
F9	40.85±0.358
F10	50.69±1.005

*Average of three readings. Data are represented as mean ± SD (n = 2).
SD: Standard deviation

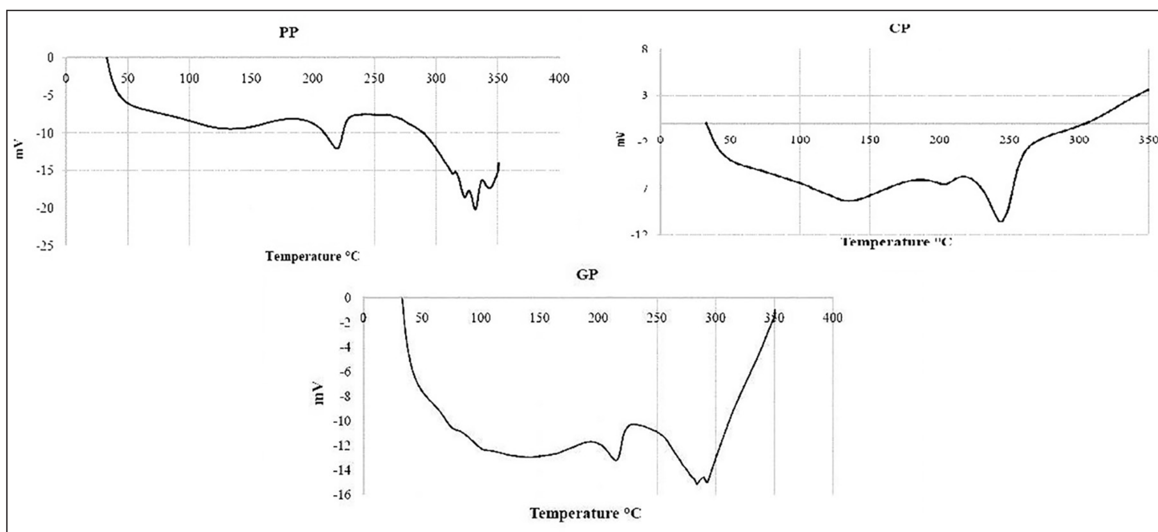

Figure 5: Differential scanning calorimetry thermogram of PP, GP, and CP

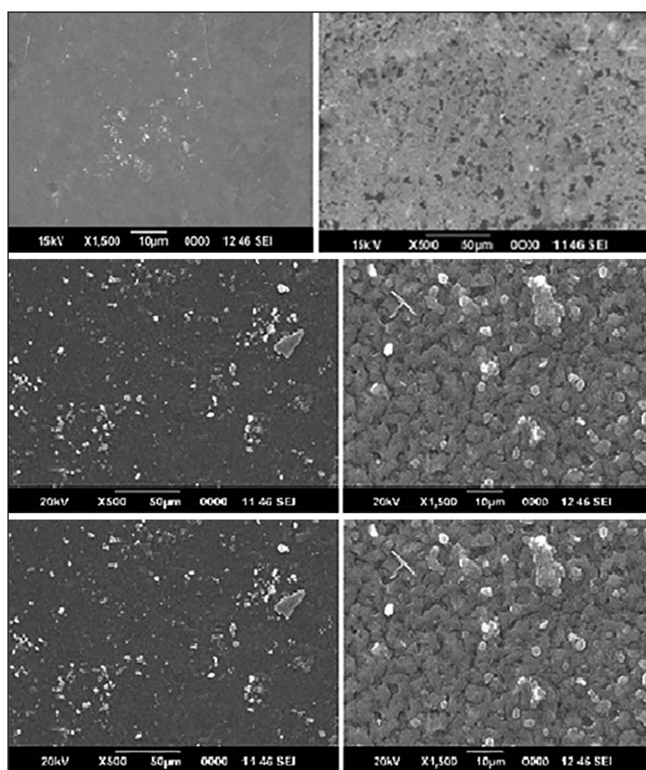

Figure 6: Scanning electron microscopy (a) CP before hydration, CP after hydration, (b) PP before hydration, PP after hydration, (c) GP before hydration, GP after hydration

Among CP, the swelling ratio of F6 is found to be more than F5 and F7, and all the three membranes were found to be stable after 24 h. As chitosan is insoluble in alkali and PVA is a water soluble polymer, due to the hydrogen bonding that occur between the functional groups of chitosan and PVA, the physically cross-linked composite. Formulation F9 showed maximum swelling compared to F8 and F10 due to the high percentage of both PVA and gelatin. As the amount of PVA in the gel decreases, the swelling ratio is also found to decrease [Figure 7a-c].

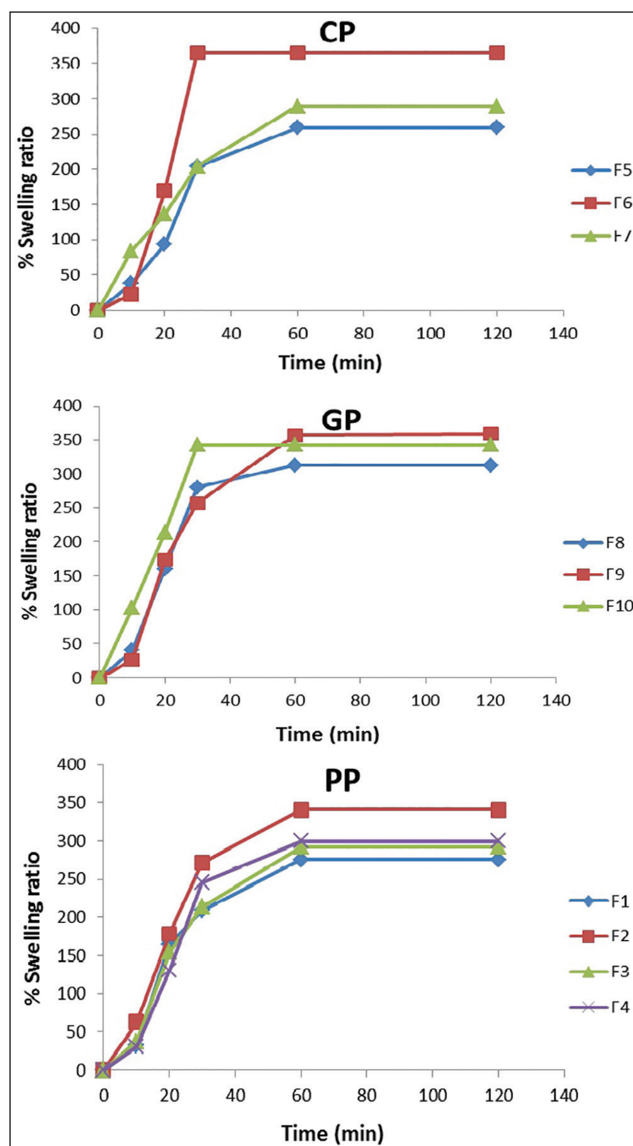

Figure 7: Percentage swelling ratio (a) CP, (b) GP, (c) PP

Drug loading

The percentage drug content of all the formulations was found to be in the range of 69.82-89.19%. Since the drug was 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 crosslinking, and percentage swelling ratio. Among PP, F3 was found to exhibit better sustained release of the drugs as compared to F1, F2, and F4. The TS, percentage elongation and drug content for the formulation F3 containing GT and DX were found to be 0.0892 ± 0.0057, 149.34 ± 0.046, 83.27%, and 81.45%, respectively. Among CP, F5 is found to exhibit better sustained release of the drugs as compared to F6 and F7. The TS, percentage elongation, and drug content of GT and DX were found to be 0.0637 ± 0.0043, 162.39 ± 0.071, 80.25%, and 83.17%, respectively. Among GP, F9 is found to exhibit better sustained the release of the drugs as compared to F8 and F10. The TS, percentage elongation and drug content of GT and DX were found to be 0.0453 ± 0.0093, 158.73 ± 0.021, 69.82%, and 75.83%, respectively. Due to its non porous nature and a mesh-like structure as confirmed by SEM the drug was released by diffusion through the mesh network. All values were found to be statistically significant ($P < 0.001$) as determined using ANOVA [Figures 8 and 9].

Kinetic analysis of *in vitro* release data

In order to determine the release mechanism that provides the best description to the pattern of drug release, the *in vitro* release data were fitted to zero order, first order, and Higuchi model. The release data were also kinetically analyzed using the Korsmeyer-Peppas model. The data were processed for regression analysis using MS-EXCEL statistical function. By using Korsmeyer and Peppas model, if $n = 0.45$ it is Case 1 or Fickian diffusion, $0.45< n >0.89$ is for anomalous behavior or non-Fickian transport, $n = 0.89$ for Case 11 transport, and $n >0.89$ for super Case 11 transport. Fickian release usually occurs by molecular diffusion of the drug due to a chemical potent gradient. Case 11 relaxation releases are the drug transport mechanism associated with the stresses and state transition in hydrophilic glassy polymers, which swell in water or biological fluids. This term also includes polymer disentanglement and erosion. In the present investigation, the release from the hydrophilic polymers followed the combination of diffusion and erosion as the '*n*' values ranged from 0.501 to 0.836 for GT and for DX values ranged from 0.543 to 0.813 for as per Korsmeyer and Peppa's model, which in turn justified the suitability of polymers for the preparation of hydrogels [Tables 3 and 4].

Test for sterility

The test was performed as per the procedure given in the methodology. Both positive and negative controls were prepared. The results of the sterility, when compared with positive and negative control, showed that the medium used was sterile and provided necessary nutrients for a 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 examination, there was no macroscopic evidence of microbial growth. Hence, it passes the test for sterility.

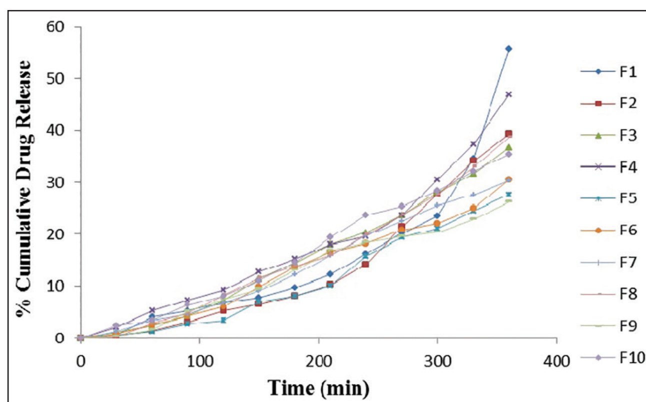

Figure 8: Drug release profile of gentamicin

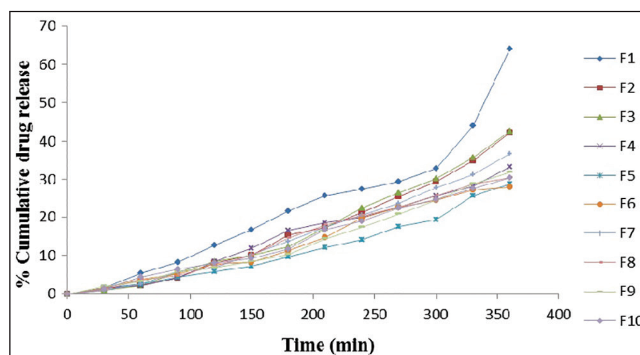

Figure 9: Drug release profile of dexamethasone

Table 3: Kinetic analysis of *in vitro* drug release data of gentamicin

Formulation code	Zero order R^2	First order R^2	Higuchi model R^2	Korsmeyer-Peppas model n	Best fitting model
F1	0.9429	0.9108	0.9126	0.569	Zero
F2	0.9873	0.9672	0.9845	0.557	Zero
F3	0.9833	0.9714	0.9821	0.543	Zero
F4	0.9952	0.9827	0.9937	0.581	Zero
F5	0.9456	0.9089	0.9107	0.707	Zero
F6	0.9672	0.9467	0.9489	0.836	Zero
F7	0.9563	0.9268	0.9559	0.871	Zero
F8	0.9753	0.9543	0.9719	0.494	Zero
F9	0.9834	0.9712	0.9689	0.501	Zero
F10	0.9332	0.9149	0.9217	0.527	Zero

Table 4: Kinetic analysis of *in vitro* drug release data of dexamethasone

Formulation code	Zero order R^2	First order R^2	Higuchi model R^2	Korsmeyer-Peppas model *n*	Best fitting model
F1	0.9825	0.9813	0.9817	0.735	Zero
F2	0.9805	0.9774	0.9791	0.758	Zero
F3	0.9754	0.9743	0.9746	0.813	Zero
F4	0.9812	0.9805	0.9808	0.693	Zero
F5	0.9846	0.9835	0.9842	0.548	Zero
F6	0.9772	0.9763	0.9770	0.689	Zero
F7	0.9801	0.9786	0.9792	0.649	Zero
F8	0.9856	0.9842	0.9854	0.593	Zero
F9	0.9789	0.9783	0.9785	0.671	Zero
F10	0.9790	0.9745	0.9756	0.699	Zero

Ocular irritation studies

The results of the ocular irritation studies indicate that all formulations are nonirritant to the eye.

Excellent ocular tolerance was noted. No ocular damage or abnormal signs to the cornea, iris, and conjunctiva was visible.

In vivo studies

In the present study, 105 mg of GT equivalent to 2.6 ml of marketed GT injection and 20 mg of DX in 1 ml buffer was used for the preparation of membranes. The initial membrane size was approximately 70 cm^2. For *in vivo* study, the membrane size was (0.2 cm^2) selected in such a way that the drug concentrations when administered into the eye, comparable with the eye drops. that is, each drop of marketed formulation contains approximately 0.3 mg of GT and 0.06 mg of DX, hence in the present study the size of the membrane was such that it contains approximately 0.3 mg of GT and 0.057 mg of DX.

Criteria of conjunctivitis response to drug therapy

ecrease in redness, mucoid discharge, lacrimal secretion, response to ocular stimulus, and swelling of an eyelid were taken as a positive response to therapy. Observations were made to note any ocular or systemic side effects in all the rabbits. Comparison of scores obtained with hydrogel formulation indicated that the hydrogel formulation is effective in relieving symptoms of conjunctivitis with the advantage of lesser frequency of administration compared to eye drops which are usually instilled into the eye at a frequency rate of 2-3 times a day [Figure 10 a-d] and [Tables 5 and 6].

Significantly better responses in redness were obtained on day 3 ($P < 0.0001$) and ($P = 0.002$) with hydrogel membrane compared to marketed eye drops. Lacrimal secretion also showed the significantly better response on day 3 ($P > 0.0169$), however, on day 5, no significant difference was obtained with hydrogel formulation. Compare to marketed eye drops with respect to mucoid discharge, response to ocular therapy, and swelling of the eyelid.

The severity of conjunctivitis was remarkably lowered at day 3 with hydrogel membrane compared to marketed eye drops.

Table 5: Grading of parameters of conjunctivitis (group I - marketed product)

Parameter	Mean score		
	Day 0	Day 3	Day 5
Redness	4±0	2.102±0.314	0.558±0.47
Lacrimal secretion	3±0	2.07±0.217	0.210±0.462
Mucoid discharge	3±0	1.88±0.340	0.87±0.311
Response to ocular stimuli	2±0	1.574±0.290	0.260±0.294
Swelling of eyelid	2±0	1.320±0.217	0.08±0.0

Data are represented as mean ± SD (*n* = 2). SD: Standard deviation

Table 6: Grading of parameters of conjunctivitis (group II - hydrogel membrane)

Parameter	Mean score		
	Day 0	Day 3	Day 5
Redness	4±0	1.500±0.223	0.07±0.0
Lacrimal secretion	3±0	1.833±0.307	0.07±0.0
Mucoid discharge	3±0	1.660±0.210	0.07±0.0
Response to ocular stimuli	2±0	1.330±0.229	0.07±0.0
Swelling of eyelid	2±0	1.160±0.166	0.07±0.0

Data are represented as mean ± SD (*n* = 2). SD: Standard deviation

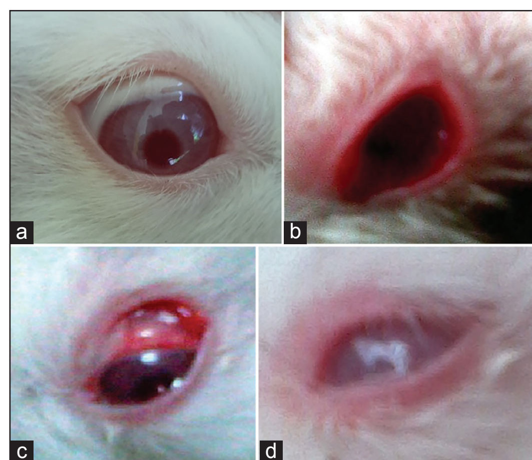

Figure 10: Rabbit's eye with infection and after treatment (a) Normal eye (control), (b) Day 1 (after inducing infection) (c) Recovery level day 3 after marketed preparation and (d) Hydrogel administration

Results of unpaired *t*-test of significance between two groups indicated that hydrogel membrane showed a better response in the treatment of conjunctivitis compared to marketed products [Table 7].

Table 7: Significance *t*-test results of group I (marketed eye drops) versus group II (hydrogel membrane)

Parameter	Significance *t*-test results of marketed eye drops versus hydrogel membrane		
	Day 0	Day 3	Day 5
Redness	*P*>0.999 *t*=0 NS	*P*<0.0001 *t*=5.990 ES	*P*=0.002 *t*=4.174 ES
Lacrimal secretion	*P*>0.999 *t*=0 NS	*P*>0.0169 *t*=2.500 S	*P*=0.2322 *t*=1.214 NS
Mucoid discharge	*P*>0.999 *t*=0 NS	*P*=0.0552 *t*=1.978 S	*P*<0.0001 *t*=9.264 ES
Response to ocular stimuli	*P*>0.999 *t*=0 NS	*P*=0.0054 *t*=2.953 MS	*P*=0.034 *t*=2.200 S
Swelling of eyelid	*P*>0.999 *t*=0 NS	*P*=0.0291 *t*=2.268 S	*P*=0.8469 *t*=0.1944 NS

Data are represented as mean ± SD (*n* = 2). ES: Extremely significant, NS: Nonsignificant, SD: Standard deviation, S: Significant, MS: Mean square

Stability studies

Stability studies were carried out for 45 days at 2-8°C (45% relative humidity [RH]), 25-30°C (60% RH). The films were observed for physical change, percentage drug content, and percentage drug release. Hydrogels containing a combination of GT and DX was found to be physically and chemically stable and showed no significant change in terms of physical characteristics, percentage drug content, and percentage drug release. However, when stored at 45-50°C for 45 days, films became brittle and showed degradation in their physicochemical properties. All the formulations showed good stability at 25-40°C/45-60% RH. There was no significant change in the drug content. The drug content did not deviate from the initial amount indicated that the drug is stable in the hydrogel formulations.

CONCLUSION

The hydrogels can be easily formulated by solvent casting method by crosslinking polymers such as PVA, gelatin, and chitosan in different polymer ratios by using propylene glycol as plasticizer. All the results were found to be satisfactory and acceptable. The hydrogel membranes containing combination of GT and DX were found to be promising ocular delivery systems for treatment of conjunctivitis. Thus, the specific objectives listed in this project were achieved. These findings with further extensive research and application of the certain concept of novel drug delivery system may help the industry to scale up for commercial production. 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.

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Conflicts of interest

There are no conflicts of interest.

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