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Formulation and in vitro evaluation of cyclosporine A inserts prepared using HPMC for treating dry eye disease

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A Thesis

entitled

Formulation and In vitro Evaluation of Cyclosporine A Inserts Prepared Using HPMC for

Treating Dry Eye Disease

by

Zahraa Hasan Falhi Al-Saedi

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Master of Science Degree in Pharmaceutical Sciences

Industrial Pharmacy

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The University of Toledo

May 2016

An Abstract of

Formulation and *In vitro* Evaluation of Cyclosporine A Inserts Prepared Using HPMC for Treating Dry Eye Disease

by

Zahraa Al-Saedi

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Aim: The aim of this study was to develop and characterize a novel sustained-release drug delivery system of cyclosporine A (CsA) using hydroxypropyl methylcellulose (HPMC) and xanthan gum (XG) for treating dry eye disease (DED).

Methods: Polymeric inserts of CsA were prepared using the solvent casting technique with a 2^3 full factorial design to evaluate the effect of HPMC, XG ratios and drug content on thickness, folding endurance, wettability and *in vitro* drug release. Inserts were also evaluated for drug content, moisture absorption and loss, and surface pH. Optimized CsA-loaded HPMC inserts were sterilized with UV light and evaluated for morphology, thermal analysis, FTIR, stability at 4°C, 25°C and 40°C, cytotoxicity in cultured bovine corneal endothelial cells, and anti-inflammatory effect in Jurkat T cells.

Results: The addition of XG increased CsA release duration and enhanced the folding endurance of films. All films showed uniformity in drug content and thickness. Formulation F4 composed of 1% w/v HPMC and 0.25% w/v XG exhibited good folding

endurance and sustained CsA release for up to 20h. Sterility testing of F4 using plate and direct inoculation confirmed the formulation sterility and validated the sterilization method. The formulation was found to be stable for at least 3 months at 4°C, 25°C and 40°C. No cytotoxicity was observed in cultured bovine corneal endothelial cells for up to 24 h. The anti-inflammatory effect of CsA was intact in ophthalmic inserts.

Conclusion: In conclusion, the combination therapy with HPMC and CsA can be a potential once-a-day formulation for treating DED.

Dedicated to my beloved mother and to my loving husband Ayad Al-Hamashi

Acknowledgements

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Chapter 1

Introduction

1.1 Dry eye disease: definition and clinical presentation

Dry eye disease (DED) is a multifactorial disorder of the tear film due to excessive tear evaporation or tear deficiency, resulting in hyperosmolarity of the tear film and inflammation to the ocular surface [1]. Symptoms include dryness, ocular surface discomfort, light sensitivity, blurred vision, redness, grittiness, foreign body sensations, pain, stinging, ocular fatigue, scratchiness, itchiness, sandiness, and soreness [2]. Although dry eye usually causes symptoms, symptoms can be absent initially or mild. Diagnostic procedures are not reproducible in mild cases of DED, even if symptoms are present [2, 3].

1.2 Anatomy of the ocular surface and production of the tear film

The ocular surface includes the corneal and conjunctival epithelia, the lacrimal gland, accessory lacrimal glands, meibomian glands, the nasolacrimal duct, the eyelashes and accompanying glands of Moll and Zeis [4]. Within the ocular surface system is the 'lacrimal functional unit' (LFU) [5]. The LFU includes the lacrimal glands (main and accessory), the meibomian glands, lacrimal drainage system, and intervening neural reflex network (Fig. 1). This system functions to synthesize and deliver tear fluid to the ocular surface, which is important for several reasons. The tear film smoothes the irregularities of

1

the cornea, supplies the cornea with oxygen [6] and creates an even surface for light refraction and good optical quality. Furthermore, it functions as a barrier to microbial infections and removes debris during the blink cycle [7]. Normally, the cornea is devoid of blood and lymphatic vessels. This absence of vascularity confers an 'angiogenic privilege' to the cornea.
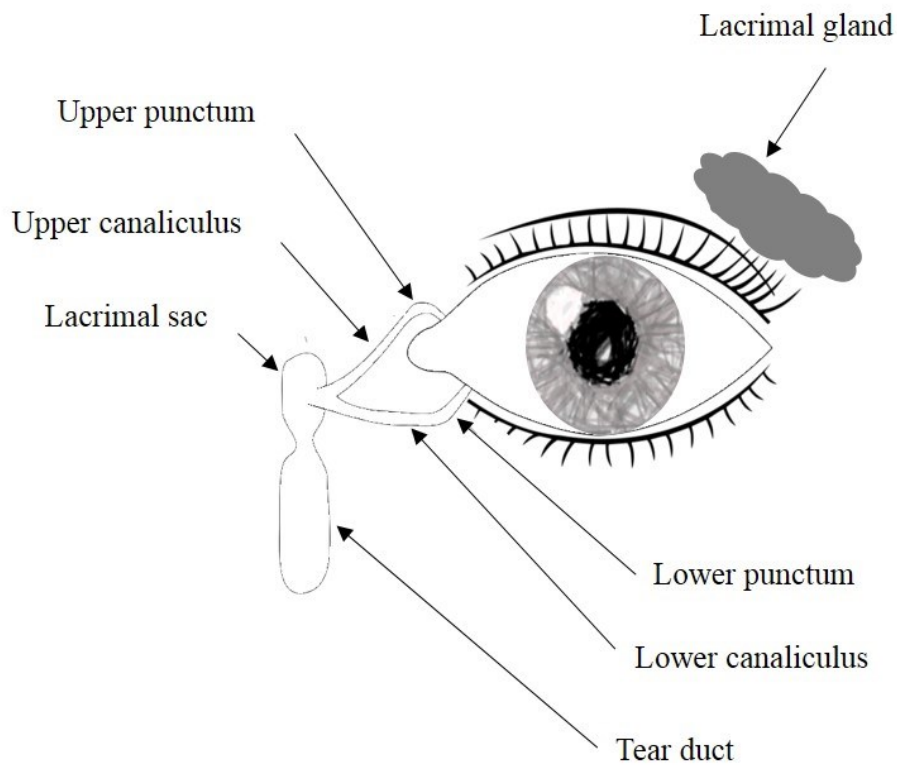
Figure 1.1: Illustration of the tear system. This figure has been modified from (https://en.wikipedia.org/wiki/Lacrimal_gland)

1.2.1. Composition of the Tear film

Tear film continuously covers the ocular surface [8]. This fluid consists of three different layers: the innermost mucin layer, a middle aqueous layer, and outer lipid layer (Fig. 2).
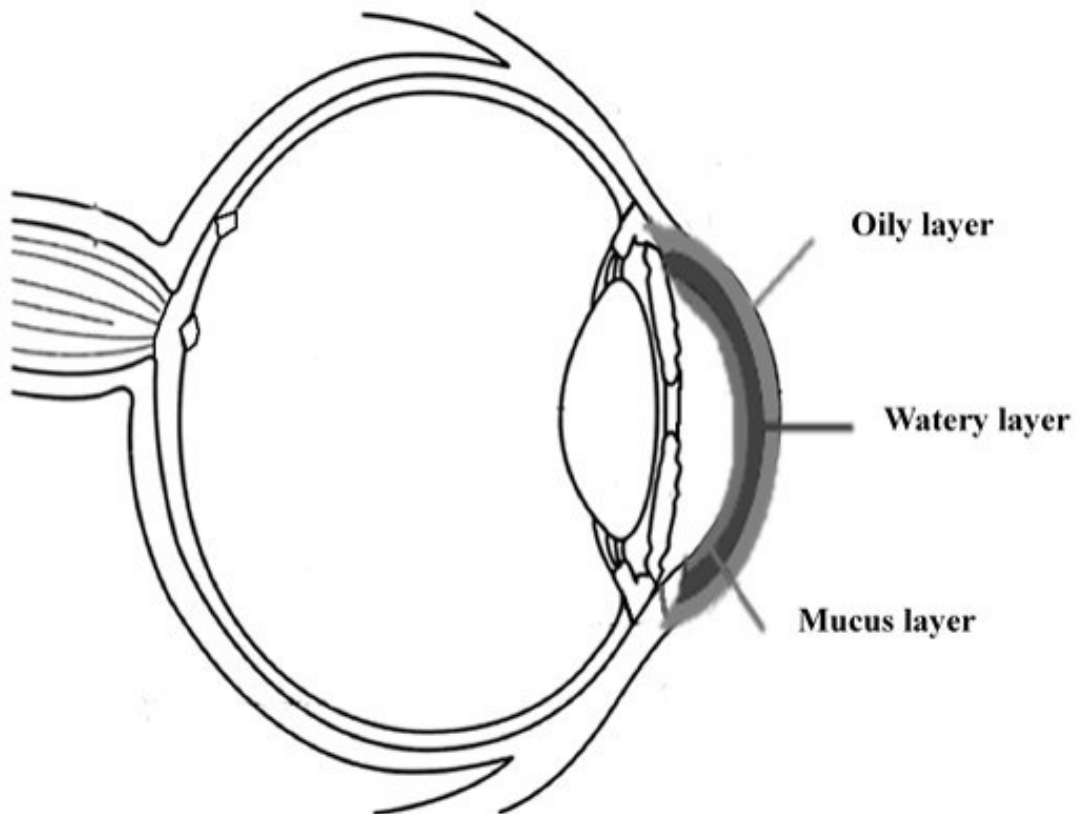
Figure 1.2: Composition of the tear film.

1.2.1.1 The lipid layer

Meibomian glands are sebaceous glands located in the tarsal plates of the upper and lower eyelids. They are simple, branched, acinar in structure and exhibit a holocrine mode of secretion. There are approximately 30-40 meibomian glands in the upper eyelid and 20-30 in the lower eyelid, with their central duct openings arranged in parallel, anterior to the

mucocutaneous junction [9, 10]. The lipids secreted by the meibomian glands form the superficial layer of the tear film. This layer, approximately 42 nm thick, forms a barrier to protect the tear film from contamination by sebaceous secretions from the skin and prevents tears from overflowing onto the eyelids. Additionally, it forms a seal during sleep and maintains tear film stability [11-13]. Most importantly, the lipid layer retards tear evaporation from the ocular surface [14]. The lipid layer is a mixture of polar and non-polar lipids, with the non-polar portion being more superficial. It consists mainly of wax cholesterol and sterol esters, membrane phospholipids and smaller amounts of triglycerides, diglycerides, monoglycerides, and free fatty acids [15].

1.2.1.2 The aqueous layer

The main lacrimal gland, located in the bony fossa of the supero-lateral orbit and the accessory lacrimal glands of Krause and Wolfring, secrete the aqueous layer of the tear film. Furthermore, the conjunctival epithelial cells secrete electrolytes, water, and mucins into the aqueous layer, and the cornea epithelial cells make a small contribution as well [16]. The aqueous layer is the largest component of the tear film. In addition to water and electrolytes, it contains a large number of proteins. The lacrimal glands secrete lysozyme, a protein that reduces the concentration of bacteria by attacking their cell walls, and lactoferrin, another protein that reduces bacterial load by making certain metals unavailable to microorganisms. Other proteins present in this layer include IgA, IgG, IgE, epidermal growth factor, along with hepatocyte growth factor, enzymes, metabolites, albumin, defensin, trefoil factor, peroxidase, and transferrin [7, 17-19].

1.2.1.3 The mucin layer

The apical surface of the corneal and conjunctival epithelia contains special membrane folds called microplicae. Emanating from these membrane specializations are membrane-associated mucins, which form the glycocalyx layer. Mucins are heavily glycosylated glycoproteins, with carbohydrates comprising 50-80% of their molecular mass [20]. Another defining characteristic of mucins is the repetition present within their coding sequence. Mucin genes contain extensive tandem repeats that code for proteins rich in proline, seronine, and threonine that serve as O-glycosylation sites [21]. The extensive O-glycosylation gives this layer a negative charge, thereby reducing pathogen adherence and entry into the corneal epithelium [22]. The secreted mucins removes debris and shed epithelial cells and inflammatory cells from the surface of the eye [23]. Within the secreted mucin subfamily, there are five polymeric, gel-forming mucins and two smaller, non-polymeric, soluble mucins. MUCs2, 5AC, 5B, 6, and 19 are gel-forming mucins, which are the largest known glycoproteins [21]. All goblet cells of the conjunctival epithelium secrete MUC5AC, the most ubiquitously expressed mucin at the ocular surface [24]. MUC7 is a small, soluble mucin secreted by the acinar cells of the lacrimal gland [25]. They lack domains rich in cysteine residues that mediate disulfide bond formation in polymeric mucins and exist predominately as monomers [26].

Membrane-associated mucins form the other major class of mucin glycoproteins. These include MUC 1, 3A, 3B, 4, 11, 12, 15, 16, 17, and 20 [19]. The ocular surface expresses MUC 1, 4, 15, 16, and 20. MUC1 is expressed by the corneal and conjunctival epithelia and the lacrimal gland [19, 27, 28]. MUC1 functions include anti-adhesion, signaling, and

providing a barrier to pathogens [19]. MUC16 expression is abundant throughout the corneal epithelium and, to a lesser extent, the conjunctival epithelium and lacrimal gland. MUC16, also known as the CA125 antigen, is an ovarian tumor cell marker and plays an important role in barrier formation and anti-adhesion [26, 29]. Evidence exists that MUC16 interacts with the cytoskeleton of the corneal and conjunctival epithelial cells by binding to actin molecules via its interactions with ERM proteins, potentially lending a role in microplicae formation [26, 30]. Finally, membrane-associated mucins contribute mucins to the tear by shedding their extracellular domains [20].

1.2.2. Pathway for tear fluid

The lacrimal gland sits in the shallow bony fossa of the supero-lateral orbit, and lacrimal fluid flows from superolateral to inferomedial canthus via the following pathway: lacrimal ducts to the superior fornix of the conjunctival sac → across the surface of eyeball → lacrimal lake → superior and inferior lacrimal puncta → superior and inferior lacrimal canaliculi → lacrimal sac → nasolacrimal duct → inferior nasal meatus.

1.2.3. Regulation by the nervous system

The ophthalmic division of cranial nerve V carries sensory fibers, by way of the nasociliary branch, from the cornea to the pons. Within the efferent limb of the lacrimation reflex, preganglionic parasympathetic fibers from the superior salivatory nucleus travel in the greater petrosal nerve (cranial nerve VII), join the nerve of the pterygoid canal, and synapse on postganglionic fibers in the pterygopalatine ganglion. Postganglionic fibers travel with

the short ciliary nerves and stimulate secretions by the lacrimal gland. The afferent and efferent limbs of the blink reflex are also mediated by cranial nerves V and VII, respectively. On the other hand, preganglionic sympathetic fibers from the upper cervical segments of the spinal cord synapse with postganglionic fibers in the superior cervical ganglion. The sympathetic postganglionic fibers follow branches of the internal carotid artery and then join the deep petrosal nerve. The deep petrosal nerve, containing the sympathetic fibers, and the greater petrosal nerve, carrying parasympathetic fibers, join each other to form the nerve of the pterygoid canal. Sympathetic fibers travel through the pterygopalatine ganglion without synapsing and travel with short ciliary nerves to reach the lacrimal gland.

1.3. Epidemiology and Classification

DED is a significant public health problem and is often unrecognized. It affects 5% to 34% of people globally [7]. One study found that DED affects as many as 17% of women and 11.1% of men in the United States, and this difference persisted across all ages [31]. In addition to being more common in women, prevalence increases with age. Among both men and women over 80 years of age, DED prevalence is slightly increased to 19% [31]. The international DED workshop identified two major classes of DED [32]. These include tear-deficient dry eye (TDDE) and evaporative dry eye (EDE), with TDDE being the more common of the two (Fig. 3). In tear-deficient dry eye, there is a reduced volume of tears produced. Lacrimal function and tear volume are normal in EDE, and the abnormality results from excessive water loss by evaporation. TDDE and EDE may also occur simultaneously.

Many systemic diseases, especially those that are autoimmune in nature, are associated with DED. TDDE is further classified as Sjogren Syndrome tear deficiency (SSTD) and non-Sjogren tear deficiency (NSTD) [1]. A European-American collaboration recognized two forms of SS [33]. Primary SS is an autoimmune disease that affects the lacrimal and salivary glands through the production of autoantibodies, and its main features are dry eye and dry mouth. One study estimated that patients with SS account for approximately 11% of DED cases [34]. In secondary SS, the symptoms of primary SS are present as a result of an autoimmune connective tissue disease, the most common being rheumatoid arthritis. Rare causes include: polyarteritis, Wegener's granulomatosis, systemic lupus erythematosus, systemic sclerosis, and primary biliary cirrhosis. Various forms NSTD include primary lacrimal disease (PLD), secondary lacrimal gland deficiency, lacrimal gland obstruction and reflex hyposecretion. PLD includes congenital alacrima and acquired PLD. The acquired form is the most prevalent cause of PLD and involves a gradual destruction of the lacrimal tissue. It is more common in women, and the risk increases with age [35, 36]. Causes of secondary lacrimal deficiency include sarcoidosis, HIV, graft vs. host disease (GVHD), vitamin A deficiency (xeropthalmia), dacryoadenitis, lymphoma, and lacrimal gland ablation [1]. Interestingly, DED is one of the most common complications of GVHD, and a study found that up to 62.5% of patients with GVHD from a hematopoietic stem cell transplant experienced ocular pathology [37]. Neural causes of NSTD can result from damage to either the sensory or motor aspects of the lacrimal reflex, leading to reflex hyposecretion. Sensory loss is common among contact lens wearers, particularly among hard and extended-wear contact lens users [38]. Neurotrophic keratitis can cause sensory loss in the cornea as a result of a loss of trophic function of the trigeminal

nerve [39]. Infections such as herpes simplex and herpes zoster and topical medications such as beta-blockers or atropine-like medications can also produce sensory loss [1]. Conditions that affect the facial nerve, cranial nerve seven, such as Bell's palsy or facial nerve paralysis, can lead to dry eye because cranial nerve seven provides secretory-motor innervation to the lacrimal gland. Lacrimal and accessory lacrimal gland obstruction occur in cicatrizing conjunctival diseases as a result of scar formation in the duct openings. Common causes of conjunctival scarring diseases include trachoma, cicatricial pemphigoid, and erythema multiforme [1]. The most common cause of EDE is meibomian gland dysfunction (MGD) [40]. Other causes include blepharitis (anterior and posterior), blink disorders, disorders of the lid aperature and lid/globe congruity, ocular surface disorders, Grave's disease, ocular rosacea, ocular allergies, laser refractive surgery, entropion, and ectropion [1]. EDE also results from vitamin A deficiency, preservatives in topical formulations, and continuous use of contact lenses [41, 42]. Contact lenses cause an increase in tear evaporation rate, which may explain the increased pervasion of DED in contact lens wearers [43, 44]. Any abnormality in the tear film lipid layer usually causes more rapid evaporation of tear from the ocular surface and is associated with EDE [45].
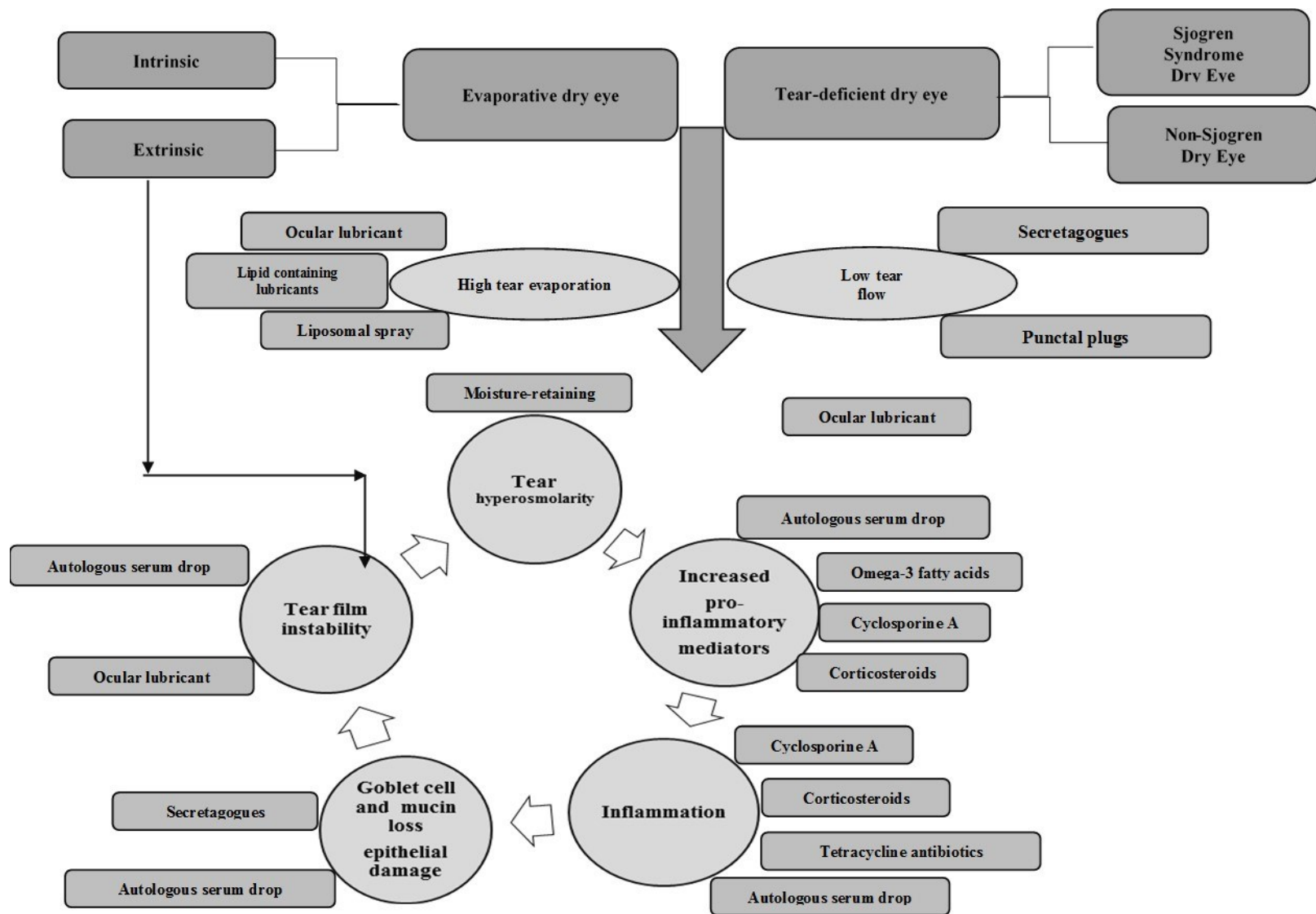
Figure 1.3: Classification of DED

1.4 Pathophysiology of DED

1.4.1. Hormones

Given that DED has a much higher prevalence in females, there is a large body of research that focuses on the role of sex hormones in the pathophysiology of DED [46]. Evidence exists that androgens, estrogens, and progestins act on many ocular structures, including the lacrimal gland, meibomian gland, conjunctival and corneal epithelia, iris, ciliary body, lens, and retina. The actions of these hormones influence structural and functional aspects of the eye, and they are implicated in other ocular diseases in addition to DED [47].

In men, anti-androgen therapy for prostatic indications is associated with alterations in meibomian lipid production and dry eye [48, 49]. Androgen deficiency is also linked to the progression of lacrimal gland inflammation, which is associated with Sjogren's Syndrome (SS). Counterintuitively, in the absence of autoimmune disease, androgen deficiency alone does not cause lacrimal gland inflammation or aqueous tear deficiency [50]. The decline in androgens that occurs in menopause, pregnancy, and lactation might be implicated in DED [47, 51]. One study demonstrated a decrease in MUC1 and MUC5AC production in women with DED as a result of androgen insensitivity syndrome [52]. Additionally, women with premature ovarian failure (POF), who suffer from androgen deficiency, exhibited an increased incidence of DED [53]. The hormonal changes that accompany the menstrual cycle, menopause, pregnancy, and contraceptive use are accompanied by corneal alterations and ocular symptoms [54-58]. Postmenopausal women receiving estrogen replacement therapy are particularly susceptible to experiencing dry eye symptoms (9.1% for those treated with estrogen and 6.7% for those treated with a

combination of estrogens and progestins) [59]. Estrogens seem to have a role in exacerbating inflammatory reactions and may be a risk factor for developing dry eye disease, but their role remains unclear. Human corneal epithelial cells showed an up-regulation of pro-inflammatory cytokines and MMPs upon exposure to estrogens [60]. On the other hand, another study demonstrated that exposure of 17-β-estradiol to human corneal epithelial cells inhibited the production of pro-inflammatory cytokines, IL-6, IL-1, and TNF-α, suggesting that estrogen may protect against inflammation [61]; thus the role of estrogen in DED remains unclear.

1.4.2. Inflammation

Exposure of the ocular surface to hyperosmotic tears activates a signaling cascade mediated by mitogen-activated protein kinases (MAPK) including c-Jun N-terminal kinase, p38, and extracellular signal-related kinase [62-64]. Activation of this pathway stimulates production of pro-inflammatory cytokines (IL-1, IL-8, TNF), chemokines, and Matrix metalloproteinases (MMPs) [63]. The pro-inflammatory environment promotes the maturation of immature antigen-presenting cells (APCs) into mature APCs. Mature APCs migrate to draining lymph nodes, where they activate CD4+ helper T cell (T_H) subtype 1 (T_H1) and subtype 17 (T_H17) [65, 66]. T_H17 antagonizes the function of immunosuppressive regulatory T cells (Tregs) in patients with DED. T_H17 secreted IL-17 and T_H1 secreted interferon (IFN) Υ further upregulate the production of pro-inflammatory cytokines, chemokines, MMPs (MMP-3 and MMP-9), cell adhesion molecules (CAMs), lymphangiogenic molecules (vascular endothelial growth factor [VEGF] D and VEGF-C.

This upregulation leads to infiltration by pathogenic immune cells and epithelial damage [67, 68]. Inflammation in the lacrimal gland and ocular surface leads to a self-perpetuating cycle that reinforces itself and increases the severity of the disease over time.

1.4.3. Mucin deficiency

Regardless of the underlying causes and subtypes of DED, tear film hyperosmolarity leads to ocular surface damage, inflammation, apoptosis, squamous epithelia metaplasia, and loss of mucin-producing goblet cells [7, 69, 70]. The tear fluid of patients with SS exhibits a reduction in MUC5AC protein and mRNA levels and a reduction in the number of goblet cells containing MUC5AC [71, 72]. One study showed that depletion of vitamin A led to a reduction in the amount of rMuc5AC and rMuc4 in rat subjects, and other studies revealed that the topical retinoid therapy is an effective treatment for keratoconjunctivitis sicca (KCS) [73-75].

There was a significant difference in the binding of antibody against the H185 antigen in patients with DED, but the mechanism behind this alteration in dry eye patients remains unknown [76]. Previous studies have shown that MUC16 is a barrier to bacterial adherence, internalization, and dye penetrance [30, 77]. A more recent study showed that knockdown of MUC16 decreased barrier functions, disturbed tight junctions, and inhibited transepithelial resistance [78]. Changes in mucin O-glycosylation have a potential role in DED, as indicated by altering H185 antibody binding, since the epitope is a carbohydrate. Patients with ocular cicatricial pemphigoid disease demonstrate an increase in expression of GalNAc-transferases during the keratinization process, implicating an initial attempt to

maintain the wettability of the epithelium since their expression is lost as the disease progresses [79]. Additionally, Versura et al., using a lectin-colloidal gold technique and transmission electron microscopy, found that there was a decrease in sialylated chains in patients with DED [80]. In another study that used HPLC and the CA 19-9 ELISA test, there was a decrease in sialic and sialyl-Lewis in the tears of patients with DED [81], but the decrease could have resulted from alterations in glycolipids and N-linked glycans, since sialyl-Lewis is not present in the O-glycans in the mucins of the tear film and conjunctiva [77, 82].

1.4.4. Lipid Abnormalities

The most common cause of evaporative dry eye disease (EDE) is meibomian gland dysfunction (MGD), defined by the International Workshop on Meibomian Gland Dysfunction as a chronic, diffuse abnormality of the meibomian glands, commonly characterized by terminal duct obstruction and/or qualitative/quantitative changes in the glandular secretion. Generally, EDE results in alteration of the tear film, eye irritation, and eye inflammation [40]. Reduced delivery of oil to the lid margin may result from congenital abnormalities in rare cases, including meibomian gland aplasia, dysplasia, or distichiasis, whereby an extra row of lashes take the place of the meibomian glands [9]. Obstructive MGD is the most common pathology and is characterized by hyposecretion due to blockage in the terminal portion of the duct by a thickened keratin containing meibum. Obstructive MGD can be further classified as cicatricial and non-cicatricial. In the cicatricial form, the duct openings are shifted posteriorly behind the mucocutaneous junction so the duct opens into the marginal conjunctiva and oil is delivered to the aqueous portion of the tear film

(termed retroplacement of the orifices). In non-cicatricial form, they remain in their normal positions, and the obstruction leads to cystic dilations and ultimately gland dropout [9, 14]. Cicatricial MGD accompanies cicatrizing conjunctivitis caused by disorders such as trachoma, erythema multiforme, cicatricial pemphigoid, chemical and thermal burns, and vernal and atopic kerato-conjunctivitis [14]. The other two main classes of MGD are hyposecretory and hypersecretory. Hyposecretory MGD is associated with medications for cystic acne, for which the goal is to cause sebaceous gland atrophy [83]. Hypersecretory MGD occurs in seborrheic dermatitis and acne rosacea [14].

1.5. Diagnosis

1.5.1. Symptom questionnaires

Although DED is typically symptomatic, approximately 40% of patients do not show symptoms [84]. Self-report surveys are commonly used to screen for DED, but a single agreed upon survey that can detect improvements over the course of treatment or one that can diagnose different subtypes of DED does not exist [46]. Schaumberg et al. proposed the most commonly used sequence of questions, which are listed below and were used in the largest population-based study of dry eye in men and women in the United States [85, 86].   1. Have you ever been diagnosed by a clinician as having dry eye syndrome?

2. How often do your eyes feel dry (not wet enough)?

3. How often do your eyes feel irritated?

More extensive questionnaires include the McMonnies questionnaire, Extensive Dry Eye Questionnaire (DEQ), the Ocular Surface Disease Index (OSDI), Contact Lens Dry Eye Questionnaire (CLDEQ), Schein, Utility Assessment, International Sjogren's Classification, the Standard Patient Evaluation of Eye Dryness questionnaire (SPEED), the Symptom Assessment in Dry Eye survey (SANDE), and the Impact of Dry Eye on Everyday Life survey (IDEEL) [33, 86-92].

1.5.2 Direct Observation

The optimal way to examine the tear film involves the use of noninvasive techniques that sample optical radiation reflected from the tear film such as interferometry, meniscometry, high speed videotopography, and optical coherence tomography (OCT) [93]. Measuring the tear meniscus with a biomicroscope provides an estimate of tear film. The normal height of the meniscus is 1mm, but it is smaller in patients with DED [94]. The corneal light reflex can be tested to measure the integrity of the tear film or ocular surface abnormalities and is determined as abnormal if the light is off center or unclear. It is also important that the clinician visually inspects the lids and lid margins since impaired lid function can affect the spreading of the tear film over the ocular surface. Signs of anterior blepharitis include debris, swelling, and erythema of the lid. Examination of the meibomian glands and the character of their secretion is important since MGD is the most common cause of EDE [40].

1.5.3 Tear osmolarity

As described by the dry eye workshop report, tear film instability and hyperosmolarity represent the two core features underlying DED [32]. Measurement of tear film hyperosmolarity, defined as 316 mOsmol/L, is a valuable method for diagnosing DED. However, the difficulties in measuring tear hyperosmolarity with previous methods such as the Ferning Test (TFT) have limited its application in a clinical setting [95]. Recently, the FDA approved a new tear osmometer (TearLab Corp, San Diego, CA) in the United States that has been in use in Canada and Europe and is undergoing clinical trials in Japan [96]. This device collects a 50nL sample in a microchannel upon contact of a disposable tip with the inferior lateral marginal tear strip (lab on a chip). It then gathers tear osmolarity measurements using an electrical impedance in three seconds. The collecting pen and tip are inserted into a desktop unit to display the measurement [94].

1.5.4 Tear Film Breakup Time (TBUT)

Fluorescein TBUT is the most common method used to measure tear film instability. Applying a flourescin strip to the inferior cul-de-sac instills a small amount of flourescin into the tear film. After several blinks, the examiner observes the tear film through a slit lamp with incident cobalt blue filtered light. The appearance of dark spots between 5-10 seconds indicates rapid tear film break up, which is an indicator of tear film instability in patients with mild to moderate DED [93].

1.5.5. Tear Secretion Rates

The Schirmer test quantifies tear production by the lacrimal gland over a fixed time interval. The examiner places a small strip (5 x 35mm) in the inferior cul-de-sac, waits for five minutes, and measures the length of the strip that is wet with tears. If an anesthetic is not instilled prior to performing the Schirmer test, then the test measures reflex tear secretion (Schirmer I). The Schirmer II test measures baseline tear secretion and is performed following anesthetic administration. Values of less than 5 mm of wetting in five minutes are indicative of DED, and normal values are greater than 10mm [93]. The phenol red thread test is similar to the Schirmer test. It involves placing a thread impregnated with phenol red dye to the lower margin of the eyelid and measuring the length of wetting after 15 seconds. Values greater than 10mm are normal [93]. Fluorophotometry is a more sensitive, yet costly, measure of tear secretion rate. It utilizes sodium fluorescein decay as a measure of tear flow and volume. It quantifies the tear turnover rate, defined as the percentage of fluorescein disappearance per minute after instillation. This value for tear turnover rate is lower in patients with DED [97].

1.5.6 Epithelial Staining

Instillation of topical dyes is an efficient way to examine the integrity of the corneal and conjunctival epithelia [93]. For fluorescein staining, a fluorescein-impregnated strip is applied to the eye, and the dye fluoresces green when excited by blue light. This technique reveals ocular surface damage. The typical staining pattern is confined to the exposed interpalpebral area of the ocular surface and may spread to the unexposed surface in

advanced DED. For optimal results, it is best to use a yellow barrier filter in combination with the standard blue excitation filter of the slit lamp [97]. With Rose Bengal staining, the amount of staining seen is dose-dependent. If the drop volume is reduced to avoid stinging, the amount of staining is reduced as well. It is best to apply anesthetic before instillation [93]. Lissamine green stains similar to Rose Bengal and is tolerated like fluorescein [97].

1.5.7 Inflammatory Molecules

Since the number of inflammatory mediators present in the tear film correlates with the severity of DED, measurement of different molecules could indicate their respective contributions to the disease. Techniques such as brush cytology, flow cytometry, and impression cytology are very effective for looking at the molecular expression of a cell, but problems arise because of the variable nature of different samples [98, 99]. A recently approved device for measuring MMP-9 expression is now available for clinical use in the United States, Canada, and Europe [100]. Detection of these inflammatory molecules may lead to earlier diagnosis and better treatment and management of DED.

1.5.8 Tear Protein Analysis

Researchers suspect that proteins play a crucial role in tear film stability and that the tears of patients with DED exhibit altered protein composition. Tear proteins are quantifiable using an ELISA assay. Colorimetric solid-phase ELISA is a good method for measuring lactoferrin. Concentrations of proteins such as aquaporin 5, epidermal growth factor (EGF), lipocalin, and IgA have been quantified by ELISA [81, 101, 102]. A lysozyme diffusion

test by van Bijsterveld et al. is used to measure lysozyme in tears, and tear lysozyme accounts for 20-40% of the protein in tears [103].

1.5.9 Sequence of Testing

The order of administration of the tests described above is important because of the potential impact one test can have on the results of another. The standard protocol is to perform the least invasive test(s), followed by more invasive test(s) as needed. The DEWS report recommends the following sequence: clinical history and symptom questionnaires, fluorescein BUT, ocular surface staining with fluorescein/yellow filter, Schirmer testing, and meibomian expression [14, 93]. Additional tests may be added according to their availability and operational factors. Of the methods discussed above, the more invasive tests include Rose Bengal staining, impression cytology, brush cytology, and flow cytometry.

1.6. Risk Factors

The most significant predisposing factors for DED include being female and being of advanced age [31, 46, 86, 104]. Research suggests that the decrease in androgens that occurs during the aging process and the increased use of hormone replacement therapy post-menopausally explains the age differential [49, 59, 105-110]. In addition to these demographic factors, environmental factors can increase the risk for DED development. Chronic visual activity such as computer use increases the risk for developing dry eye symptoms [111, 112]. Low environmental humidity increases the tear evaporation rate in

dry eye subjects and exacerbates their discomfort and irritation [113]. Other environmental factors such as air pollution, cigarette smoking, allergic conjunctivitis, high temperature and air velocity contribute to dry eye symptoms [31, 114]. Lifestyle factors such as alcohol consumption, multivitamin use, caffeine consumption, and topical use of anesthetics and preservatives also show an association with DED [31].

Contact lens-wearing, associated with decreased blink and evaporation rates as well as tear film instability, predisposes many patients to contact lens-related DED [115-117]. When describing their discomfort, contact lens-wearers often complain of dryness and decreased corneal sensitivity, and 50% of contact lens discontinuations are associated with this discomfort [90, 116, 118-120]. One study showed that an increase in tear osmolarity accompanied a period of contact lens wear among subjects who had not worn them before [121]. A reduction in the tear film lipid layer might result from contact lens wear as well [115].

Systemic diseases like arthritis, thyroid disease, diabetes, and autoimmune disorders are associated with DED [31]. Of the autoimmune diseases, Sjogren's Syndrome underlies a very large proportion of DED cases, as discussed previously [34]. A comparative study among patients with Sjogren Syndrome, patients with rheumatoid arthritis, and healthy controls revealed that patients with Sjogren Syndrome had higher dry eye severity scores and tear osmolarity, but elevated tear osmolarity persisted across all groups [7]. This supports the notion that DED is more severe in Sjogren's Syndrome compared to other patients with TDDE [122]. Another study found ocular disease and hyperosmolarity to be associated in approximately half of patients with Grave's Disease, and the increase did not result from proptosis and increased palpebral fissure, despite the fact that those factors may

contribute to DED through evaporative tear loss [123]. Undergoing refractive surgery, cataract surgery, or glaucoma therapy elevates the risk of developing DED. A recent survey demonstrated that DED is one of the most common complications following refractive surgery, and many of these patients exhibit changes in corneal sensitivity and tear stability [124]. A study evaluating patients that underwent LASIK eye surgery found that the mean tear osmolarity was elevated a year following the procedure [125]. Cataracts and DED are very common in the elderly population. A retrospective study indicated that following intraocular lens implantation, DED accounted for 15% of patients complaining of blurred vision post operationally [126]. Another study demonstrated that patients who underwent phacoemulsification experienced an adverse effect on corneal sensitivity and tear physiology, with tear physiology recovering after one month and corneal sensitivity improving after 3 months [127]. Topical glaucoma medications increase tear osmolarity and induce a variety of inflammatory reactions, possibly because glaucoma therapies contain preservatives such as benzalkonium chloride [128, 129].

1.7. Management of DED

DED disease management focuses on identifying the underlying etiology and treating the cause. Though there is no cure for dry eyes, a number of steps can be taken to control the dryness of the eye, relieve symptoms, minimize risk factors, prevent ocular damage and improve the quality of life. Several modalities exist for treating symptoms and consequences of dry eye [130]. These include: artificial tears, lipid-containing lubricants, liposomal spray, ophthalmic inserts, anti-inflammatory or immunosuppressant drops, antibiotics, dietary omega-3 essential fatty acids, autologous serum, intense-pulsed-light

(IPL), punctual plugs, moisture-retaining eyeglasses, hydrophilic bandage contact lenses and secretagogues (Fig .3). Treatment recommendations are based on the type and severity of dry eye disease.

1.7.1. Artificial tears (Ocular lubricants)

Artificial tears remain as the mainstay of treatment for dry eye, with global total annual sales of at least $540 million [131]. The ingredients used in the preparation of artificial eye drops act as artificial tears. Artificial tears are preferred, as they have a low toxicity profile and can be self-administered by patients. They contain water-soluble polymers that act as active ingredients for reducing the eye irritation. Polymers widely used in artificial eye drops include: cellulose derivatives, polyol liquids, polyvinyl alcohol, povidone, hyaluronic acid and polyacrylic acid. These polymers are used in varying concentrations either alone or in combination. These polymers act as demulcents and viscosity-enhancing agents. The derivatives and properties of these polymers are highlighted in Table 1.

These polymers also have the advantage of smoothing corneal surface irregularities, which leads to enhanced visual function [132, 133]. Viscosity is an important property of artificial tears. Higher viscosity increases the retention time of artificial tears on the ocular surface and enhances the level of comfort for patients by decreasing application frequency, but results in blurred vision [134]. For example, carboxymethyl cellulose (CMC), a viscosity-enhancing agent in artificial tears, binds to the epithelial layer and enhances re-epithelialization in cases of corneal damage [135, 136]. These polymers thicken the tear film and decrease the rate of tear loss. Hydroxypropyl methylcellulose (HPMC), another common ingredient, restores corneal density, which is low in dry eye patients [137].

Hydroxypropyl-guar (HP- gar) is used as a gelling agent in lubricant eye drops and can bind preferentially to the damaged area in the epithelial cells, providing protection to the ocular surface [138]. Hyaluronic acid is used as a viscosity-enhancing agent. Some studies have demonstrated its role in relieving the symptoms and improving vision in dry eye patients [139] by increasing tear breakup time [140, 141] and resulting in decreased rose bengal staining [141].

Artificial tears containing hyaluronic acid, such as Blink Tears[®,] are slowly gaining popularity. Hyaluronic acid, which is present in the aqueous humor, is in contact with both the corneal endothelium and vitreous humor. The viscoelastic property of hyaluronic acid reduces dry eye symptoms by increasing tear stability and reducing tear removal, thus exhibiting protective effects on the corneal epithelium [142]. Further, the viscoelasticity of hyaluronic acid could be enhanced by covalently crosslinking hyaluronic acid. A recent study by Willams and Maan concluded that the cross-linked hyaluronic acid applied t.i.d. significantly reduced the clinical signs associated with dry eye compared to a standard hyaluronic acid in a dog model [143]. In a different study, the efficacy of three types of ocular lubricants (0.1% or 0.3% sodium hyaluronate, carboxymethylcellulose, or HPMC) in protecting corneal epithelial cells was tested in dry eye animal models. The authors concluded that 0.3% sodium hyaluronate retained significantly longer than the other lubricants (all $P < 0.01$) [144]. Newer agents have been developed to reduce the dosing frequency and prolong the lubricating effect without compromising visual acuity. They are available as more viscous gel forms like Tears Naturale PM[®] (Alcon) lubricant eye ointment and Tears Again[®] Liquid Gel Drops (Cynacon/Ocusoft) [145]. Nevertheless, higher viscosity increases the shear on the ocular surface, resulting in discomfort.

Multidose bottles of artificial tears are generally preserved with preservatives such as benzalkonium chloride (BAK), chlorobutanol, ethylenediaminetetraacetic acid (EDTA), parabens, polyquaternium, sodium perborate, and stabilized oxychloro complex (SOC). However, preservatives can exacerbate the inflammation associated with dry eye [146]. The toxicity of BAK and chlorobutanol is well documented, and they are known to cause corneal neurotoxicity and accelerate the dryness of the ocular surface [147]. Polyquad is a quaternary ammonium preservative that is less toxic to the corneal and conjunctival epithelium than BAK [148]. Currently, single-dose, preservative-free artificial tears are available, like Tears Naturale free® and Bion Tears®, for patients who are hypersensitive to preservatives. Sodium perborate and SOC are categorized as oxidative preservatives. They are less toxic to the ocular surface and are sometimes called "vanishing preservatives". They decompose rapidly into salt and water in the presence of air and/or tear fluids. A list of commonly used preservatives is shown in Table II.

Electrolytes are yet another important component of artificial tears, in addition to polymers and preservatives. The surface of the ocular epithelium is devoid of blood supply, and tear fluids supply the necessary electrolytes and oxygen to the cornea. The amount and ratio of electrolytes in artificial tears reflect those of tear film for a better therapeutic outcome. The electrolytes in artificial tears also play an important role in healing corneal damage in DED [149]. When tested in a rabbit dry eye model, electrolytes increased the corneal collagen and conjunctival goblet cell density and reduced tear osmolarity [150]. Bicarbonates can boost the recovery of the damaged corneal epithelial barrier and protect the mucin layer [151]. Potassium helps maintain corneal thickness, which tends to decrease as a result of lacrimal dysfunction [152]. Hyperosmolar tear fluids is a common feature of most forms

of dry eye disease. Hypotonic artificial tears such as HypoTears® are used in patients producing hypertonic tears.

Table 1.1: List of polymers used in artificial tears

Type of polymer	Derivatives/concentration used % w/v	Properties
Substituted cellulose ethers	Hypromellose (0.2-2.5%) Hydroxyethylcellulose (0.2-2.5%) Methylcellulose (0.2-2.5%) Carboxymethylcellulose (0.2-2.5%)	• Cellulose derivatives act as demulcents • Act as viscosity-enhancing agent when concentration is increased • Viscoelastic property of polysaccharides increases the viscosity of tears
Polyols	Polyethylene glycol 300 (0.2-1%) Polyethylene glycol 400 (0.2-1%) Propylene glycol (0.2-1%) Glycerine (0.2-1%) Polysorbate 80 (0.2-1%)	• Used as a lubricant • Do not have viscosity property
Polyvinyl alcohol (PVA)	0.1-4%	• PVA has low viscosity • Used as a wetting agent at 1.4%
Povidone (polyvinyl pyrrolidone)	0.1-2%	• Wetting is improved when combined with polyvinyl alcohol
Carbomers (polyacrylic acid)	~0.2%	• High molecular weight polymers of acrylic acid • Mainly used for their viscoelastic property • Possess high viscosity when eye is stationary, and viscosity decreases dramatically during blinking • Longer retention time on ocular surface than polyvinyl alcohol

Table 1.1 (continued)

Dextran 70	0.1%	• Used in combination with other demulcents
Hyaluronic acid, autologous tears [153].	0.1-2.5%	• Biologically occurring polymer • Glycosaminoglycan biopolymer that shows long retention time on the ocular surface • Used a viscosity-enhancing agent

Table 1.2. List of commonly used preservatives in artificial tears

Preservatives	Concentration (% w/v)	Mechanism of action
Benzalkonium chloride	0.01-0.02%	Changes cell membrane permeability by lysing cellular membrane lipid bilayers [148]
Chlorobutanol	Up to 0.5%	Alcohol-based preservative that perturbs the microbial cell membrane lipid configuration [154]
Ethylenediaminetet raacetic acid (EDTA)	0.01-0.1% [155]	Chelates with trace amounts of heavy metals
Parabens	Methylparaben (0.015-0.2%) Propylparaben (0.005-0.01%)	Disrupts cell membrane and cause cell lysis
Polyquaternium-1 (Polyquad)	0.001% w/v	Detergent preservative that is derived from BAK but is less toxic to the cornea and the conjunctiva than BAK [156]
Sodium perborate	0.02% [157]	Oxidizes cell membrane, disrupts protein synthesis and affects membrane-bound enzymes
Stabilized oxychloro complex (SOC) (Purite)	0.005% [157]	Oxidizes glutathione and unsaturated lipids in the cell and disrupts protein synthesis

1.7.2 Lipid-containing lubricants

In dry eye, there is a significant decrease in the aqueous tear secretion from the lacrimal gland, while the evaporative form is mainly due to deficient or unstable tear film lipid layer, resulting in excessive evaporation of the aqueous tear layer [158]. The lipid layer of tears is generated by the meibomian gland and helps control tear evaporative rate. Lipid-containing lubricants are intended to replace the lipid entities in tear fluids. The most commonly used physiological lipids include phospholipid, triglyceride and fatty acids [159]. Some marketed lipid-containing lubricants are identified in Table III. Many studies have demonstrated the advantages of lipid containing lubricants over conventional tear supplements for the treatment of dry eye by improving lipid layer structure and decreasing tear evaporation rate compared with the conventional tear supplement [160]. However, a high concentration of lipid eye drops tend to cause blurred vision, resulting in patient dissatisfaction. The treatment of meibomian gland dysfunction (MGD) using emulsion-based eye drops has been reported to be safe and effective. Goto et al. [161] showed that low-concentration homogenized castor oil eye drops significantly improved the signs and symptoms of MGD without blurring the vision.

Table 1.3. List of lipid-containing lubricant eye drops

Name	Active ingredient	Concentration (% w/v or %v/v)	Lipid	Preservative
Soothe XP® Emollient eye drops (Bausch & Lomb).	Light mineral oil Mineral oil	1.0% 4.5 %	Mineral oil	Polyquaternium-1
Refreshs Optive Advanced Lubricant Eye Drops (Allergan)	CMC Sodium Glycerin Polysorbate 80	0.5% 1% 0.5 %	Castor Oil	Preservative-free
SYSTANE® BALANCE Lubricant Eye Drops (Alcon)	Propylene Glycol	0.6%	Mineral oil	Polyquaternium-1
Retaine® MGD™ Lubricant Eye Drops (OCuSOFT)	Light Mineral Oil Mineral Oil	0.5% 0.5%	Mineral oil	Preservative-free

1.7.3 Liposomal spray

More recently, a phospholipid liposomal spray (Tears Again®, Optima Pharmazeutische GmbH) was developed as a potential therapy for EDE. This formulation is sprayed onto the closed eyelids, and the liposomes migrate into the tear film via the lid margins. A study was conducted by Craig et al. [162] to investigate the short-term effect of a single application of the liposomal spray in subjects with normal and mildly symptomatic dry eye. The results demonstrated the ability of the liposomal spray to improve the stability of the tear film and increase the thickness of the lipid layer, with symptomatic improvement. The

use of the liposomal spray improved lid margin inflammation and visual acuity and increased patient adherence over conventional therapy [163-165].

1.7.4 Ophthalmic inserts

Ophthalmic inserts of hydroxypropyl cellulose ophthalmic insert (Lacrisert®) are used widely in treating moderate to severe dry eye syndrome. Lacrisert® is a water soluble, rod-shaped ocular insert (1.27 mm in diameter and 3.5 mm length) administered into the cul-de-sac of the eye once daily to protect and lubricate the eye [130]. Studies conducted to evaluate the efficacy of Lacrisert® have shown significant symptomatic improvement in dry eye patients, with improvement in quality of life and ability to perform daily activity [166, 167]. Hydroxypropyl cellulose ophthalmic inserts improve conjunctival staining and tear volume and disease index score [167]. They are relatively safe for long term use; however, blurred vision has been reported by some patients [166, 167]. For detailed information on hydroxypropyl cellulose ophthalmic inserts, readers can refer to the review article by Nguyen and Latkany [130].

1.7.5. Punctal plug

Occlusion of the tear drainage route as a method of increasing tear volume in severe dry eye was first described by Beetham in 1935 [168]. Punctal occlusion, which is considered when non-prescription or prescription eye drops fail to relieve dry eye condition, is the most common non-pharmacological treatment [169]. Punctal occlusions are also known as lacrimal plugs or occluders. Lacrimal punctal occlusion maximizes the contact time of tears with the ocular surface [170]. A punctal plug exerts its action by inhibiting the drainage of

natural and artificial tears through the ocular canaliculi [171]. Occulders have the ability to reduce patient dependency on tear supplements and improve visual acuity in dry eye patients [161, 171-173]. There are generally two types of punctal plugs: dissolvable plugs and semi-permanent plugs [174, 175]. Dissolvable plugs are made of absorbable materials such as collagen, while the latter are made of long-lasting materials such as silicone. Depending on the shape, punctal plugs are also classified into umbrella, tapered, hollow, reservoir, and slanted or low-profile caps. The most common problems associated with punctal plugs include: displacement or loss of the plug [176, 177], punctal scarring [178], epiphora [179], and formation of pyogenic granuloma [180]. Gupta and Chauhan [181] have developed punctal plugs of cyclosporine A (CsA) to offer a dual mechanism for treating dry eye syndrome. The system consisted of drug microparticles in a cylindrical hydroxy ethyl methacrylate core, surrounded by an impermeable silicone shell that covers half of the core. In addition to blockage of tears, this system releases CsA for up to three months in a zero-order fashion at a rate of ~3 μg/day. Though punctual plugs improve dry eye symptoms and prevent complications, they do not treat dry eye-dependent inflammation. Thus, alternate therapies involving anti-inflammatory drugs such as topical corticosteroids and nonsteroidal anti-inflammatory drugs, oral tetracyclines, and cyclosporine A (CsA) are being considered.

1.7.6 Anti-inflammatory treatment for dry eye

1.7.6.1 Cyclosporine A (CsA)

CsA is a neutral cyclic undecapeptide consisting of 11 amino acids with lipophilic property. CsA acts as immunosuppressant and many studies have shown that topical CsA reduces

conjunctival inflammation in moderate to severe dry eye patients [182-184]. CsA is effective in addressing the immune-mediated inflammatory processes associated with dry eye disease rather than being assuasive like lubricants. Topical CsA has the ability to decrease interluekin-6 (IL-6) in the conjunctival epithelium of dry eye patients [185], increase goblet cell numbers in Sjögren's Syndrome (SS) and non-SS dry eye patients, diminish epithelial turnover in those with non-SS dry eye [186], and significantly decrease the number of activated lymphocytes in the conjunctiva of dry eye patients [187]. Many studies have demonstrated the effectiveness of topical CsA in treating MGD (posterior blepharitis) mainly by enhancing the stability of tear film [188, 189]. The delivery of CsA in the form of eye drops is challenging due to its low water solubility and permeability. CsA ophthalmic emulsion 0.05% (Restasis[®], Allergan, Irvine, CA) is the only medication approved by the Food and Drug Administration (FDA) for treating the dry eye. However, many side effects such as blurred vision and itching have been reported with the use of Restasis[®] [190]. In a retrospective clinical comparative analysis, the effect of topical corticosteroids administered prior to topical CsA emulsion in patients with chronic dry eye disease was assessed. A group of 36 dry eye patients received loteprednol etabonate prior to instillation of CsA emulsion and a control group received CsA emulsion without concomitant loteprednol etabonate. The administration of loteprednol etabonate prior to CsA reduced the side effects like stinging that are associated with CsA emulsion, which is the most common reason for discontinuation of CsA treatment [191].

In an attempt to reduce the toxicity and increase the residence time of Restasis[®], many alternate strategies for delivering CsA have been reported in the literature and is

summarized in several reviews [192, 193]. Preservative free cationic and anionic emulsions of CsA were developed by Daull and coworkers for ocular delivery [194]. Cationic emulsion of CsA was found to be more effective than anionic emulsion and Restasis® in delivering the drug to the target tissues. This study confirmed the potential advantage of cationic emulsion as a vehicle for ocular drug delivery for treating surface ocular diseases. A novel microemulsion *in situ* electrolyte-triggered gelling system of CsA has been developed and tested by Gan et al. [195] for ocular delivery in an animal model. The system was prepared using Solutol HS 15 (as a surfactant), castor oil, glycerol and water and dispersed in Kelcogel® solution. Therapeutic levels of CsA remained in the cornea for up to 32 h with good ocular tolerance and no toxicity from the microemulsion system. This study showed the ability of microemulsions dispersed in an *in situ* gel to prolong precorneal residence time of drugs. In a different study CsA emulgel has been developed using polycarbophil as gelling agent. *In vivo* results showed that polycarbophil has the ability to prolong the retention time and enhance the bioavailability of CsA without ocular irritation [196].

Many researchers have attempted to prepare nanoparticles for CsA with synthetic and natural polymers for treating inflammatory and autoimmune ocular disease. Further, the ocular retention and drug availability can be altered by changing the surface properties of nanoparticles. CsA-loaded nanoparticles prepared using poly-lactic-co-glycolic acid (PLGA) or a mixture of PLGA and positively charged Eudragit®RL showed higher uptake in L929 cells compared to Carbopol® coated nanoparticles [197]. Poly-ε-caprolactone (PCL) is yet another biodegradable synthetic polymer widely used in ophthalmic drug delivery. CsA-loaded in PCL nanospheres were coated with hyaluronic acid and tested in

healthy rabbit corneas. Coated nanospheres showed about 10-15 fold higher drug concentration compared to CsA in castor oil and uncoated nanospheres [198]. Phenylboronic acid modified PLGA nanoparticles of CsA developed via nanoprecipitation method exhibited higher efficacy in a mice dry eye model [199]. Chitosan, a naturally occurring polymer, is used in ophthalmic preparations due to its mucoadhesive property. Nanoparticles prepared using chitosan exhibit longer contact time with corneal and conjunctival surfaces [200]. Hermans et al. [201] prepared CsA-loaded PLGA nanoparticles coated with chitosan and confirmed anti-inflammatory activity by inhibiting interleukine-2 in concanavalin A stimulated Jurkat T cells.

In addition to polymeric nanoparticles, solid lipid nanoparticles (SLN) and surfactants have been exploited as carriers for ophthalmic delivery of CsA. CsA-loaded liquid crystalline nanoparticles prepared using glyceryl monooleate (GMO) and poloxamer 407 showed higher penetration in rabbit corneas compared to oil solution with low ocular irritation [202]. CsA nanospheres made using lecithin, trilaurin, cremophor, polysorbate 20, sorbitan monooleate 80, and propylene glycol demonstrated increased penetration and lower irritation in a rabbit model compared with Restasis® [203]. Gökçe et al. [204, 205] reported higher uptake of CsA-loaded SLNs prepared using Compritol 888 ATO (Glyceryl behenate), Tween 80 and Poloxamer 188 in rabbit corneal epithelial cells. In the same study the authors have confirmed higher CsA concentration in aqueous humor of rabbit eyes. The modified Draize test confirmed no significant irritation in the rabbit eyes. SLNs with positive charge on the surface have been shown to interact better with corneal epithelial cells. CsA-loaded solid lipid nanoparticles (SLNs) when coated with chitosan resulted in

higher penetration and permeation in excised porcine corneas [206]. Battaglia et al [207] prepared CsA-loaded SLNs and coated them with stabilizers with varying charge. Cationic SLNs obtained by coating of SLNs with chitosan showed higher drug accumulation and higher permeability compared to non-ionic and anionic SLNs in corneal epithelial cells.

Nanocarrier systems such as liposomes, microemulsions and nanomicelles have also been reported for CsA. Karn et al. [208] prepared CsA encapsulated liposomes with phosphatidylcholine from soybean (Lipoid S100) and egg yolk (Lipoid E80) using supercritical fluid of carbon dioxide ($SCF-CO_2$) as an antisolvent. The SCF-mediated liposomes have been tested *in vivo* in a rabbit dry eye model. The result showed that CsA encapsulated liposomes caused significant improvement in Schirmer tear test values and have significantly higher tear film concentration of CsA as well as $AUC_{0-24\,h}$ with lower irritation compared to Restasis® [209]. Prolonged precorneal residence of CsA was achieved using microemulsion prepared using Solutol HS 15 as surfactant, castor oil, glycerol and water and dispersed in Kelcogel® solution [195]. When tested in New Zealand albino rabbits, CsA microemulsion maintained therapeutic concentration level in the cornea after 32h with good ocular tolerance. Tommaso et al. [210] developed methoxy poly (ethylene glycol)-hexylsubstituted poly (lactides) (MPEG-hexPLA) micelles of CsA using co-solvent method for ocular delivery. CsA micelles were found to be biocompatible in human corneal epithelial cells and the ocular tolerance has been confirmed in a rabbit model. CsA micelles also maintained therapeutic drug concentrations in cornea for a prolonged time period in a rat model [211]. Cholkar et al. [212] prepared CsA-loaded nanomicelles using polyoxyethylene hydrogenated castor oil 40 and octoxynol 40 by

solvent evaporation and film rehydration technique. The results from cytotoxicity study and *in vivo* study in New Zealand White albino rabbits demonstrated that the nanomicellar formulation of CsA is safe and effective in delivering the drug to anterior and posterior segments of the eye.

Prodrug strategy has also been widely used to design a water soluble prodrug of CsA in an attempt to overcome its low water solubility. Lallemand et al. [213] synthesized water soluble ester prodrugs of CsA for ocular delivery according to Wenger et al. [214]. The prodrugs were readily converted into CsA in the presence of human and rabbit tears, while the chemical conversion in phosphate buffer solution (pH 7.4) was negligible. These findings make this prodrug a good candidate for the treatment of dry eye syndrome and corneal graft rejection. Rodriguez-Aller et al. [215] synthesized CsA prodrugs and compared them with Restasis® in rabbit and rat models. The result showed that CsA prodrugs achieved higher bioavailability and lower elimination of CsA in anterior and posterior segments of the eye compared with Restasis®.

Collagen particles, collagen shields, contact lenses and implants of CsA have been developed for sustained delivery of the drug in DED therapy [216]. Peng and Chauhan [217] developed vitamin E loaded silicone-hydrogel contact lenses for delivering CsA in a sustained manner for up to a month. Kapoor et al. [218-220] developed surfactant-laden p-HEMA poly (2-hydroxyethyl methacrylate) hydrogels contact lenses for extended ophthalmic delivery of CsA. Episcleral CsA implants using silicone-based matrix design have been developed for treating lacrimal gland graft versus-host disease. These implants

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were found to be effective in a dry eye animal model by maintaining normal Schirmer scores over a 6-month follow-up with good safety profile [221, 222]. Pehlivan et al. [223] developed CsA-loaded nanodecorated biodegradable subconjunctival implants. Two kinds of CsA nanoparticles have been prepared using PLGA and PCL and loaded in implants prepared by electrospinning or molding techniques using two different polymers, PCL or poly-L-(lactide-co-caprolactone) (PLCL). *In vitro* release study revealed that implant prepared by molding method was able to sustained CsA release up to 2 months, while electrospun implants release CsA for up to a month. *In vivo* studies in Swiss Albino mice with DED following subconjunctival implantation showed that healing is significantly faster with implant formulation.

1.7.6.2 Corticosteroids

The efficacy of corticosteroids in treating dry eye disease has been supported by many clinical trials. The steroids have an inhibitory effect on the inflammatory cytokine production in human corneal cells. They inhibit IL-1 β, IL-6, IL-8 , MCP-3, RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted) and granulocyte macrophage colony-stimulating factor (GM-CSF) [224]. In a single-masked, randomized, prospective clinical trial in moderate-to-severe dry eye patients, the investigators found that the subjective and objective clinical parameters were improved by using topical corticosteroids, and this is accompanied by reduction of inflammation markers in the conjunctival epithelial cells [225]. In a randomized, double-masked, placebo-controlled clinical trial, topical loteprednol etabonate 0.5% administered four times a day was found to be effective in patients with dry eye disease [226]. However, long term administration

of corticosteroids is associated with several side effects, such as cataract and increased intraocular pressure, although this can be mitigated by pulse therapy [227]. The results from randomized clinical trials using ocular iontophoresis to deliver dexamethasone phosphate solution in dry eye patients showed significant improvement in signs and symptoms of DED. This technique acts by enhancing the mobility of charged particles by a small applied current field, and that increases drug concentration and enhances duration of action compared to the conventional dosage form [228].

1.7.6.3 Tetracycline antibiotics

Besides antibiotic activity, tetracyclines exhibit anti-inflammatory activity and that makes them good candidates for many ocular surface inflammatory diseases. Tetracycline antibiotics' ability to treat ocular surface disease has been attributed to inhibition of interleukin (IL-1) in the human corneal epithelium [229] and to the inhibition of MMP-9 production in the corneal epithelium, which further diminishes MMP-9 mediated corneal matrix degradation [230, 231]. In addition, tetracycline antibiotics have the ability to diminish the degradation products of the meibomian gland lipid by inhibiting lipase [232] [233] or by inhibiting bacteria responsible for its degradation [232]. Ocular surface inflammation caused in benzalkonium chloride in an induced murine dry eye model was successfully treated with doxycycline [234]. Doxycycline, a derivative of tetracycline, is a potent corticosteroid with partial absence of side effects [229]. The results of a randomized prospective study in 150 patients with chronic MGD showed statistically significant differences after treatment in tear breakup time test and Schirmer test results. This study concluded that low dose doxycycline (20 mg twice a day) therapy was effective

in patients refractory to conventional therapy [235]. Combination therapy of 0.025% doxycycline with 1% methylprednisolone has been shown to be effective in improving corneal smoothness and decreasing involucrin and small proline-rich protein immunoreactivity compared to physiologic saline solution [236].

1.7.7 Essential fatty acids

Omega-3 fatty acids are generally recommended as a dietary supplement for patients who do not respond to topical eye drops alone. Omega-3 and omega-6 fatty acids have the ability to reduce the inflammation and improve the symptoms of dry eye disease [237-239]. A study was conducted to evaluate the effect of topical omega-3 and omega-6 fatty acids in a mouse dry eye model. Mice were treated with alpha-linolenic acid (ALA), linoleic acid (LA), combined ALA and LA, or vehicle alone. Results from this study demonstrated that ALA significantly inhibited corneal IL-1α, tumor necrosis factor α (TNF-α), conjunctival TNF-α, and CD11b+ cell number associated with dry eye disease compared to other vehicles and control [240]. Many studies have demonstrated a decrease in inflammation of the ocular surface in DED with higher dietary intakes of omega-3 fatty acids [241]. For example, systemic linoleic acid (LA) and gamma-linoleic acid (GLA) were found to inhibit inflammation in a randomized clinical trial in patients with aqueous-deficient DED [242]. A recently published study by Tanaka et al. [243] showed that the deficiency of omega-3 can lead to an increase in the evaporation of the tear film due to inhibition of meibomian gland and oily tear secretion. Orally taken omega-3 fatty acids have shown significant improvement in the dry eye condition, with more prominent symptom reductions in patients with meibomian gland disease and blepharitis [244]. Omega-3 fatty acids also

reduced the dry eye symptoms in regular contact lens wearers [245]. Omega-3 fatty acid in combination with lipid emulsion eye drops and eyelid cleansing wipes significantly improved meibomian gland functionality in patients with lipid-deficient/evaporative dry eye disease (LDDE) [246].

Resolvin E1 is a metabolite of eicosapentaenoic acid (EPA), an omega-3 polyunsaturated fatty acid found in cold water fatty fish. The efficacy of RX-10005, a methyl ester prodrug of resolvin E1, was investigated in a murine model of dry eye [247]. This study concluded that RX-10005 improved the goblet cell density, indicating the potential utility of resolvin and its analogues in the treatment of dry eye.

1.7.8 Autologous serum

Tear fluids contain several growth factors such as transforming growth factor-ß (TGF), epidermal growth factor (EGF), and endothelin-1, which play an important role in wound healing [248-250]. Tear deficiency leads to a decrease in growth factor production. Autologous serum provides essential components like epidermal growth factor (EGF) and other kinds of growth factors and vitamin A that help in the wound healing process in response to corneal abrasion or ulceration [251]. Autologous serum is generally prepared by centrifuging venous blood and further diluting it to 20% with balanced salt solutions [252]. The efficacy of autologous serum in dry eye was first reported by Fox et al. in 1984 [253]. Unlike artificial tears, autologous serum provides both lubrication and growth factors, mimicking natural tears more closely. The results of a randomized, controlled crossover trial have demonstrated significant improvement upon treatment with serum eye drops compared to conventional therapy [254]. Serum eye drops were found to be

beneficial over artificial tears for the treatment of severe dry eye [255] and dry eye after laser-assisted *in situ* keratomileusis (LASIK) [256, 257]. Autologous serum is generally used as a second-line therapy, and published studies demonstrate its long-term safety in dry eye patients [258]. Autologus serum drops have shown objective improvement in signs such as tear breakup time, corneal staining, Schirmer values and subjective symptoms associated with corneal disease [259].

1.7.9 Intense-Pulsed-Light (IPL) Treatment

The meibum is a lipid-rich secretion produced by the meibomian glands. In chronic eye inflammation, abnormal blood vessels surround the meibomian glands and secrete inflammatory mediators that cause MGD, leading to the formation of an abnormal meibum [260]. IPL can effectively provide heat to meibomian secretions and seal abnormal telangiectatic vessels that discharge inflammatory mediators. In IPL, powerful and transitory bursts of light with wavelengths ranging between 500 and 800 nm are flashed using a xenon flash lamp. Several studies have confirmed the efficacy of IPL treatment in MGD [261, 262].

1.7.10 Moisture-retaining eyeglasses

The moisture-retaining eyeglasses act by increasing periocular humidity, which leads to a decrease in tear evaporation [263]. The use of moisture-retention eyewear is a time-honored approach, and ophthalmologists have consistently touted their potential in alleviating the signs and symptoms of dry eye [264]. According to report of the Management and Therapy Subcommittee of the International Dry Eye Workshop (2007),

despite the longstanding and persistent claims that using Moisture Chamber Spectacles improves dry eye symptoms, there is no high level of evidence supporting their efficiency for dry eye treatment [265].

1.7.11 Contact lenses

Gas-permeable scleral lenses have the ability to oxygenate, protect and moisturize the epithelium and promote the healing of corneal epithelial defects [266]. They provide protection and hydration to the ocular surface and, because of their oxygen permeability, gas permeable scleral lens can be considered for overnight wear [267]. In addition, gas permeable scleral lenses can also improve visual acuity and symptoms associated with dry eye like ocular discomfort [268].

Dimit et al.[269] reported an improvement in visual acuity in moderate to severe dry eye patients after wearing a scleral device (Prosthetic Replacement of the Ocular Surface Ecosystem – PROSE) without reporting any serious complication. Scleral contact lenses have diminished the visual discomfort associated with dry eye, decreased the need for artificial tears, and improved the quality of life in moderate to severe dry eye patients [270]. However, there are some drawbacks associated with scleral lenses, including difficulty in handling, cost [270], microbial keratitis [271], and corneal vascularization [272], but the occurrence of these side effects is not common.

1.7.12 Secretagogues

A secretagogue is a substance that results in the secretion of another substance. They are used very commonly in the management of dry mouth. Many compounds have the ability

to increase tear and mucin secretion in humans. One such compound is diquafosol tetrasodium, a P2Y2 agonist [273]. The P2Y2 receptor gene is present in the conjunctiva, corneal epithelia, meibomian glands and ductal cells [274]. The safety and efficacy of diquafosol tetrasodium was investigated in a randomized, double-masked trial consisting of 527 dry eye patients. This study concluded that 2% diquafosol was well tolerated and had significantly lower corneal staining scores compared to the placebo [275]. DIQUAS is a 3% ophthalmic solution of diquafosol tetrasodium approved in Japan for dry eye treatment in 2010. It is currently not marketed in the United States and is in a Phase III clinical trial [276].

15(S)-HETE (hydroxyeicosatetraenoic acid) has the ability to increase mucin secretion and stimulate MUC1, which is widely expressed in the conjunctiva, cornea, and mucus layer to protect the ocular surface [277]. The results of a study conducted in a rabbit model of desiccation-induced injury demonstrated that 15(S)-HETE stimulates ocular mucin secretion *in vitro* and effectively alleviates corneal injury and restores corneal integrity [278]. Rebamipide is a quinolinone derivative with mucin secretagogue activity [279]. In Japan, rebamipide is marketed under the trade name Mucosta® in two forms: 100-mg tablets and 20% granules for gastric mucosal disorders and gastritis. When administered to rabbits following the completion of N-acetylcysteine treatment, rebamipide increased the production of mucin-like substances in the cornea and conjunctiva [280]. The results of a randomized, multicenter, active-controlled, parallel-group study revealed that 2% rebamipide ophthalmic suspension improves the signs and symptoms of dry eye effectively with a well-tolerated profile [279]. A 2% rebamipide ophthalmic suspension is currently approved for the treatment of dry eye in Japan. Gefarnate is yet another mucin secretagogue

used to treat gastric ulcers in Japan. Studies have shown that gefarnate stimulates secretion of mucin like glycoprotein in rabbit conjunctival tissue and improves corneal damage in rabbit and cat dry eye models [281, 282]. Pilocarpine is a parasympathomimetic alkaloid widely used in treating dry mouth and glaucoma. Many studies have demonstrated the ability of pilocarpine to improve signs and symptoms of dry eye associated with Sjögren's Syndrome [283]. In a randomized placebo-controlled study in 256 patients with Sjögren's-related dry mouth and dry eye symptoms, oral pilocarpine significantly increased salivary flow at 20 mg/day and ocular symptoms, including a lower artificial tear requirement at a slightly higher dose of 30 mg/day [284]. However, a recent study by Hua et al. [285] conducted in just thirty-six adult subjects showed that topical pilocarpine (2% ophthalmic solution) positively increases salivary volume but adversely affects the visual acuity, contrast sensitivity, visual field and thus the overall visual function of participants. Further studies should be conducted to clarify pilocarpine use in dry eye management.

Cevimeline is approved by the FDA for treating dry mouth in Sjögren's Syndrome. Currently, studies are underway to evaluate the potential of cevimeline in improving dry eye symptoms and tear dynamics [286]. Ecabet sodium, sulfodehydroabietic acid monosodium salt pentahydrate, has successfully completed Phase IIb randomized clinical trial in 144 patients. The patients treated with ecabet sodium have demonstrated improvement in Schirmer's test results, which indicates an increase in the quantity of tears produced and in tear film breakup time.

Table 1.4. List of secretagogues
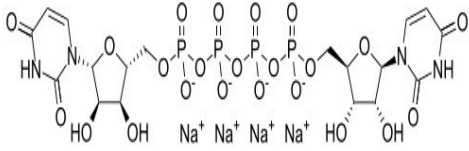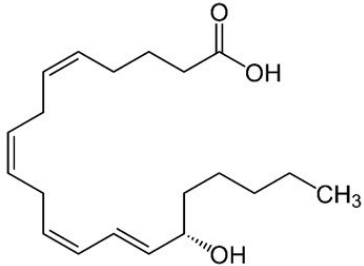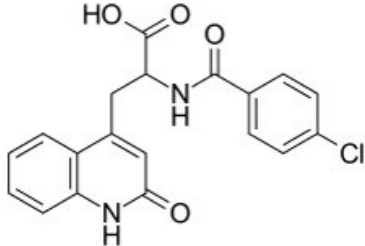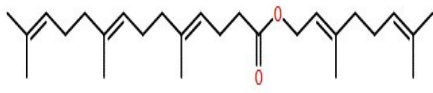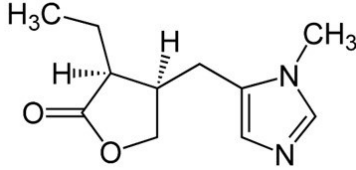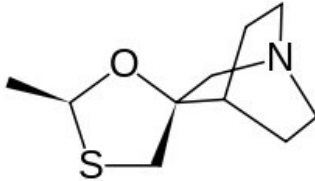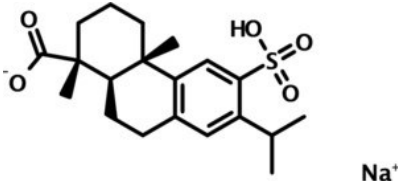
Name	Structure	Properties
Diquafosol tetrasodium		P2Y2 purinogenic receptor agonist.
15(S)-HETE (hydroxyeicosatetraenoic acid)		The exact mechanism is not clear but may be due to prostaglandin-like activity. [287]
Rebamipide		The exact mechanism is unknown but may be due to enhancing secretion of endogenous prostaglandin and prostaglandin independent mechanism [287].
Gefarnate		The mechanism is not clear but may be due to increased secretion of endogenous prostaglandin [287].
Pilocarpine		cholinergic agonist

Table 1.4 (continued)

Cevimeline		cholinergic agonist
Ecabet sodium		A new class of molecules that have the ability to increase the quantity and quality of mucin produced by conjunctival goblet cells and corneal epithelia. The mechanism may be due to stimulation of prostaglandin E2.

1.8. Challenges and future trends in the management of DED

With DED being a multifactorial condition, the primary challenge is the disease diagnosis. Around 40% of dry eye disease patients are asymptomatic. The lack of correlation between signs (tear breakup time, Rose Bengal staining, fluorescein staining, Schirmer test) and symptoms (dryness, soreness, redness, ocular surface discomfort, light sensitivity, and blurred vision) of DED poses a major challenge and affects both diagnosis and management of the disease [84]. The overlap of the symptoms of DED with those of conjunctivochalasis (gradual thinning and stretching of the conjunctiva with age) further

complicates the clinical picture. Most commonly used diagnostic techniques in DED are invasive in nature and fail to produce reproducible results, while the noninvasive tests developed over the last decade are not readily available in all clinics [288]. The lack of uniform criteria for assessing disease severity makes it difficult for an ophthalmologist to compare the results from different studies by different authors or to evaluate the usefulness of medications in DED treatment [289, 290].

To date, the only FDA-approved medication for treating DED is Restasis®. The continual advancement in the understanding of the pathogenesis of DED may open the door for finding new diagnostic tools and novel mechanisms for the treatment of this disease. With the increase in prevalence of DED, many pharmaceutical companies are focusing their efforts on finding a new medication. Many pharmacological treatments have been patented for the treatment of DED and are now in clinical trials. As inflammation plays a key role in the pathogenesis of DED, newer drugs are now being designed to combat inflammation. Some of these drugs act by inhibiting Janus kinase-3 ("Jak3") for treating inflammation [291], or by inhibiting matrix metalloproteinase-9 (MMP-9) and inflammation-mediated cytokines like interleukin (IL-1β) and tumor necrosis factor-α (TNF-α) in corneal epithelia of dry eye [292], or by inhibiting TNF-α to prevent the series of events that cause inflammation and apoptosis [293], or by acting as anti-lymphangiogenic agents to inhibit ocular inflammation associated with DED [294]. Other drugs that exert action on DED by increasing tear and mucus secretion, such as 3′,4′,5-trimethoxy flavone derivatives [295], purinergic receptor agonists [296], anti-KLK-13 antibody, which showed activity in increasing tear secretion [297], are also gaining popularity.

1.8.1. Novel therapies in clinical trials

A comprehensive discussion of novel therapies currently under investigation is beyond the scope of this review; however, a few will be reviewed to illustrate the spectrum of agents in the pipeline. Lifitegrast Ophthalmic Solution (5%) is currently undergoing a Phase III, multicenter, randomized, double-masked, and placebo-controlled trial in dry eye patients (ClinicalTrials.gov Identifier: NCT02284516). Lifitegrast is a novel, small-molecule LFA-1/ICAM-1 antagonist, and it is considered as the first integrin anti-inflammatory specifically developed for ocular use [298]. The results from Phase I [299], Phase II [300], and Phase III trials for efficacy and safety, as well as one long-term Phase III safety study [275, 301], revealed that lifitegrast is well tolerated by dry eye patients and also showed improvement in the signs and symptoms of the disease. Eleven Biotherapeutics is conducting a clinical trial for EBI-005 topical ophthalmic solution (ClinicalTrials.gov Identifier: NCT02405039). EBI-005 is a novel IL-1 receptor blocker for topical treatment of inflammation in dry eye [302, 303], and the results of Phase I and Phase II clinical trials showed promising results [304].

Cis-urocanic acid (cis-UCA) is shown to have anti-inflammatory and cytoprotective properties in UV-B-induced inflammation in the human cornea and conjunctiva [305]. The topical treatment with cis-UCA in a murine model of dry eye showed significant reduction in corneal staining [306] and good tolerability in a Phase I study. This molecule is currently in a Phase II clinical trial (ClinicalTrials.gov Identifier: NCT02326090). SYL1001 is found to be effective in treating the ocular pain associated with dry eye by targeting the transient receptor potential vanilloid 1 (TRPV1) receptor on the ocular surface [307]. Following

promising results from a Phase I study [308], SYL1001 is undergoing a dose efficacy study in patients with ocular pain (ClinicalTrials.gov Identifier: NCT02455999). Bromfenac is an nonsteroidal anti-inflammatory drug (NSAID) marketed in the US as an ophthalmic solution for treating ocular inflammation and pain after cataract surgery. Ista Pharmaceuticals is evaluating the safety and efficacy of bromfenac in DuraSite® ophthalmic solution (ISV-101) in DED patients in a Phase II study (ClinicalTrials.gov Identifier: NCT01478555) InSite Vision.

Recombinant Human Deoxyribonuclease Eye Drops (DNase eye drops) are being evaluated for the preliminary efficacy in patients with Sjogren's and Non-Sjogren dry eye disease in a Phase II study (ClinicalTrials.gov Identifier: NCT02193490). DNase eye drops act by decreasing extracellular DNA (eDNA), which is abundant in tear fluids of patients with DED [309, 310]. Kissei Pharmaceutical Co., Ltd. is conducting a Phase II clinical trial for KCT-0809, a thromboxane A2 synthase inhibitor with anti-inflammatory property.

Ocular Technologies SARL is planning a Phase II/III study for OTX-101 (ClinicalTrials.gov Identifier: NCT02254265). Dompé Farmaceutici S.p A has recently completed its phase I study for human nerve growth factor (rhNGF), a neurotrophin with beneficial effects in treating damage or degeneration of the cornea, conjunctiva, retina, or optic nerve in diseases such as neurotrophic keratitis, corneal ulcers, dry eye, retinitis pigmentosa, and glaucoma [311]. Currently, it is recruiting participants for a Phase II study to assess the efficacy and safety of different doses of rhNGF when administered as eye drops to dry eye patients (ClinicalTrials.gov Identifier: NCT02101281) [311]. In place of serum, autologous blood from a fresh finger prick is being studied for treatment of dry eyes and corneal ulcers/epithelial defects by Bedford Hospital NHS Trust (ClinicalTrials.gov

Identifier: NCT02153515). The results from unpublished case reports conclude that fresh blood is an effective tool for treating the corneal pathology. Universidad Nacional de Colombia is conducting a phase 3 study of Platelet-Rich Plasma (PRP) for cellular restoration and its effects in lacrimal production in patients with severe dry eye (ClinicalTrials.gov Identifier: NCT02257957). Civamide is a TRPV-1 receptor modulator and neuronal calcium channel blocker. Intranasal formulation of civamide in a Phase III clinical trial for treating various types of pain has resulted in increased tear production in more than 50% of the patients. This finding has prompted Winston Laboratories to conduct a open-label, Phase II study to assess the tolerability and efficacy of civamide nasal solution 0.01% in patients with dry eye syndrome (ClinicalTrials.gov Identifier: NCT02116244).

Alcon Research is currently in a Phase II study (ClinicalTrials.gov Identifier: NCT02365519) with LME636, a TNF-alpha inhibitor. DH Bio Co., Ltd. has recently completed a multicenter Phase III study to determine the efficacy and safety of Haporine-S eye drops for patients with moderate to severe dry eye disease (ClinicalTrials.gov Identifier: NCT01804361). OTX-DP is a dexamethasone-eluting punctum plug that releases dexamethasone in a sustained manner for four weeks. OTX-DP was initially developed for the treatment of post-operative inflammation and pain; however, it is also being investigated for allergic conjunctivitis and inflammatory dry eye by Ocular Therapeutix, Inc (ClinicalTrials.gov Identifier: NCT02468700). Vitamin D and Omega-3 (VITAL) is being studied for its potential to reduce the incidence and/or progression of dry eye disease by Brigham and Women's Hospital (ClinicalTrials.gov Identifier: NCT01880463). Amnion-derived Cellular Cytokine Solution (ACCS) eye drops are currently undergoing a Phase I study (ClinicalTrials.gov Identifier: NCT02369861).

Allergan is carrying out a Phase I trial for AGN-232411 topical ophthalmic solution (ClinicalTrials.gov Identifier: NCT02420730) and Phase II trial for AGN-223575 Ophthalmic Suspension (ClinicalTrials.gov Identifier: NCT02435914). Other formulation beings investigated by Allergan include 0.1% CsA, Restasis® X, which is currently in Phase II (ClinicalTrials.gov Identifier: NCT02013791)

Table 1.5: US patents issued between January 1st, 2010 and June 30th, 2015 that contain the words "dry eye" or "keratoconjunctivitis sicca" in the title.

US patent number	Issued date	Title
7,691,811	April 6, 2010	Transporter-enhanced corticosteroid activity and methods and compositions for treating dry eye
7,745,461	June 29, 2010	Method of treating dry eye disorders
7,759,322	July 20, 2010	Method of treating dry eye disease with purinergic receptor agonists
7,825,102	November 2, 2010	Treatment of dry eye conditions
7,897,642	March 1, 2011	Compositions and methods for dry eye syndrome
7,923,471	April 12, 2011	Method of treating dry eye disorders and uveitis
8,114,420	February 14, 2012	Composition for treating dry eye and related methods of manufacture and methods of use
8,207,226	June 26, 2012	Use of FAAH antagonists for treating dry eye and ocular pain
8,211,434	July 3, 2012	KLK-13 antibody inhibitor for treating dry eye
8,211,942	July 3, 2012	Compositions and methods for dry eye syndrome
8,293,713	October 23, 2012	Beta-turn peptidomimetic cyclic compounds for treating dry eye
8,314,118	November 20, 2012	Methods and compositions for treating dry eye
8,491,905	July 23, 2013	KLK-13 antibody inhibitor for treating dry eye
8,541,426	September 24, 2013	Pharmaceutical compositions and methods of treating dry eye disorders
8,614,178	December 24, 2013	Pharmaceutical composition for treatment of dry eye syndrome
8,722,728	May 13, 2014	Composition and method for treating dry eye syndrome

Table 1.5 (continued)

8,748,391	June 10, 2014	.beta.-turn peptidomimetic cyclic compounds for treating dry eye
8,821,874	September 2, 2014	KLK-13 antibody inhibitor for treating dry eye
8,828,412	September 9, 2014	Stable polyphenol containing ophthalmic emulsion for treating dry eyes
8,871,995	October 28, 2014	Method for screening an agent for its usefulness in the treatment of dry eye and/or corneal and conjunctival lesion and pharmaceutical composition obtained by the method
8,918,181	December 23, 2014	Systems and methods for treatment of dry eye
8,957,048	February 17, 2015	Compositions for the treatment of dry eye
8,980,898	March 17, 2015	Dendrimer-like amino amides possessing sodium channel blocker activity for the treatment of dry eye and other mucosal diseases
8,987,241	March 24, 2015	Time-release and micro-dose formulations for topical application of estrogen and estrogen analogs or other estrogen receptor modulators in the treatment of dry eye syndrome, and methods of preparation and application
9,006,208	April 14, 2015	Agent for treatment of dry eye characterized by combining P2Y.sub.2 receptor agonist and hyaluronic acid or salt thereof, method for treating dry eye, and use of the P2Y.sub.2 receptor agonist and hyaluronic acid or salt thereof
9,011,861	April 21, 2015	Therapeutic compositions for the treatment of dry eye disease
9,044,388	June 2, 2015	Dry eye treatment

Chapter 2

Significance of Research

Thus far, artificial tears are considered the cornerstone treatment of dry eye disease (DED). The water-soluble polymers in artificial tears act as demulcents and lubricant for reducing the eye irritation. These polymers also have the ability to enhance visual function via smoothing the corneal surface irregularities [132, 133]. Hydroxypropyl methylcellulose (HPMC) is one such polymer that has the ability to restore corneal density, which is low in dry eye patients [137].

Cyclosporine A (CsA) is yet another treatment option effective in addressing the immune-mediated inflammatory processes associated with DED. Restasis® is the marketed ophthalmic emulsion of CsA and is the only medication approved by the Food and Drug Administration (FDA) for treating DED. The daily dose of CsA ophthalmic emulsion is about 25 μg administered twice a day. In addition to the drug, Restasis® contains inactive ingredients such as glycerin, castor oil, polysorbate 80, and carbomer copolymer type A. Many side effects including blurred vision and itching have been reported with the use of Restasis® [190]. Moreover, topically administered drugs in the form of eye drops are eliminated quickly due drainage and tear turnover resulting in low ocular bioavailability [312-314].

This work intends to develop a once-a-day ophthalmic insert that combines the lubricating effect of HPMC and the anti-inflammatory effect of CsA. We hypothesized that combination treatment with HPMC and CsA would be beneficial in treating DED. An ophthalmic insert can increase the residence time of CsA in the precorneal area or cul-de-sac and thus enhance bioavailability and reduce the adverse effects associated with Restasis® eye drops. In addition, ophthalmic inserts will enhance patient adherence by reducing dose frequency. It is also possible to reduce the exposure of the eye to toxic preservatives used in artificial tears [315]. Xanthan gum (XG) has been used as a release-retarding agent. XG is used in ophthalmic preparations to prolong the contact time of drugs with the ocular surface [316]. A recently published article also highlighted the potential activity of XG as an antioxidant in DED [317]. The mixture of polymers used in our study are biocompatible, inert, biodegradable and non- immunogenic [318].

In this work, we prepared and characterized ophthalmic inserts of CsA using HPMC and XG. To our knowledge, this is the first *in vitro* study to design ophthalmic inserts containing both CsA and HPMC for treating DED by a dual approach, CsA to reduce the immune-mediated inflammatory processes and HPMC as a demulscent. Inserts can increase the ocular bioavailability of CsA by enhancing its residence time in the precorneal area.

Chapter 3

Formulation and *in vitro* evaluation of cyclosporine A inserts prepared using HPMC for treating dry eye disease

3.1. Abstract

Aim: The aim of this study was to develop and characterize a novel sustained-release drug delivery system of cyclosporine A (CsA) using hydroxypropyl methylcellulose (HPMC) and xanthan gum (XG) for treating dry eye disease (DED).

Methods: Polymeric inserts of CsA were prepared using the solvent casting technique with a 2^3 full factorial design to evaluate the effect of HPMC, XG ratios and drug content on thickness, folding endurance, wettability and *in vitro* drug release. Inserts were also evaluated for drug content, moisture absorption and loss, and surface pH. Optimized CsA-loaded HPMC inserts were sterilized with UV light and evaluated for morphology, thermal analysis, FTIR, stability at 4°C, 25°C and 40°C, cytotoxicity in cultured bovine corneal endothelial cells, and anti-inflammatory effect in Jurkat T cells.

Results: The addition of XG increased CsA release duration and enhanced the folding endurance of films. All films showed uniformity in drug content and thickness. Formulation F4 composed of 1% w/v HPMC and 0.25% w/v XG exhibited good folding endurance and sustained CsA release for up to 20h. Sterility testing of F4 using plate and

direct inoculation confirmed the formulation sterility and validated the sterilization method. The formulation was found to be stable for at least 3 months at 4°C, 25°C and 40°C. No cytotoxicity was observed in cultured bovine corneal endothelial cells for up to 24 h. The anti-inflammatory effect of CsA was intact in ophthalmic inserts.

Conclusion: In conclusion, the combination therapy with HPMC and CsA can be a potential once-a-day formulation for treating DED.

3.2. Introduction

Dry eye disease (DED) is a significant public health concern and is often unrecognized. DED affects 5% to 34% of people around the world [7]. A study found that DED affects as many as 17% of women and 11.1% of men in the United States, and this difference persisted across all ages [31]. DED is a disease of the tear film resulting from tear deficiency or extreme tear evaporation causing damage to the interpalpebral ocular surface [1], dryness, light sensitivity, blurred vision, redness, grittiness, foreign body sensations, pain, stinging, ocular fatigue, scratchiness and itchiness [2]. DED is usually associated with hyperosmolarity of the tear film and inflammation of the ocular surface [41]. DED is mainly treated using cyclosporine A (CsA) ophthalmic emulsion and artificial tears.

CsA is a lipophilic, neutral cyclic undecapeptide consisting of 11 amino acids. CsA acts as an immunosuppressant and reduce conjunctival inflammation in moderate to severe dry eye patients [182-184]. The delivery of CsA in the form of eye drops is difficult due to its low water solubility. Restasis® (Allergan Inc., Irvine, CA) is a 0.05% cyclosporine ophthalmic emulsion approved by the US FDA for treating DED. However, many side

effects such as blurred vision, itching, conjunctival hyperemia, discharge, foreign body sensation, and stinging have been reported with the use of Restasis® [190]. Moreover, drugs applied topically as eye drops are eliminated quickly from the precorneal area due to the protective mechanisms of the eye resulting low bioavailability (< 5%). To compensate for the drug loss by protective mechanisms, many drugs are either applied in high concentrations or administered frequently which increases the risk of systemic toxicity [312, 313, 319]. Researchers have tried to reduce the toxicity and increase residence time of Restasis® using novel delivery strategies such as prodrugs [213], liposomes [208], nanoparticles [199-202], nanospheres [198, 203], solid lipid nanoparticles (SLNs) [204-207], micelles [210, 212], collagen particles and collagen shields [216, 320], contact lenses [217-220], ocular films [321], implants [221-223], and emulgels [196]. However, none of these strategies have succeeded in becoming a clinical reality [322]. Another approach for treating DED is by using artificial tears which contain water-soluble polymers such as hydroxypropyl methylcellulose (HPMC) that reduce the eye irritation by smoothing the corneal surface irregularities [132, 133]. Higher viscosity increases the retention time of artificial tears on the ocular surface and enhances the level of comfort in patients by decreasing application frequency, but results transient blurring of vision [134]. The results of a randomized study has demonstrated the ability of HPMC to restore corneal density in dry eye patients [137]. Lacrisert® (hydroxypropyl cellulose ophthalmic insert) is a sterile, rod-shaped, preservative-free, slow-release, lubricant which is placed into the inferior cul-de-sac of the eye in patients who do not respond to artificial tears [131].

An ocular insert is a solid devise placed in the conjunctival sac. Ocular inserts are known to enhance the bioavailability of drugs by increasing the contact time and releasing the drug in a sustained manner [323-325]. They have numerous advantages over conventional eye drops such as: enhance patient adherence by decreasing dose frequency, provide accurate dosing and minimize systemic side effects in comparison with eye drops, release the drug in a sustained manner, enhance the bioavailability by increasing the contact time of the drug with ocular surface, do not require preservatives, increased shelf life, and possible inclusion of microparticulate, prodrug and mucoadhesives [315]. The most common disadvantage associated with inserts is the foreign body sensation with initial administration and difficulty in placement and removal of inserts [315]. This could be mitigated by using a biocompatible hydrophilic polymer and not requiring removal from the eye after use [326]. To date, many research groups have reported delivery of several drugs using ocular inserts such as dexamethasone [327], dexamethasone and gentamicin [328], ofloxacin [329-332], pefloxacin mesylate [333], ciprofloxacin [334-337], diclofenac [323], lysozyme [338], phenylephrine and tropicamide [339], valacyclovir HCl [340], brimonidine tartarate [341-343], gatifloxacin sesquihydrate [344], tilisolol [325, 326, 345], cyclosporine A [321, 346], lidocaine HCl [347], acyclovir [348], flurbiprofen sodium [349], piroxicam [350], moxifloxacin [351], and azithromycin [352].

In this work we propose the delivery of CsA using ophthalmic inserts of HPMC, which is a cellulose derivative polymer widely used in artificial tears with good mucoadhesive and film forming properties. We hypothesize that combination treatment with HPMC and CsA would be beneficial in treating DED. We also added xanthan gum (XG) as a mucoadhesive

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polymer and release-retarding agent. XG is a high molecular weight extracellular anionic polysaccharide produced by the fermentation of Xanthomonas campestris. XG has been successfully employed as a mucoadhesive controlled-release excipient for buccal drug delivery [353-357] and oral tablets [358-360]. XG is used in ophthalmic preparations to prolong the contact time with the ocular surface. XG, along with guar gum (GG) and poloxamer, are used with *in situ* gel-forming eye drops. The results showed that addition of XG: GG (in 3:7 ratio) can decrease the release rate of atropine sulphate and increase bioavailability by enhancing precorneal residence time [316]. XG is present in marketed formulations such as Lubristil® Gel (Moorfields Pharmaceuticals, UK) indicated for use in treating DED. A recently published article highlighted the potential activity of XG as an antioxidant in DED [317]. In this study, we prepared and characterized polymeric inserts of CsA using the solvent casting technique with a 2^3 full factorial design to evaluate the effect of HPMC and XG ratios and drug content on thickness, folding endurance, wettability and *in vitro* drug release. The optimized insert was further evaluated for morphology, thermal analysis, FTIR, stability at 4°C, 25°C and 40°C, cytotoxicity in cultured bovine corneal endothelial cells, and anti-inflammatory effect in Jurkat T cells.

3.3. Materials and Methods

3.3.1. Materials

Cyclosporine A (Lot C143639) was procured from PCCA (Houston, TX). Hydroxypropyl methylcellulose (HPMC) (Methocel® E4M premium CR (hypromellose USP) (Lot C143851) was purchased from PCCA (Houston, TX). Xanthan gum (Lot XV0506) was

purchased from Spectrum Chemical (Gardena, CA). Polyethylene glycol 400 (Lot 2603104) was procured from Hampton Research (Aliso Viejo, CA). Ethanol (Lot B0522876) was supplied by ACROS (Fair Lawn, NJ). Sodium hydroxide (Lot YI0086) was procured from Spectrum Chemical (Gardena, CA). Potassium phosphate monobasic KH_2PO_4 (Lot 103497), polysorbate 80 (Lot 20589), tryptic soy broth (Soyabean Casein Digest medium-BactoTM, Lot 2030828), DMSO (Lot 104549) and Mueller-Hinton agar (Lot 3240477) were procured from Fisher Scientific (Pittsburgh, PA). Phosphoric acid (Lot A0305025) was procured from ACROS (Fair Lawn, NJ). TACS® MTT reagent (Lot 31205J14) was supplied from Trevigen. RPMI Medium 1640 (Lot 1738144) was supplied from (Life technologies, NY). DuoSet IL-2 ELISA kit (Lot 1376605) was procured from (R&D Systems, MN). T cell activator concanavalin A (Lot SLBK7858V) was supplied from Sigma-Aldrich (St. Louis, MO). High Performance Liquid Chromatography (HPLC) solvents, including acetonitrile (Lot 121151) were purchased from Fisher Scientific (Pittsburgh, PA). Distilled deionized water was used throughout the study.

3.3.2. *Preparation of CsA ophthalmic insert*

3.3.2.1. *Experimental design*

A three-factor two-level (2^3) full factorial experimental design was used to evaluate the effect of different parameters on the properties of inserts [361]. The three factors (X) studied were concentrations of HPMC (X1), XG (X2), and CsA (X3). Response variables were folding endurance, wettability, film thickness and *in vitro* release. The design resulted in $2^3 = 8$ different insert formulations (Table 3.1). All preparations were made in triplicate and the Design of Experiments (DoE) data analysis was done using JMP Statistical

Discovery v7.0.1 (SAS, USA). Each variable to be optimized was coded at two levels, -1 and 1 (Table 3.2). Data from the factorial experimental design was subjected to multiple regression analysis using the least square regression method to obtain parameters of mathematical models. Analysis of variance (ANOVA) was applied to evaluate the statistical significance of the model. A value of $p < 0.05$ was considered significant. R^2 and adjusted R^2 were also calculated for the regression model. Significant factors and interactions were identified by t test at 95% significance level. The model was validated by checking model assumptions and actual by predicted plot.

Table 3.1: Quantitative composition of ophthalmic inserts

Formulation code	Pattern[a]	HPMC (%, w/v) X_1	XG (%, w/v) X_2	CsA (%, w/v) X_3	PEG 400 (%, w/w) relative to dry weight of polymer
F1	---	1	0	0	30
F2	--+	1	0	0.024	30
F3	-+-	1	0.25	0	30
F4	-++	1	0.25	0.024	30
F5	+--	2	0	0	30
F6	+-+	2	0	0.024	30
F7	++-	2	0.25	0	30
F8	+++	2	0.25	0.024	30

[a]: (-) low, (+) high

Table 3.2: Experimental values and coded levels of the independent variables used for the 2^3 full factorial design.

Independent variables	Code units	Coded variable level	
		-1	**1**
HPMC% w/v	X_1	1	2
XG% w/v	X_2	0	0.25
CsA% w/v	X_3	0	0.024

3.3.2.2. Preparation of blank and CsA loaded ophthalmic insert

CsA ophthalmic inserts were prepared using the solvent casting evaporation technique. HPMC E4M solutions (1% or 2% w/v) with or without XG (0.25% w/v) were prepared in distilled water (40 ml) using a magnetic stirrer. The mixture was stirred for 1 h. CsA was dissolved in ethanol and added to the mixture resulting in 0.024% w/v drug concentration. PEG 400 (30% w/w relative to the dry weight of the polymer) was then added as plasticizer and the mixture was stirred for 3 h and sonicated for 30 minutes (FS140H, Fisher Scientific) to remove any air bubbles and disperse the drug uniformly. The films were cast into polystyrene dishes (100 x 15mm) and allowed to dry at room temperature for 48 h. Inserts of 6mm diameter (~50 µg of CsA/insert) were punched out and stored in a sealed dark container. The blank inserts were also prepared by the same method without using CsA.

3.3.3. HPLC analysis of CsA

HPLC (Waters Alliance e2695 separation module, Milford, MA), equipped with a 2998 PDA detector and reverse-phase C18 column (Dimension mm: 250 x 4.6 mm) (5 µm, Hypersil™ ODS, Thermos scientific, MA), was used to analyze CsA in the formulation.

An isocratic method was used for analysis with mobile phase containing acetonitrile/1 mM H$_3$PO$_4$ in water, pH 3.1 (70:30) pumped at a flow rate of 1.3 ml/min. The column was maintained at 65°C and injection volume was 20 µl. The absorbance of CsA was measured at 210 nm. The drug content was determined quantitatively by plotting a calibration curve. A stock solution (1 mg/ml) of CsA was prepared in methanol and calibration standards ranging from (0.195 - 50 µg/ml) were prepared in the mobile phase. Each standard was analyzed in triplicate and average peak areas were plotted against concentration to obtain the calibration curve.

3.3.4. Drug content

CsA inserts were dissolved in 1 ml of ethanol in an Eppendorf tube and vortex thoroughly. The drug content was analyzed using HPLC. Drug content was measured in triplicate [335, 345].

3.3.5. Thickness measurement

The thickness of the inserts was determined using a digital micrometer (Mitutoyo Absolute, model PK-0505CPX, Japan) and reported as the mean value of ten independent measurements. The inserts were randomly selected for thickness measurement [335, 350].

3.3.6. Folding endurance

The folding endurance is the number of times that the insert could be folded without breaking. The folding endurance was carried out to determine the mechanical properties of

the inserts. It was determined by repeatedly folding inserts at the same place until they broke. The mean value of three measurements was recorded.

3.3.7. Wettability study

Contact angle measurement is used to determine the surface wettability and hydrophilicity /hydrophobicity of the surface and surface energy. Simulated tear fluid (STF) composed of sodium chloride (0.670 g), sodium bicarbonate (0.200 g), calcium chloride dehydrate (0.800 g) in 100 ml of distilled deionized water was used. The contact angle of inserts was measured by Half-Angle™ technique using a contact angle meter (CAM-PLUS MICRO model, Tantec INC, IL) (Figure 3.1). A droplet of STF was deposited on the surface of inserts from a microsyringe needle and the angle of droplet was measured immediately from the droplet image projected on the screen [362]. The average of ten independent measurements was reported.

Figure 3.1: Contact angle measurement

3.3.8. In vitro drug release study

The *in vitro* release of CsA from inserts was performed using a dialysis membrane (Fisherbrand® Dialysis tubing, MWCO: 12,000-14,000Da, 0.45µm pore size) and two-chamber side- by- side diffusion cell. The dialysis membrane containing an insert was sandwiched between the two half-cells of a two-chamber side-by-side diffusion cell (Figure 3.2). The phosphate buffer (pH 7.4) containing 0.025% (w/v) Tween 80 was used in order to maintain sink conditions. The content in both chambers was stirred continuously using a magnetic stirrer at 60 RPM. Temperature was maintained at 34±0.5°C by circulating water jacket to mimic the eye temperature [363, 364]. At predetermined time intervals, 300 µl of sample were withdrawn and replaced with an equal volume of fresh buffer. The study was carried out in triplicate under sink condition and samples were analyzed using HPLC.

Figure 3.2: Two- chamber side- by- side diffusion cell set-up

3.3.9. Moisture absorption

Percent moisture absorption was carried out to check the physical stability and integrity of CsA inserts in humid condition. The inserts were weighed and placed in a desiccator

containing a saturated solution of aluminum chloride (80% RH). After three days the inserts were taken out and weighed. This test was done in triplicate [365]. The percent moisture absorption was determined using the following equation.

$$\text{Percent moisture absorption} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \; x \; 100$$

3.3.10. Moisture loss

Percent moisture loss was carried out to check the physical stability and integrity of CsA-loaded HPMC inserts in dry conditions. The inserts were weighed and placed in dedicator containing anhydrous calcium chloride. After three days the inserts were taken out and weighed. This test was done in triplicate [365]. The percent moisture loss was determined using the following equation.

$$\text{Percent moisture loss} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \; x \; 100$$

3.3.11. Surface pH

The pH of inserts was determined using an Accumet$^®$ excel XL 25 pH meter (Fisher Scientific, Pittsburgh, PA). The pH meter was calibrated with standard buffer solutions of pH 4, 7, and 10 before each use. The inserts were placed in vials containing double distilled water. The inserts were allowed to swell for 30 minutes. The pH meter was held close to the surface of the insert and pH values were recorded after equilibration for 1 minute. This test was done in triplicate [349, 366].

3.3.12. Differential Scanning Calorimetry (DSC)

Physical state and thermal properties of CsA, HPMC, XG, CsA-loaded HPMC insert (formulation F4), and its corresponding blank (formulation F3) were characterized using a Differential Scanning Calorimeter (DSC) (822e Mettler Toledo) equipped with a TS0800GCI gas flow system attached to a nitrogen gas cylinder and TSO801RO sample robot. Samples of 5 mg were weighed using a Mettler MT 5 microbalance and placed in an aluminum pan and sealed. The samples were heated at a constant rate of 10°C/min over a range of 30 - 300°C. Nitrogen, at a rate of 20 ml/min, was used as purged gas. Star-e software V8.10 was used to obtain the scans.

3.3.13. Fourier transform infrared spectroscopy (FTIR)

The spectra for pure CsA, HPMC, XG, F3 and F4 were recorded at room temperature using a Thermo Scientific NICOLET iS5 Fourier Transform Infrared Spectrometer (iD3 ATR) equipped with a Zinc-Selenium crystal on which the samples were mounted directly and 16 scans were collected. The spectra were recorded in the range of 4000-400 cm^{-1}.

3.3.14. Scanning Electron Microscopy (SEM)

The surface morphology of CsA, F3, and F4 was carried out using scanning electron microscope (SEM) (JEOL JSM-7500F, USA). The inserts were attached to a double-sided adhesive tape fixed on stubs and sputtered with gold using a Denton Vacuum Desk II Sputter coater and then observed at suitable accelerating voltage.

3.3.15. Sterility Test

The formulation F4 was sterilized by exposing to UV light on both sides for 10 minutes and was tested for sterility in order to verify the suitability of sterilization method to produce a microbial free insert. Sterilized inserts were also evaluated for changes in surface morphology by SEM and *in vitro* drug release before and after sterilization [367, 368]. The sterility testing was performed using plate and direct/tube inoculation methods as per our published protocol [369, 370]. Trypsin soy broth (TSB) was used for direct/tube inoculation method. Liquid culture of *Staphylococcus aureus* Rosenbach ATCC BAA 1692 was grown in TSB at 37°C for 24 h in a shaker water bath. After a serial dilution with autoclaved sterile water, the final concentration of liquid culture was 10^2 CFU/ml. The concentration was determined using Spectronic 20 Genesys spectrophotometer (Spectronic instruments, USA) using sterile water as blank and compared to 1 McFarland standard having a concentration of (3×10^8) CFU/ml. Samples for direct inoculation included a negative control, positive control, positive sample control, and sterile sample vials. The negative control vial contained 9 ml of un-inoculated medium and 1 ml of sterile water. The positive control vial contained 9 ml of un-inoculated medium and 1 ml of water containing 10^2 CFU/ml. The positive sample control vial contained 9 ml of un-inoculated medium, non-sterile insert, and 1 ml of water containing 10^2 CFU/ml. The sterile sample vial contained 9 ml of un-inoculated medium, sterile insert, and 1 ml of sterile water. All vials were tested in duplicate and incubated at 37°C to speed up the growth of bacteria. For the plate inoculation, samples of 100 µl were withdrawn from the vials prepared by the direct inoculation method on 0, 7 and 14 days. These samples were uniformly spread on

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Mueller Hinton (MH) agar plates, incubated at 37°C for 24h and checked for bacterial growth visually at room temperature. The study was carried out in aseptic conditions in a laminar air flow hood. Glassware was autoclaved and non-autoclavable materials were disinfected with isopropyl alcohol. The study was carried out in duplicate.

3.3.16. Harvesting bovine corneal endothelial cells and cytotoxicity study

The cytotoxicity of formulation F4 was evaluated using bovine corneal endothelial cells. The bovine eyes were obtained from a slaughterhouse (Kastel's Slaughter House & Processing Center, Riga, MI). The corneal cells were harvested according to a published procedure within 2-5h following sacrifice [371]. The harvested cells were plated in a sterile petri dish with Dulbecco's modified Eagle's medium (DMEM), 10% Fetal Bovine Serum (FBS) and 100 µg/ml penicillin-streptomycin solution and incubated at 37°C. The medium was changed every other day until the culture is ~80-90% confluent. Cells were then harvested and the final cell concentration was adjusted to 2×10^6 cells/ml. A 200 µl aliquot of cell dispersion was added to each well of a 96 well plate (Corning, Inc.) resulting in a concentration of ~15,000 cells/well. The plate was incubated at 37°C in a 5% CO_2 environment for 24 h to assist cell attachment. After 24 h of incubation, media from the wells was carefully removed. Cells were treated for 24 h with negative control containing 200 µl of DMEM medium, positive control containing DMEM with 5% DMSO, and DMEM exposed to blank (F3) and drug-loaded insert (F4) for 24 h. After 24 hours, media was aspirated carefully; fresh DMEM media was added with 10% MTT reagent. The cells incubated at 37 ºC for 2-3 h. The yellow medium was aspirated and 150 µl of 100% DMSO was added to each well to allow dissolution of the formazan salt formed. The absorbance

was measured using a microplate reader at 570 nm to determine the cytotoxicity of each sample.

3.3.17. Anti-inflammatory study

The anti-inflammatory activity of F4 was carried out in Jurkat T cells in order to evaluate the effect of polymers and preparation conditions on the biological activity of cyclosporine A. The study was conducted as per the procedure published by Hermans et al. [201, 361]. In brief, cells were grown in RPMI Medium 1640 supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml) and incubated at 37°C. The medium was replaced every other day. Cells were passed after reaching 80-90% confluency. Jurkat T cells were seeded in a 96-well plate at density 2×10^4 cells/well. After 24 h of seeding, cells were exposed to blank insert (F3), drug-loaded insert (F4) and pure drug at concentrations 10 and 25 ng/ml. After 45 minutes, T cell activator concanavalin A (20 µl) was added at a final concentration of 37.5 µg/ml to all wells except for the negative control. After 20 hours, the concentration of interleukin-2 (IL-2) in supernatant was evaluated using DuoSet IL-2 ELISA kit according to the manufacturer's instructions. The absorbance was measured using a Synergy H1 microplate reader with Gen5 Data Analysis Software at 450 nm with wavelength correction at 540 nm.

3.3.18. Stability test

Inserts were packaged in aluminum foil and stored at 4°C and 25°C and 40°C (70% RH) for 3 months. Samples were examined for drug content and physical appearance at regular time intervals [372].

3.3.19. Statistical analysis

The data presented are expressed as means and standard deviations (mean± SD) with the number of data replicates in each study. Analysis of variance (ANOVA) was applied to evaluate the statistical significance followed by Tukey test to compare the means of more than two samples using SPSS software, IBM SPSS Statistics 21 (IBM, Armonk, NY, USA). A value of $p < 0.05$ was considered significant. The experimental design and data analysis were done using JMP Statistical Discovery v7.0.1 (SAS, USA).

3.4. RESULTS

CsA-loaded ophthalmic inserts with varying ratios of HPMC and XG (F2, F4, F6 and F8) and corresponding blank inserts (F1, F3, F5 and F7) were successfully prepared. Inserts were evaluated for thickness, folding endurance, contact angle, and *in vitro* drug release using 2^3 full factorial experimental design. The solvent casting technique produced colorless, translucent and homogeneous inserts. Inserts of 6mm diameter (~50 µg oCsA/insert) were punched out for physicochemical characterization. All inserts were smooth and flexible without any sharp edges (Fig. 3.3). The physicochemical parameters of inserts such as percent drug content, film thickness, folding endurance, contact angle, percent moisture absorption, percent moisture loss, and surface pH are shown in Table 3.3. The effect of varying HPMC and XG ratios on thickness, folding endurance, contact angle, and *in vitro* drug release is shown in Table 3.4. The master model was found to be significant for response variables such as film thickness, folding endurance and *in vitro*

release with p values of 0.0320, 0.0139, and <0.0001 respectively, and not significant for wettability (p=0.6293) (Table 3.5). The correlation coefficient (R^2) for the regression models were 0.999709, 0.999945, 0.865047, 0.976471 for film thickness, folding endurance, contact angle and *in vitro* release suggesting that the model explains 99.9%, 99.9%, 86.5%, and 97.64% of variation with respective response variables. The model was also validated based on the plot of actual vs. predicted plot (Fig 3.4).
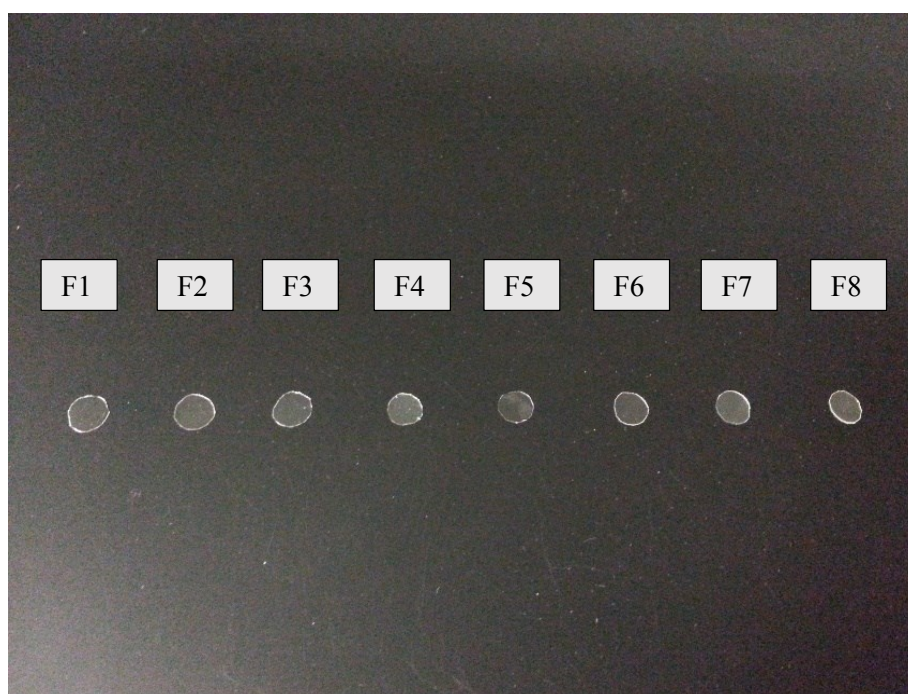
Figure 3.3: Picture of ophthalmic inserts

Table 3.3: Physicochemical parameters of ophthalmic inserts such as drug content (µg/insert), film thickness (mm), folding endurance, contact angle (o), percent moisture absorption, percent moisture loss (%), and surface pH. Values are expressed as mean ± SD.

Formulation	Drug content (µg/insert)	Film thickness (mm)	Folding endurance	Contact angle (º)	Percent moisture absorption %	Percent moisture loss %	Surface pH
F1		0.072 ± 0.009	485.7±12.5	56.0 ± 4.32	1.01 ±0.18	2.17 ±0.11	6.82 ± 0.075
F2	50 ± 2.65	0.073 ± 0.007	480.3±1.50	62.4 ± 2.95	1.00 ±0.14	2.25 ±0.31	7 ± 0.021
*F3		0.086 ± 0.007	593.3±7.60	62.6 ± 1.90	1.63 ±0.19	2.09 ±0.11	6.73 ± 0.208
*F4	50.7 ± 1.47	0.088 ± 0.008	591.3±1.50	62.0 ± 1.33	1. 78 ±0.40	2.03 ±0.52	6.98 ± 0.056
F5		0.146 ± 0.005	69.7±4.60	56.8 ± 2.35	1.08 ±0.08	1.68 ±0.16	6.86 ± 0.04
F6	52.5 ± 2.60	0.139 ± 0.024	67 ±6.2	60.0 ± 1.33	1.00 ±0.08	1.64 ±0.54	7.03 ± 0.026
F7		0.165 ± 0.014	216±5.30	59.6 ± 2.80	1.06 ±0.09	1.76 ±0.09	6.68 ± 0.176
F8	50.2 ± 3.71	0.154 ± 0.005	229± 25.4	63.4± 3.41	1.02 ±0.4 0	1.77 ±0.33	6.99 ± 0.032

Table 3.4: Summary of the model

Independent variable	Thickness		Folding endurance		Contact angle		Percent cumulative release (after 12 h)	
	Parameter estimate	**Prob>|t|**	**Parameter estimate**	**Prob>|t|**	**Parameter estimate**	**Prob>|t|**	**Parameter estimate**	**Prob>|t|**
HPMC	0.035625	0.0112*	-196.1125	0.0050*	-0.4	0.7463	15.409167	<.0001*
XG	0.007875	0.0504	65.8625	0.0149*	1.55	0.3500	-4.604167	0.0017*
CsA	-0.001875	0.2048	0.3625	0.8526	1.6	0.3411		
HPMC*XG	0.000625	0.5000	11.2125	0.0868	0	1.0000	8.5291667	<.0001*
HPMC*CsA	-0.002625	0.1488	2.2125	0.3866	0.15	0.9003		
XG*CsA	-0.000375	0.6560	2.3875	0.3642	-0.8	0.5544		

* indicate $p < 0.05$

Table 3.5: p-value (ANOVA), and correlation coefficients for the master model

Response variable	p-value(ANOVA)	R^2	Adjusted R^2
Thickness	0.0320	0.999709	0.997963
Folding endurance	0.0139	0.999945	0.999615
Contact angle	0.6293	0.865047	0.055327
Percent cumulative release (after 12 h)	<0.0001	0.976471	0.967648
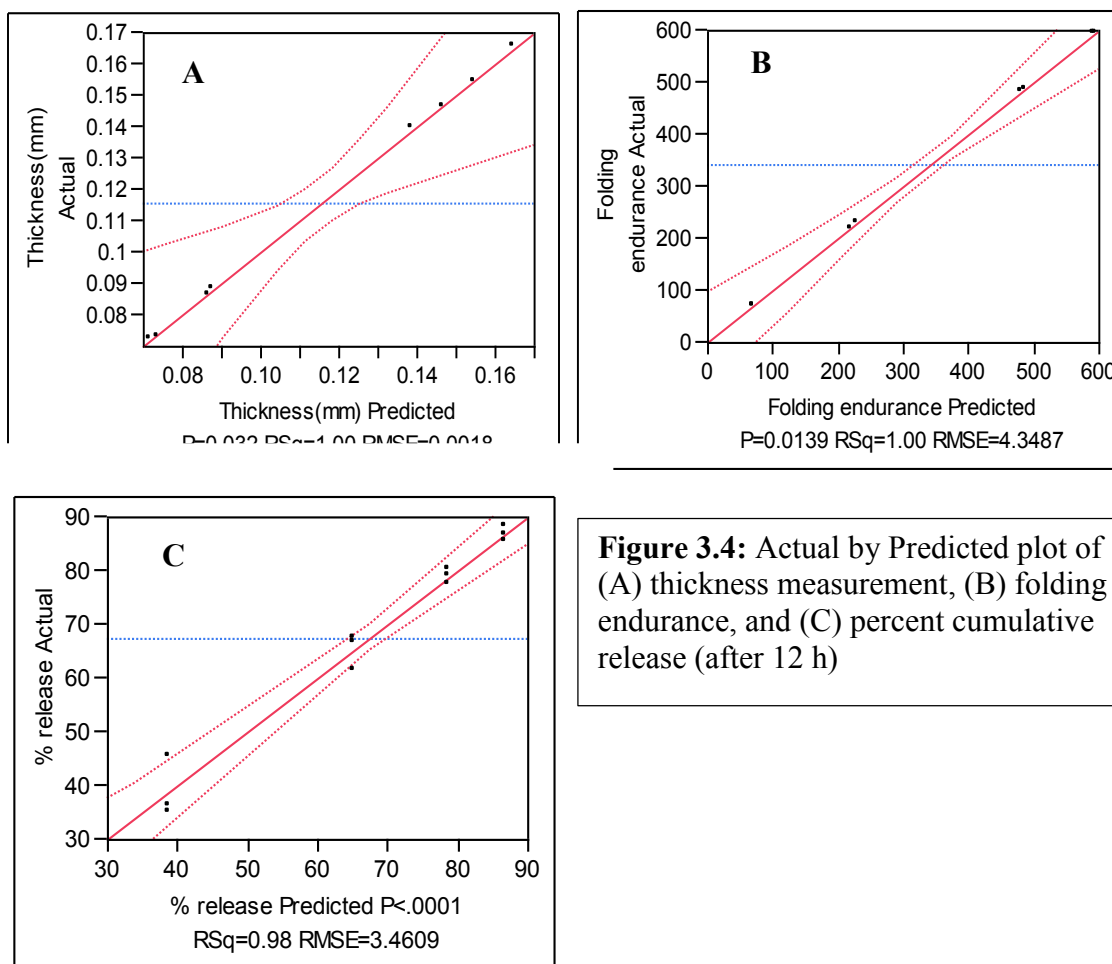
Figure 3.4: Actual by Predicted plot of (A) thickness measurement, (B) folding endurance, and (C) percent cumulative release (after 12 h)

HPLC method for analysis and quantification of CsA concentration was successfully developed and validated for linearity, accuracy, limit of detection (LOD), limit of quantification (LOQ), and precision. The retention time of CsA was found to be 8.522 minutes. The calibration curve resulted in a straight line (y = 1472.3x + 295.73) with a correlation coefficient value (r^2) of 0.9999 (Figure 3.5). A sample chromatogram of CsA is shown in Fig. 3.6. LOD and LOQ were found to be 1.315 ng and 3.987 ng, respectively. The percentage recovery of CsA ranged from 98.5%-100.2% indicating the method accuracy. The developed method also showed good intraday precision and suitability with (RSD) less than 2% .
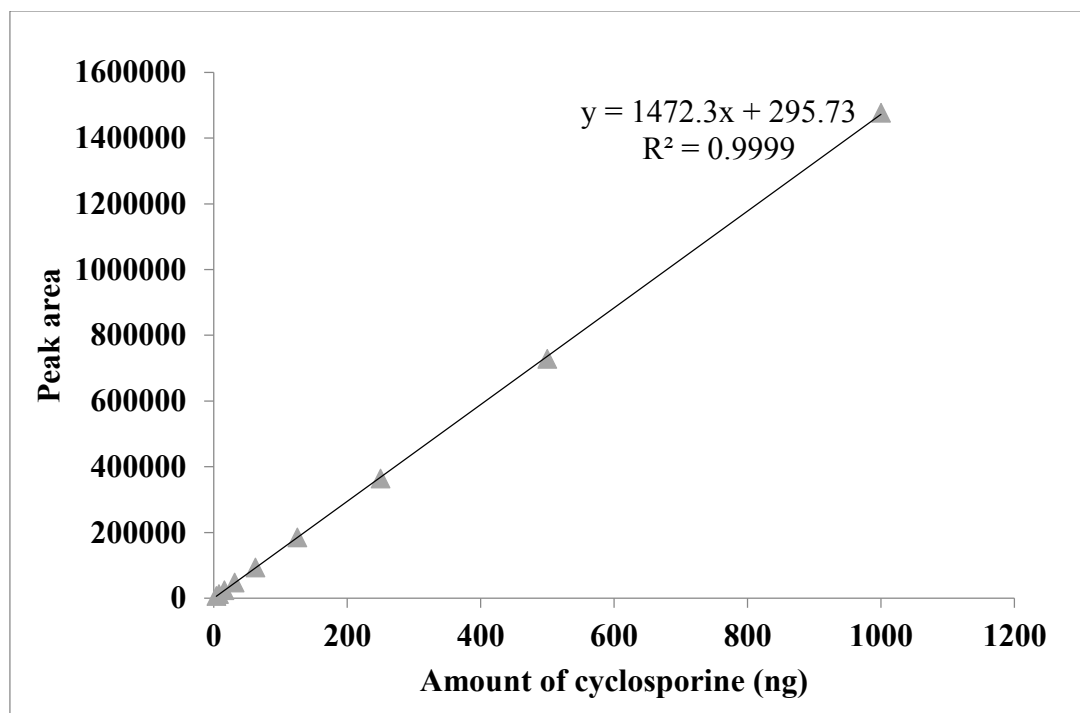
Figure 3.5: Calibration curve of cyclosporine A
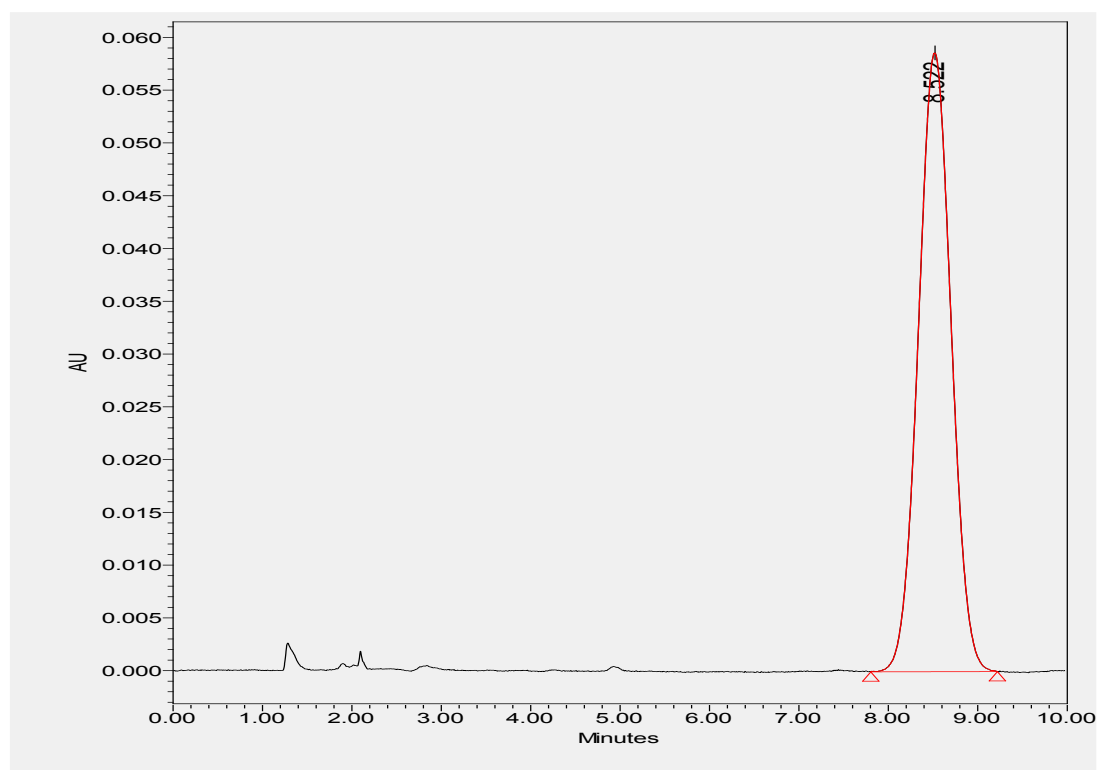
Figure 3.6: A sample HPLC chromatogram of cyclosporine A

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The drug content of all formulations was found to be in the range of 50 ± 2.65 µg/insert to 52.5 ± 2.60 µg/insert. This study indicated that CsA is uniformly distributed in the films and the solvent casting technique used in the preparation of inserts is effective. The thickness values of inserts ranged from 0.072 ± 0.009 mm to 0.165 ± 0.014 mm (Table 3.3). The thickness of inserts was found to be uniform with a very low standard deviation. The folding endurance is a measure of breaking strength of inserts. The folding endurance of all films was found to be in the range of 67 ± 6.2 to 593.3 ± 7.6 (Table 3.3). The results revealed that the concentration of HPMC had a negative effect on the mechanical strength of inserts and XG increased the folding endurance and mechanical strength of inserts. The surface hydrophilicity of the inserts was evaluated by contact angle measurement. The contact angle of inserts was in the range of $56.0 \pm 4.32°$ to $63.4\pm 3.41°$ (Table 3.3). The release profiles of CsA from inserts are shown in Fig. 3.7. After 12 h, formulations F2, F4, F6, and F8 released about 65%, 39%, 79%, and 87% of CsA, respectively. All inserts released 100% of CsA after 16 h except for F4, which sustained the release of CsA for up to 20 h.
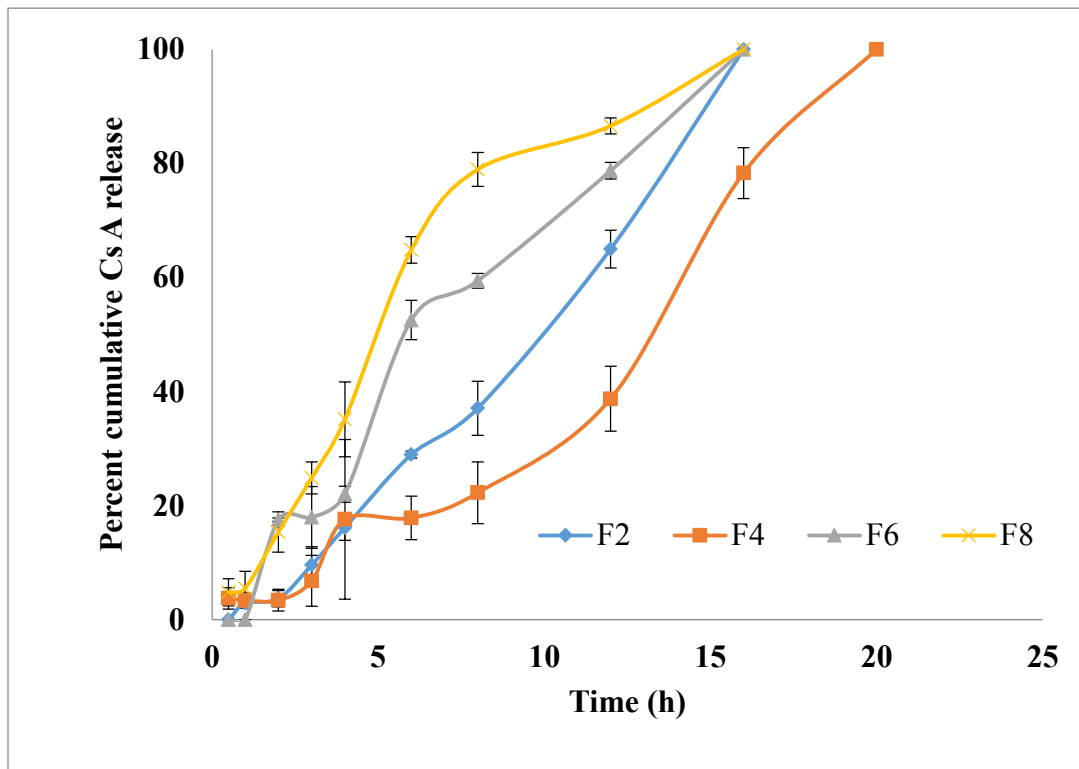
Figure 3.7: *In vitro* release of cyclosporin A from ophthalmic inserts. Values are expressed as mean (n=3) and error bars represent standard deviation.

The percent moisture absorption of inserts ranged between 1.00 ±0.08% to 1.78±0.40% and the percent moisture loss ranged between 1.64 ±0.54% to 2.25 ±0.31% (Table 3.3). There was no change in the integrity of inserts. This indicates good stability and physical integrity of inserts in both dry and humid conditions. Changing the concentrations of HPMC and XG didn't show any significant effect on the percentage of moisture absorption or percentage of moisture loss. However, with the increase in film thickness the percentage of moisture loss in films decreased [349]. The surface pH of inserts was found to be in the range of 6.68 ± 0.17 to 7.03 ± 0.03 (Table 3.3).

Based on the drug content, thickness, folding endurance, surface pH, and *in vitro* release data, formulation F4 was considered optimum and used for further studies. DSC thermograms of XG, HPMC, CsA, blank insert, and CsA-loaded HPMC insert are shown in Figure 3.8. XG showed an exothermic peak with peak onset at 259.72°C and peak temperature at 285.43°C corresponding to the decomposition of XG [373]. HPMC showed endotherm peak between 36°C and 100°C corresponding to the evaporation of moisture content [374]. CsA exhibited a thermal event with peak onset at 126.51°C and a peak temperature at 130.44°C [375]. The characteristic sharp endothermic peak of CsA was completely absent in drug loaded insert. This indicated the absence of any un-dissolved CsA in the formulation.
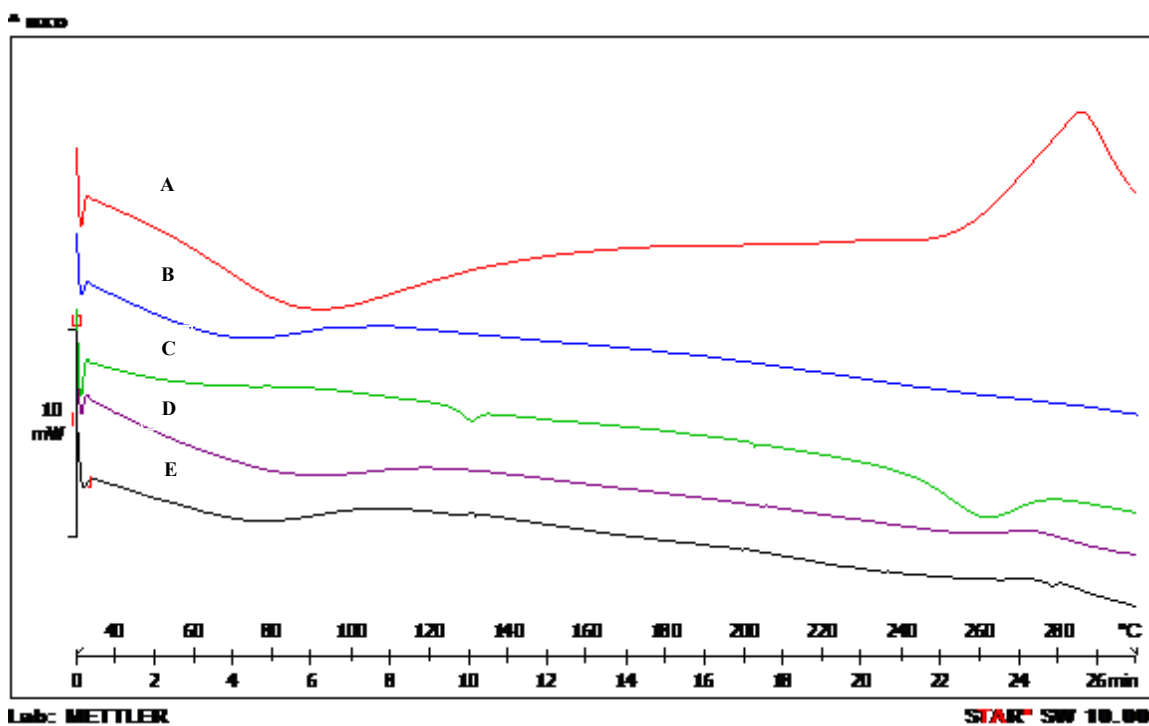
Figure 3.8: Differential scanning thermograms of (A) xanthan gum, (B) hydroxypropyl methylcellulose, (C) cyclosporine A, (D) blank insert, and (E) formulation F4-drug loaded insert

Figure 3.9 displays the infrared spectra of HPMC, XG, CsA, F3 and F4 over the range 400-4000 cm $^{-1}$. The IR spectrum of HPMC shows characteristic peaks at 3445.83 and 1045.79 cm^{-1} attributed to the stretching vibration of O-H and C–O, respectively, and C-H stretching at 2894.44cm^{-1} [376]. The IR spectrum of XG shows O-H stretching vibration at 3243.32 cm^{-1}, -CH2 at 2884.18 cm^{-1}, and carboxylate group (COO−) asymmetric stretching at 1602.49 cm^{-1}. The appearance of peaks at 1253.27 cm^{-1} in the spectra of XG indicates the presence of a C-O-C group [373, 377]. The IR spectrum of CsA shows an intense amide I carbonyl band at 1624.92 cm^{-1}, N-H stretching vibration at 3304.96 cm^{-1}, C-H stretching vibration at 2948.74 cm^{-1}, and C-N stretching band of amine at 1250 cm^{-1} [378, 379]. The IR spectra of the blank formulation showed contributions of peaks from both polymers with a little peak at 1635 due to H_2O bending [380, 381]. The IR spectra of formulation F4 also showed contributions from the polymers. and CsA.
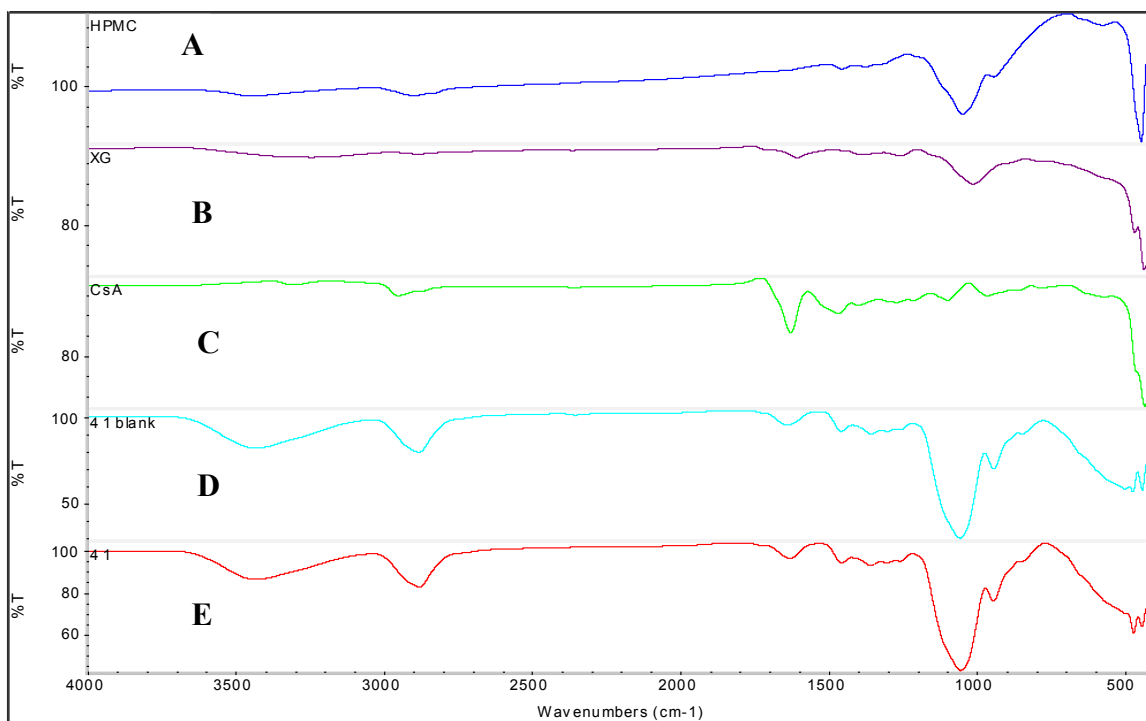
Figure 3.9: FTIR spectra of (A) hydroxypropyl methylcellulose, (B) xanthan gum, (C) cyclosporine A, (D) blank insert, and (E) formulation F4-drug loaded insert.

The SEM was used to evaluate the surface morphology of ophthalmic inserts. Pure CsA showed rod shaped particles with a diameter approximately 50 µm[382] (Fig. 3.10). Blank insert exhibited a smooth surface morphology, while CsA-loaded insert exhibited uniform drug distribution in the matrix. The particles of CsA in inserts were circular with an approximately size of 5 µm.
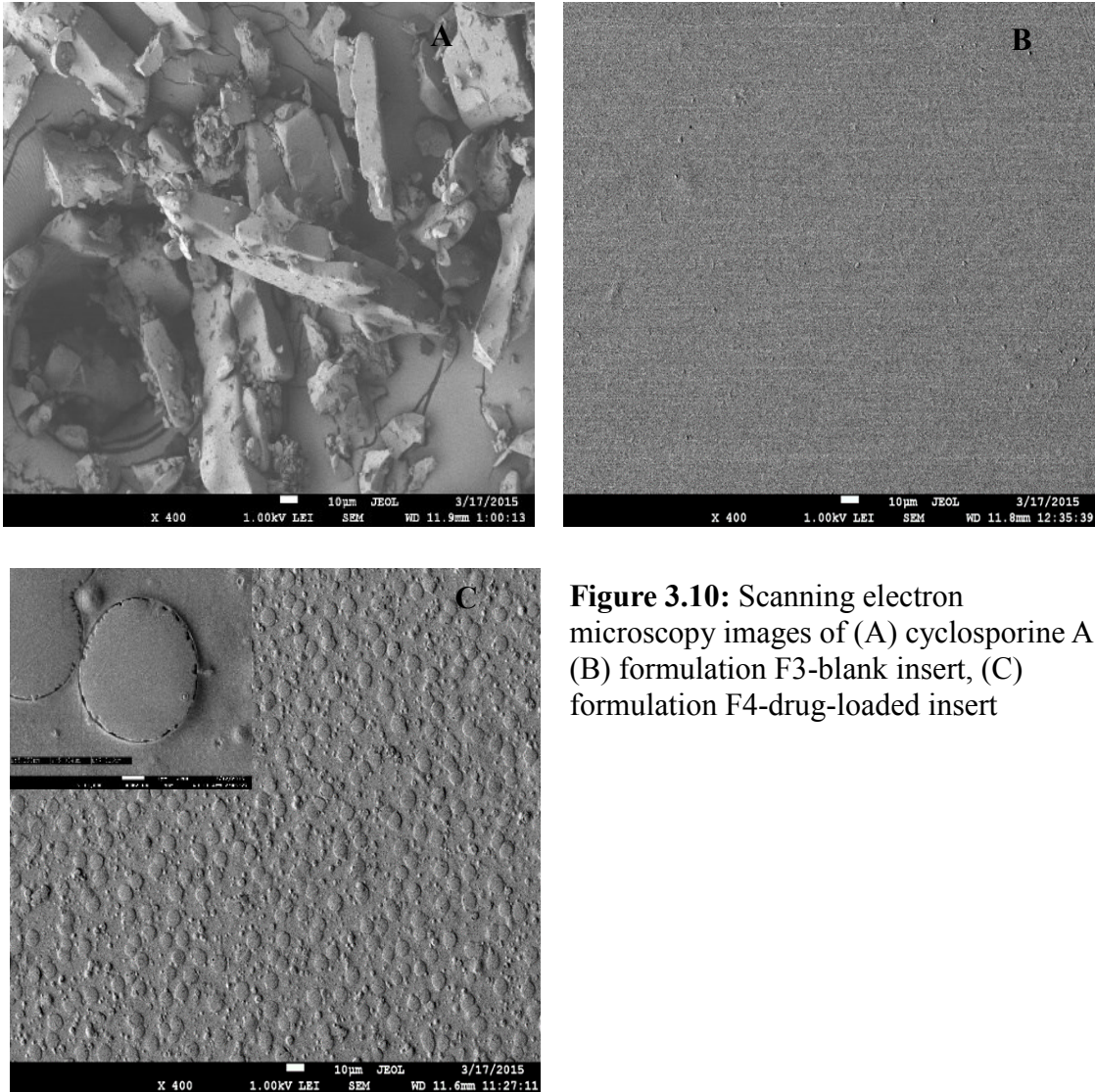
Figure 3.10: Scanning electron microscopy images of (A) cyclosporine A, (B) formulation F3-blank insert, (C) formulation F4-drug-loaded insert

Based on USP guidelines; the sterility of CsA-loaded HPMC insert exposed to UV light was analyzed using direct inoculation and plate inoculation techniques in a TSB broth. Samples of 100 µl were withdrawn by the direct inoculation method from each of the vials on days 0, 7 and 14 and spread over Mueller-Hinton agar plates for the plate inoculation method. The plates were examined for presence/absence of microbial growth after incubation for 24h at 37°C. As shown in Table 3.6, negative control and sterile CsA insert

were clear and did not show any bacterial growth during the experimental period of 14 days. Positive control and positive sample control showed turbidity indicating microbial growth. This experiment indicates that UV light could be used for sterilizing inserts and sterilized inserts maintained the sterility for at least 14 days (Figs. 3.11 and 3.12). No change in the surface morphology of F4 and release of CsA from the insert was observed after sterilization (Fig. 3.13).

Table 3.6: Sterility validation test performed on MH Agar plates indicating the presence (+) or absence (-) of microbial growth on days 0, 7 and 14

No. of Days	Sterile ophthalmic insert	Negative Control	Positive Control	Positive Sample Control
Day 0	-	-	+	+
Day 7	-	-	+	+
Day 14	-	-	+	+
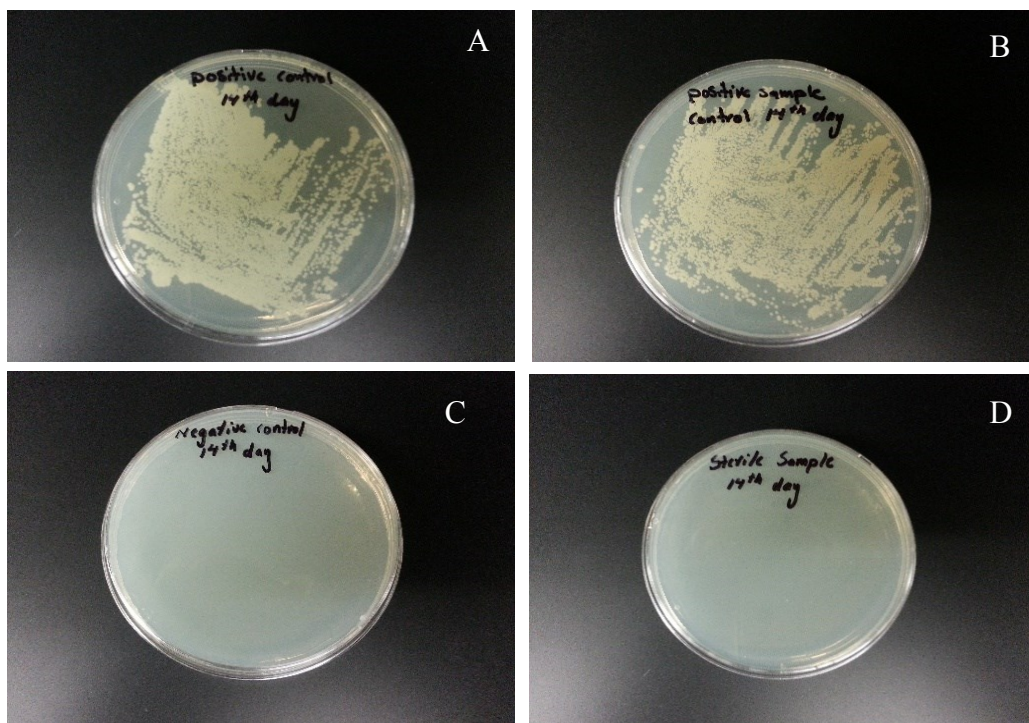
Figure 3.11: Images of agar plates after 14days showing presence/absence of microbial growth. (A) positive control, (B) positive sample control, (C) negative control, and (D) sterile sample, formulation F4.
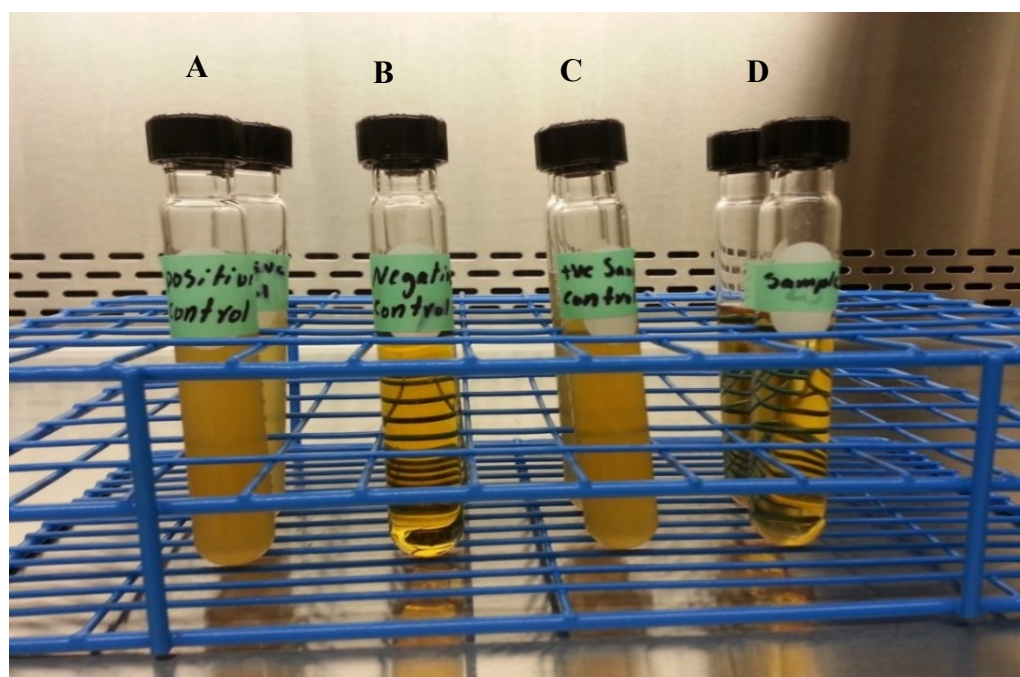
Figure 3.12: Represented picture of direct inoculation tubes (A) positive control, (B) negative control, (C) positive sample control, and (D) sterile sample, formulation F4, after 14 days of inoculation
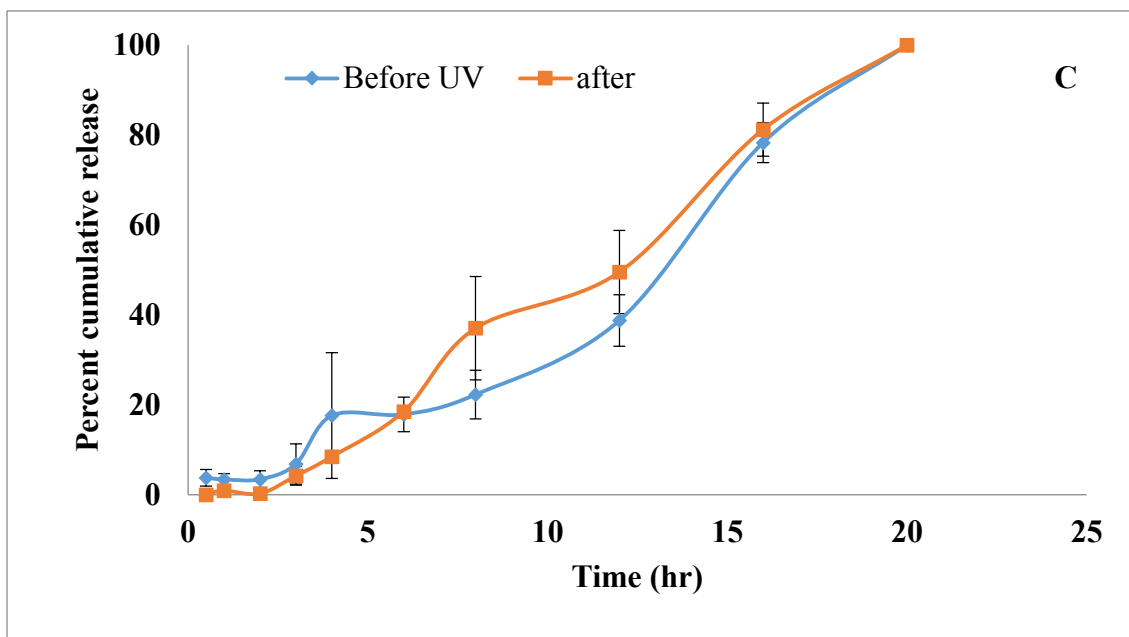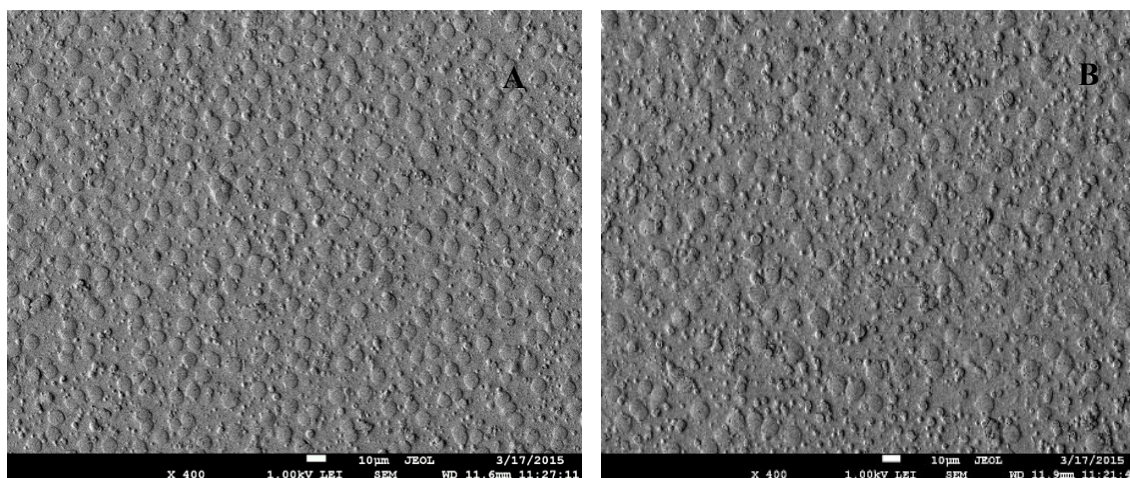
Figure 3.13: SEM images of formulation F4 (A) before sterilization and (B) after UV sterilization, (C) *in vitro* release of cyclosporine A from sterile insert. Values are expressed as mean (n=3) and error bars represent standard deviation.

The cytotoxicity of blank (F3) and CsA-loaded HPMC insert (F4) was compared with negative and positive controls. DMSO is considered as a positive control. In the presence of DMSO, only 25.9% cell viability was observed. The percentage cell viability of F3 and F4 inserts was 89.4% and 94.2%, respectively compared to that of negative control (Fig. 3.14).
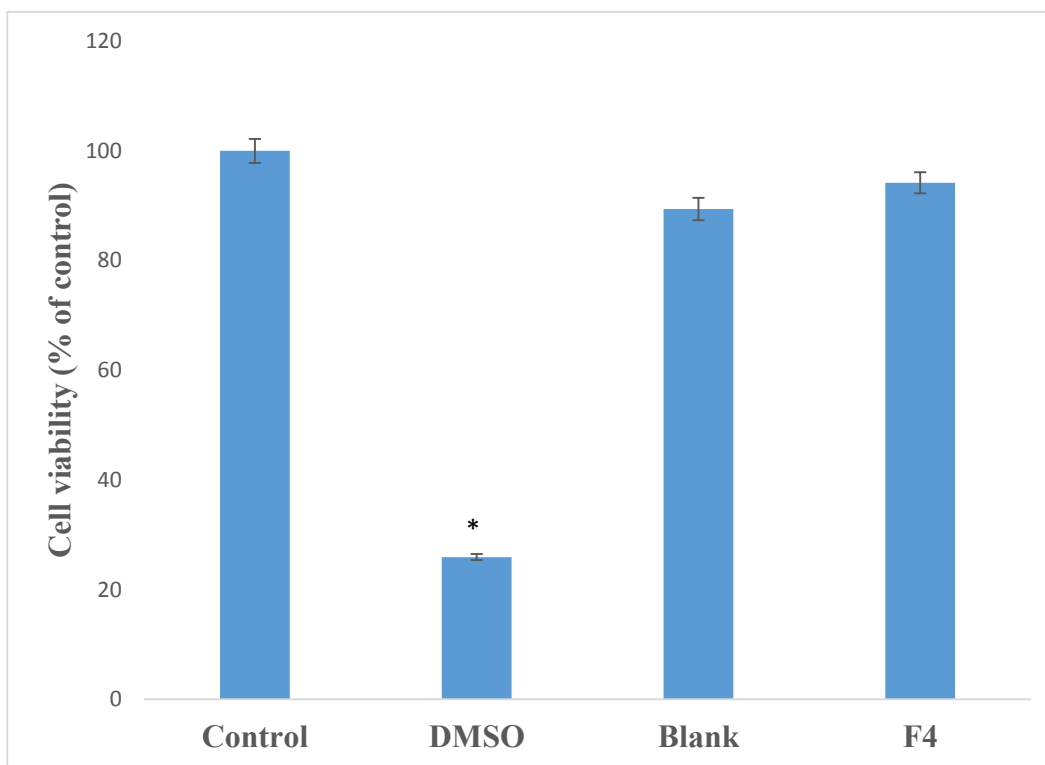
Figure 3.14: Cell viability of formulation F4 in bovine corneal endothelial cells (n=5). Error bars represent standard deviation. No significant difference was observed between F4 and the control.

The anti-inflammatory activity of CsA in F4 was assessed on interleukin-2 (IL-2) expression in activated Jurkat T cells and the activity was compared with pure CsA (Fig. 3.15). Both the pure CsA and CsA-loaded HPMC insert showed concentration dependent anti-inflammatory activity. In comparison with the positive control, pure CsA and CsA-

loaded HPMC insert at 25 ng/ml significantly suppressed the IL-2 expression. Blank HPMC insert did not show any significant difference from the positive control indicating that the anti-inflammatory effect was due to CsA released from the insert.
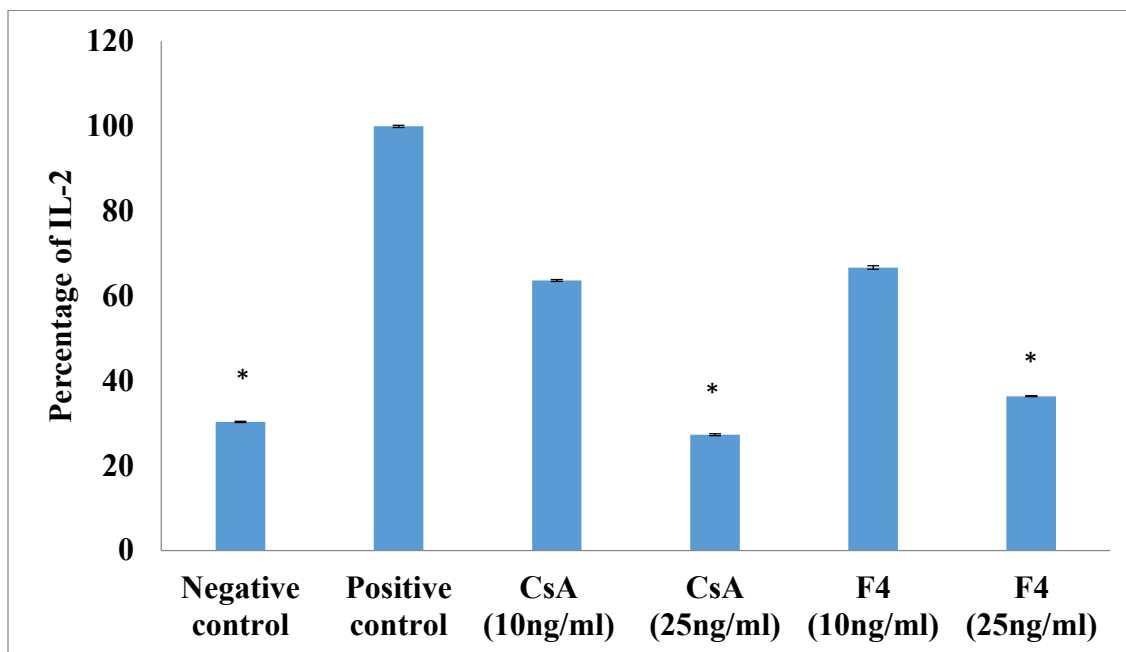
Figure 3.15: Interleukin-2 secretion in Jurkat T cells activated by concanavalin A (n=3). Negative control (cells without activator), positive (cells with activator), Error bars represent standard deviation. No significant difference was observed between the anti-inflammatory effect of the pure drug and drug incorporated in the formulation F4.

CsA-loaded HPMC inserts were stored at 4°C, 25°C and 40°C for 3 months. At regular time intervals samples were evaluated for changes in physical appearance and drug content. No visible change was observed in the physical appearance and the drug content during the three-month evaluation. This indicated the physical and chemical stability of CsA-loaded HPMC inserts for at least 3 months at 4°C, 25°C and 40°C (Table 3.7).

Table 3.7: Stability of cyclosporine A ophthalmic inserts

Temperature	Parameters	1 month	2 months	3 months
4°C	Drug content (%)	98.7 ±1.40	104.7±0.41	103.9±1.6
	Physical appearance	Colorless and Translucent	Colorless and Translucent	Colorless and Translucent
25°C	Drug content (%)	96.8±2.94	100.2±0.62	103.6±0.5
	Physical appearance	Colorless and Translucent	Colorless and Translucent	Colorless and Translucent
40°C	Drug content (%)	95.9±3.84	95.0±3.07	102.1±0.4
	Physical appearance	Colorless and Translucent	Colorless and Translucent	Colorless and Translucent

3.5. DISCUSSION

In this work, we have successfully prepared CsA-loaded ophthalmic inserts with varying HPMC and XG ratios and characterized them for thickness, folding endurance, contact angle, and *in vitro* drug release using 2^3 full factorial experimental design. Using a factorial design helps in the evaluation of the effect of one variable when others are changed, this cannot be done using traditional methods [361]. The smooth surface and homogenous nature of inserts indicates miscibility of HPMC, XG and PEG400. The homogenous nature also indicates the uniform dispersion of CsA in inserts. The absences of sharp edges on inserts make them suitable for placement in the precorneal area [383]. Uniform thickness inserts were obtained with a very low standard deviation. Ocular inserts reported in literature for animal and human use showed a thickness of 0.070 to 0.500 mm [384] and

the thickness of inserts developed in our work are within the range of acceptable values. Thinner inserts are suitable for ophthalmic delivery as they cause less irritation to the eye. The results from factorial design showed that the film thickness increases significantly by increasing the concentration of HPMC ($p < 0.05$), while an increase in the concentration of XG and CsA didn't show any significant effect on the film thickness. This might be due to the presence in XG and CsA in low concentrations. The concentration of HPMC had a negative effect on the mechanical strength of inserts. The mechanical strength of inserts decreased with an increase in HPMC concentration. While, XG significantly increased the folding endurance and mechanical strength of inserts. CsA concentration didn't show any significant effect on the mechanical strength of inserts. The increase in folding endurance of inserts with XG could be partly due to greater bonding strength of gums. The contact angle values indicated the hydrophilicity and wettability of inserts. The hydrophilicity of inserts could be due to the availability of hydroxyl groups in HPMC, XG, and PEG 400 [334]. *In vitro* release study indicated that the drug release from inserts was retarded in presence of XG, suggesting its role as a release retardant.

Contact angle measurement is used to determine the surface wettability and hydrophilicity /hydrophobicity of the surface. The addition of HPMC, XG and CsA has no significant effect on the contact angle ($p > 0.05$). The pH of inserts should be close to the physiological pH of lachrymal fluids 7.31-7.62 [385], suggesting the suitability of inserts for ophthalmic use without causing irritation to the eye. DSC was conducted to identify the physical state of CsA in the formulation and to detect any physicochemical interaction between the drug and polymers used in the formulation. The endothermic peak observed in CsA disappeared

in the thermogram of F4, indicating the stable maintenance of a CsA amorphous state and suggesting no chemical or physical interaction between the polymers or between the polymers and the drug. FTIR analysis was performed to check the interaction between CsA and polymers in the formulation. FTIR spectrum of F4 clearly shows the characteristic peaks of CsA like amide I at 1626 cm^{-1}, and C-N stretching band of amine (of NHC=O) at 1254 cm $^{-1}$. This study indicated the absence of significant interactions between the drug and the polymers in the insert. However, we cannot exclude some minor chemical interactions such as hydrogen bond and van der Waals interaction between the drug and polymers used in the preparation of inserts [379]. The surface morphology of blank (F3) and drug-loaded (F4) inserts was studied using SEM. Rod shaped particles with diameter approximately 50 μm were observed with pure CsA[382]. The SEM image of CsA in F4 exhibited a clear change compared to the pure CsA. The particles of CsA in inserts were circular and uniformly distributed throughout the matrix. The size of CsA particles in F4 was approximately 5 μm, which result in a higher specific surface area than pure CsA.

According to the USP guidelines, the ophthalmic preparations should be sterile [386]. In the present study, UV light was used for sterilization of formulation F4 for 10 minutes on both sides. Direct inoculation and plate inoculation methods in a Tryptic soy broth (TSB) as described in USP guidelines were used to test the sterility of inserts. The results obtained after days 0, 7 and 14 successfully validated the UV sterilization method used for sterilizing inserts. The cytotoxicity of formulation F4 was measured using MTT assay. MTT is a colorimetric test used to determine the cell viability by measuring the cellular metabolic activity through NAD(P)H-dependent cellular oxidoreductase enzymes. Adult bovine eyes

were obtained from a nearby slaughterhouse within 2-5 h following sacrifice and the corneal cells were harvested according to a published procedure [371]. The assay is based on the ability of viable cells to form a purple colored formazan product after metabolizing tetrazolium salt present in the MTT reagent. The higher absorbance value indicates less toxicity and more cell viability. No significant toxicity was observed from F3 and F4 for up to 24h ($p > 0.05$) compared to negative control.

Jurkat T cells activated with concanavalin A was used to evaluate the anti-inflammatory activity of CsA released from F4. Jurkat T cells express IL-2 when activated by concanavalin A and the IL-2 levels decrease in the presence of CsA. The anti-inflammatory activity of CsA in F4 was compared with the pure drug to assess the effect of polymers and conditions used in the preparation of inserts on biological activity of CsA. At the tested concentrations of 10 and 25 ng/ml, no significant loss in the activity of CsA was observed in HPMC inserts during the preparation of inserts. CsA-loaded HPMC insert was found to be stable with respect to physical appearance and drug content at 4°C, 25°C and 40°C for up to three months.

3.6. CONCLUSION

In this study, we report the preparation and physicochemical evaluation of CsA-loaded HPMC inserts for treating DED. CsA-loaded inserts prepared using HPMC (1% w/v) and XG (0.25% w/v) showed good mechanical properties, wettability and thickness to ensure no eye irritation during use. CsA was uniformly distributed in the polymer matrix and the *in vitro* release study showed release of the drug in a sustained manner for up to 20 h. XG showed significant effects in sustaining the release of CsA and enhancing folding endurance. The inserts were successfully sterilized using UV light. The cytotoxicity study showed no sign of toxicity in bovine corneal cells in comparison with the control. The anti-inflammatory study in Jurkat T cells showed that CsA retained its biological activity when loaded in HPMC inserts. In conclusion, CsA inserts were successfully prepared using HPMC, XG and PEG 400. The novel combination therapy with HPMC and CsA could simultaneously provide lubrication and anti-inflammatory effects and could be used as a potential once-a-day formulation for treating DED.

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