A Thesis

entitled

Development and Evaluation of a Novel Microemulsion of Dexamethasone and
Tobramycin for Topical Ocular Administration

by

Rinda Devi Bachu

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the
Master of Science Degree in Pharmaceutical Sciences

Industrial Pharmacy

Sai Hanuman Sagar Boddu, PhD., Committee Chair

Jerry Nesamony, PhD, Committee Member

Caren L. Steinmiller, PhD, Committee Member

Dr. Amanda Bryant-Friedrich, PhD, Dean
College of Graduate Studies

The University of Toledo

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Aim: The drug absorption from an ophthalmic suspension dosage form is highly unpredictable. Identical formulations with similar concentrations of active and inactive ingredients tend to exhibit differences in absorption. These differences in absorption could be due to varying physicochemical properties such as pH, particle size, zeta potential, size distribution and viscosity. These properties affect the precorneal residence time, drug release and ocular drug absorption [1, 2]. Drug delivery to the eye using microemulsions has drawn significant attention due to their intrinsic properties and ability to solubilize both hydrophilic and lipophilic drugs. They offer several advantages including thermodynamic stability combined with improved dose uniformity [3], ocular retention, permeation and absorption of drugs [4]. The present study involves the development and evaluation of a novel dexamethasone (0.1%) and tobramycin (0.3%) loaded microemulsion with potential for treating anterior segment eye inflammation and infection.

Methods: The microemulsion was evaluated for pH, particle size, zeta potential, light transmittance, morphology, and *in vitro* drug release. Sterility of microemulsion was evaluated by direct as well as plate inoculation methods. Anti-inflammatory activity of dexamethasone, bactericidal activity of tobramycin, and cytotoxicity of the microemulsion were evaluated and compared with that of marketed eye drop suspension (Tobradex[®]). Histological evaluation was performed in bovine corneas in order to assess the safety of microemulsion in comparison to Tobradex[®] suspension. In-addition, the stability of the microemulsion was also studied at 4°C, 25°C and 40°C.

Results: The pH of microemulsion was close to the pH of tear fluid. The microemulsion displayed average globule size under 20 nm with light transmittance around 95-100%. Aseptically prepared microemulsion remained sterile for up to 14 days. The cytotoxicity of microemulsion in bovine corneal endothelial cells was comparable to that of Tobradex[®] suspension. Anti-inflammatory activity of dexamethasone and anti-bacterial activity of tobramycin from the microemulsion were significantly higher than those of Tobradex[®] suspension ($p < 0.05$). Histological evaluation showed intact corneal epithelium without any signs of toxicity and the developed microemulsion was found to be stable at 4°C and 25°C for 3 months.

Conclusion: In conclusion, the microemulsion developed could be a suitable alternative to the currently marketed Tobradex[®] suspension for treating anterior segment infection and inflammation. Further *in vivo* studies in animals are warranted to evaluate the clinical utility of the microemulsion.

Dedicated to my Family

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Chapter 1

Introduction

The human eye is an intricate organ with a unique anatomy and physiology. The eye is composed of two major parts: anterior and posterior segments. The anterior segment makes up one-third of the eye and consists of the cornea, conjunctiva, aqueous humor, iris, ciliary body and lens. The remaining two-thirds of the eye is occupied by the posterior segment of the eye and consists of the sclera, choroid, retinal pigment epithelium, neural retina, optic nerve and vitreous humor. Both anterior and posterior segments are affected by several vision threatening diseases. Some of the diseases affecting the anterior segment of the eye include glaucoma, anterior uveitis, allergic conjunctivitis and cataract while age-related macular degeneration and diabetic retinopathy are the most common diseases affecting the posterior part of the eye [5]. The estimated number of people with visual impairment globally are 285 million, while 246 million are with low vision and 39 million are blind which raises the need for drug delivery to the eye [6].

Drug delivery to the eye is a very challenging task due to its inherent and intricate anatomical and physiological barriers. These barriers are also specific to the route of administration i.e., topical, systemic and injectable [7]. Topical instillation of dosage

forms including solutions, suspensions and ointments is the highly preferred route for anterior segment delivery due to its ease of administration and high patient adherence [8]. However, the major concern with these conventional topical delivery systems is low bioavailability [9]. In addition, numerous precorneal factors also influence the bioavailability of topical dosage form due to which less than 5% of the instilled dose reaches the deeper ocular tissues [10].

1.1 Barriers to topical ocular drug delivery

1.1.1 Precorneal factors

The composition and the amount of tear film determines the health of the ocular surface and offers the first line of defense for both cornea and conjunctiva because of its high turnover rate [11]. The tear film is composed of the outer lipid layer, middle aqueous layer and an inner mucin layer [12]. Mucin present in the tear film forms a hydrophilic layer that extends over the glycocalyx, protecting the ocular surface from debris and pathogens [11]. Precorneal factors such as tear turn over, nasolachrymal drainage, reflex blinking, and induced lacrimation pose challenges and impede the permeation of drug molecules into deeper ocular tissues [7]. Tear volume in humans is estimated to be 7 μ l, and the cul-de-sac can transiently hold approximately 30 μ l of the instilled eye drop, which induces lacrimation and increases the rate of reflex blinking. The immediate loss of the drug by spillage [13] and reflex blinking induced nasolacrimal drainage reduces the ocular bioavailability of the drug. Therefore, most of the topically instilled solutions are washed out within 15-30s after administration and all of these factors contribute towards the low bioavailability of drugs (< 5%) to the deeper ocular tissues [10].

1.1.2 Cornea as a barrier

The cornea is multilayered, avascular and highly innervated tissue, which prevents the transport of topically administered exogenous compounds into the conjunctival cul-de-sac. It is composed of five layers namely outer lipophilic epithelium, Bowman's layer, hydrophilic stroma, Descemet's membrane and the endothelium. Specific arrangement and interaction of these layers are essential for proper functioning and integrity of the tissue [13]. The outer layer i.e., corneal epithelium is in turn composed of 5-6 layers of columnar cells (superficial, wing and basal cells) which limit the drug absorption from the lacrimal fluid into the eye [14]. The corneal barrier is formed upon the maturation of epithelial cells. These epithelial cells migrate from the limbal region to the apical surface wherein they form tight junctions thereby limiting the paracellular permeation of the compounds [15]. Due to the lipoidal nature of the epithelium, hydrophobic compounds are relatively more permeable into the cornea than the hydrophilic compounds. However, the permeation of hydrophobic drugs in to deeper ocular tissues is further prevented due to the presence of hydrophilic stroma. Another barrier of minor importance associated with the cornea is the monolayered endothelium with leaky tight junctions sandwiched between the aqueous humor and the hydrophilic stroma [13].

1.1.3 Conjunctiva as a barrier

The conjunctiva is a highly vascular tissue internally lining the upper and lower eyelids, anterior sclera and constitutes about 80% of the ocular surface. It is composed of outer 2-10 layered stratified epithelial cells and inner stroma (substantia propria) [13]. The apical

epithelial cells of the conjunctiva also form tight junctions limiting the paracellular permeation of the instilled compound to the deeper tissues [16]. Embedded in the conjunctiva are numerous goblet cells, which are involved in the secretion of mucin and tear formation. The secreted mucin performs various functions such as: aiding in adhesion and maintenance of tear film, providing protection and nourishment to the avascular cornea [17]. The physiochemical properties including molecular weight and hydrophilicity play a major role in the conjunctival permeation of drugs. The hydrophilic compounds of low molecular weight (<20 kDa) can pass more easily across the conjunctiva when compared to lipophilic high-molecular weight compounds [16, 18]. In addition, the conjunctival blood capillaries and lymphatics further limit the ocular bioavailability through conjunctiva due to the drug loss into systemic circulation [19].

1.1.4 Efflux transporters as a barrier

Efflux transporters are membrane-associated proteins that belong to the ATP-binding cassette (ABC) superfamily. These transporters actively translocate molecules out of the cytoplasm and cell membrane, resulting in low ocular bioavailability. The efflux transporters such as P-glycoprotein, multidrug resistant protein (MRP), Breast cancer resistance protein (BCRP) were identified on various ocular tissues [20]. P-glycoprotein effluxes hydrophobic molecules from both normal and malignant cells and is the main reason behind drug resistance. Also, P-glycoprotein has been expressed on various ocular tissues including the cornea, conjunctiva and retinal pigment epithelium (RPE) [21]. Three isoforms of MRP have been reported in ocular tissues, which extrude organic anions and conjugated compounds. MRP1 is identified on rabbit conjunctival epithelial

cells and RPE whereas MRP2 and MRP5 have been expressed in the corneal epithelial cells [7]. Transport studies indicate that BCRP was identified in human corneal epithelial cells wherein it acts as a drug flippase, effluxing out the conjugated and unconjugated anions [22].

1.2 Conventional delivery systems

Topical instillation is the most commonly preferred, non-invasive route of administration for treating anterior segment eye diseases. Conventional topical delivery systems including solutions, ointments and suspensions account for 90% of the commercial ophthalmic formulations [23] with individual shares of 62.4% (solutions), 17.4% (ointments) and 8.7% (suspensions) [24].

1.2.1 Eye drops

Upon eye drop instillation, pulsating drug permeation occurs, due to which the concentration of the drug decreases rapidly, following first-order kinetics. Therefore, to enhance the residence time, permeation and bioavailability, various additives are added to eye drops including viscosity enhancers, permeation enhancers and cyclodextrins [5]. Viscosity enhancers such as carboxymethyl cellulose, hydroxyl methylcellulose, hydroxyl propyl methylcellulose, and polyalcohol increase the formulation viscosity and therefore enhance the drug residence time and ocular bioavailability [25-27]. Sasaki et al, investigated the effect of carboxymethyl cellulose on the ocular absorption of tilisolol in a rabbit model. This study concluded that the presence of viscosity enhancer, increased the levels of tilisolol in both tear fluids and aqueous humor [28].

Permeation enhancers, enhance the cellular uptake of drugs by modifying the corneal integrity. Additives such as sodium taurocholate, saponins, cremophor EL, EDTA, benzalkonium chloride were investigated as possible permeation enhancers for ocular delivery [29-31]. The general classes of compounds used as permeation enhancers include surfactants, calcium chelators, bile acids and salts. The surfactants increase the drug permeation by readily forming mixed micelles, which leads to membrane solubilization, thereby increasing the transcellular transport of therapeutic compounds. The bile salts act by producing a transitory change in the lipid membrane structure, thereby affecting the cell membrane integrity, which eventually leads to enhanced drug permeation. The chelators on the other hand, act by binding to the calcium ions and loosening the epithelial junctions, facilitating an increased transport of compounds across the membrane [32]. A study indicated that the permeation of fluorescein increased by 5-fold when Tween 20 and Brij 35 were used at a concentration of 1%, without causing any adverse effects [33]. Another study conducted by Newton et al., showed that therapeutic concentration of cyclosporine was achieved with Azone[®] in a rabbit corneal model [34]. Also, the corneal absorption of macromolecules including thyrotropin-releasing hormone and luteinizing releasing hormone were reported to be higher in the presence of saponins, EDTA, benzalkonium chloride and parabens [35]. However, a few studies indicate that ocular solutions containing permeation enhancers are associated with adverse effects such as local toxicity [36].

Cyclodextrins (CDs) are cyclic oligosaccharides composed of an inner hydrophobic core surrounded by a hydrophilic corona with attached hydroxyl groups. Depending upon the

number of sugar units, cyclodextrins are of three kinds: α , β and γ with 6, 7, 8 sugar units respectively [37]. CDs are generally used as solubilizers and stabilizers in pharmaceutical compositions. They also have the ability to sustain drug release, reduce drug-induced irritation and enhance *in vivo* performance [38, 39]. Zhang et al., investigated the ocular pharmacokinetics of ketoconazole aqueous drops containing hydroxyl propyl beta-cyclodextrin (HP- β -CD) and compared with 1.5% ketoconazole suspension. The area under the curve (AUC) in the aqueous humor and cornea was reported to be approximately 8 and 13 times higher with ketoconazole-CD solution when compared to the drug suspension. Also, significantly higher drug levels were observed in the aqueous humor and cornea with the addition of HP- β -CD to ketoconazole solution [40]. However, all of these approaches suffer from a few disadvantages including precorneal loss in the case of viscosity enhancers & cyclodextrins and high sensitivity of ocular tissues with permeation enhancers. Therefore, conventional approaches of formulation with inert carrier systems including emulsions, suspensions and ointments were investigated [41].

1.2.2 Suspensions and ointments

Suspensions can be defined as coarse dispersions of finely divided insoluble drug in an aqueous phase with a suspending agent. They are another class of non-invasive drug delivery systems in which the insoluble drug particles retain in the precorneal region thereby increasing the contact time, when compared to the drug solution. The duration of action with suspensions depends on the particle size; smaller particles replenish the drug absorbed from the precorneal region into the ocular tissues, whereas the larger particles remain for a longer time and show a slower dissolution profile. Therefore, an optimum

particle size is necessary for optimal drug activity [42]. A randomized, double masked, multicenter phase II clinical trial was conducted by Kinoshita et al., to investigate the efficacy of 1% and 2% rebamipide ophthalmic suspension for treating dry eye. The efficacy of suspensions was evaluated and was compared to the placebo. A dose-dependent response was demonstrated with the formulations and the placebo for both fluorescein corneal staining and lissamine green conjunctival staining tests. A significant difference in tear film break up time was observed with the suspensions compared with the placebo. The results indicate that both the formulations were effective in treating dry eye and showed an improvement of about 64.1% and 54.9% respectively [43].

Ophthalmic ointments are mixtures of hydrocarbons that melt at physiological temperature (34 °C) and the selection of hydrocarbon is generally based on its biocompatibility. Ointments show advantages such as enhanced drug retention and are capable of sustaining the drug release [44]. These carriers also inhibit the drug dilution by tears and are less affected by nasolacrimal drainage [45]. However, when compared to solutions, ointments show slow onset of action and higher dosage variability [42]. A study was conducted to evaluate the inhibitory effect of vancomycin ointment on methicillin-resistant staphylococcus aureus (MRSA) keratitis. Four ointment formulations were prepared using vancomycin of 0.03%, 0.1%, 0.3% and 1% concentrations, liquid paraffin and vaseline. The efficacies of the ointments were examined in a rabbit model induced with MRSA keratitis infection. The results of this study revealed that ointment containing 0.3% vancomycin was effective and sufficient against MRSA keratitis and showed no recurrence of infection over the 14-day study period in rabbits [46]. Several ointments are now available commercially for administration during night time such as

erythromycin (Ilotycin[®]), gentamicin (Gentak[®]) and tobramycin (Tobradex[®]) for treating various ocular conditions [32].

Although extensive efforts are being put in improving the efficacy of the conventional formulations, still the above-mentioned formulations: suspension and ointments usually interfere with the vision, and cause ocular side effects such as irritation and redness of the eye [5]. Also, chronic administration may increase the availability of API in systemic circulation leading to severe complications [47]. Therefore, to overcome these drawbacks and deliver therapeutic amounts of drugs to ocular tissues, the current research is focused on exploring other novel strategies for topical ocular drug delivery.

1.3 Novel ocular drug delivery strategies

To improve the bioavailability and overcome various ocular barriers, alternative novel drug delivery systems including nanomicelles, nanoparticles, nanosuspensions, liposomes, dendrimers, implants, contact lenses, and *in situ* thermosensitive gels are being investigated [5].

1.3.1 Vesicular systems

Vesicular drug delivery systems, also referred to as liquid-retentive systems, consist of a drug enclosed in a vesicle. Numerous promising agents were unsuccessful in clinical trials because of limited cell permeation, which can be addressed by the vesicular systems. These also provide sustained drug release, therefore, eliminating the need for frequent administration. Vesicular systems are prepared using lipids and have the ability to encapsulate both hydrophilic and lipophilic drugs. They also prevent the metabolism of

drugs by tear fluids and corneal enzymes [48]. The types of vesicular systems investigated as ocular colloidal carriers include liposomes, niosomes, discomes and pharmacosomes [49, 50].

1.3.1.1 Liposomes

Liposomes are microscopic vesicles containing aqueous core surrounded by one or more phospholipid bilayers. Hydrophilic drugs can be enclosed in the aqueous core while the lipophilic drugs can be encapsulated in the lipid bilayer [51]. Liposomes are generally made of natural phospholipids including egg phosphatidylethanolamine and dioleoylphosphatidylethanolamine (DOPE), which make them the ideal carriers for ocular delivery [52]. The efficiency of liposomes depends upon various factors such as size, charge, stability, entrapment efficiency, retention time and affinity towards the cornea [53]. The precorneal residence time of liposomes is further enhanced by the use of positively charged lipids generating cationic liposomes. The positively charged liposomes show greater affinity towards the negatively charged corneal surface when compared to negatively charged or neutral liposomes. A few examples of positively charged lipids include stearylamine and didodecyldimethylammonium bromide [20].

In a study, liposomes containing ciprofloxacin were formulated using the reverse phase evaporation technique and were evaluated for *in vivo* pharmacokinetic studies in albino rabbits. The ocular bioavailability of liposomal formulation was compared with ciprofloxacin eye drops (Ciprocin®). The bioavailability and the area under the aqueous humor concentration time curve were 3 and 2.5-fold higher than the eye drop solution [54]. Kouchak et al., prepared liposomes of dorzolamide using reverse phase evaporation

vesicle (REV) and thin layer hydration techniques (TLH). The entrapment efficiency was found to be higher in liposomes prepared by TLH when compared to the REV method. The study evaluated the *in vivo* intraocular pressure (IOP) by treating the rabbits with TLH nanoliposomes, dorzolamide solution and marketed dorzolamide formulation (Biosopt®). Liposomes prepared by the TLH method decreased IOP and also exhibited prolonged efficacy compared to the drug solution and Biosopt® [55]. Despite of their potential as ocular delivery systems, liposomes have few drawbacks, including low stability, low entrapment efficiency, difficulties in sterilization and degradation by lysosomes [20]

1.3.1.2 Niosomes and Discomes

Niosomes are bilayered surfactant vesicles with an inner aqueous core. Similar to liposomes, these can encapsulate both hydrophilic and lipophilic drugs [56]. Niosomes are generally made of biocompatible and non-immunogenic materials and are chemically more stable. Also, niosomes can be manufactured using low cost which makes them advantageous over other delivery systems [57]. Discomes are another novel delivery system, which are obtained by the incorporation of non-ionic surfactants such as solutan C24 in niosomes. These are large disc shaped structures with a size range of 12-16 μm [58]. Niosomal formulations of selected beta-blockers (PP-24, PP-34, PP-41, PP-48, PP-50) were investigated for topical delivery. The niosomal formulation of PP-24 showed high *ex-vivo* permeability across pig corneas resulting in significant enhancement of ocular bioavailability and decrease in IOP of rabbits when compared to the free drug solution [59]. Novel elastic niosomes (ethoniosomes) were developed by Gaafar et al., for

topical ocular delivery of corticosteroids including prednisolone acetate and prednisolone sodium phosphate. Ethoniosomes were prepared by ethanol injection method and evaluated for ocular irritation, bioavailability and anti-inflammatory activity. The evaluations were compared with the commercial Prednisol[®] eye drops and Predforte[®] suspension. Ethoniosomes showed no ocular irritation, and the bioavailability was 1.75 and 1.54 times higher than the Prednisol[®] eye drops and Predforte[®] suspension respectively. The healing time of clove oil induced inflammation was reduced to half with ethoniosomes. Moreover, IOP elevation generally observed with the commercial products was significantly lower with the prepared ethoniosomes [60]. In-addition, discoms are less affected by systemic drainage and show a longer residence time in the cul-de-sac of the eye due to their large size and disc shaped structure [61]. Naltrexone hydrochloride encapsulated in discoms showed 5-fold increase in the entrapment efficiency when compared to niosomes. However, the niosomes prepared were found to be non-irritant on chorioallantoic membrane and were capable of enhancing permeability of naltrexone hydrochloride across the bovine cornea [57].

1.3.2 Nanoparticles

Nanoparticles can be defined as particles with less than 1 μ m diameter and are comprised of various polymers, lipids/ phospholipids and metals. They are categorized into nanospheres or nanocapsules, depending on whether drug molecules are homogenously dispersed or coated within a polymeric substance [62]. Nanoparticles offer several advantages such as increased stability of the encapsulated drug and carrier system, reduced drug metabolism and clearance, improved bioavailability, and low ocular

irritation [20]. They also have the ability to sustain the drug release and this can be achieved by embedding the nanoparticles in biocompatible thermosensitive gels [63]. Most commonly used polymers for the preparation of nanoparticles include albumin, hyaluronic acid (HA), sodium alginate, chitosan and poly(lactide-co-glycolic acid) (PLGA). Nanoparticles, when administered, are eliminated rapidly, similar to any aqueous solutions. To overcome this issue and improve the retention time, surface-modified nanoparticles are prepared using chitosan, PEG and HA [20]. Modification by positively charged chitosan produces cationic nanoparticles which effectively bind to the negatively charged corneal surface thereby improving the residence time [64].

Solid lipid nanoparticles of indomethacin were formulated by Hippalgaonkar et al., using hot homogenization method and evaluated for *in vitro* permeability across rabbit cornea. The evaluation was compared with the indomethacin solution and indomethacin hydroxypropyl-beta-cyclodextrin based preparation. A dramatic increase in the chemical stability and corneal permeability ~4.5 and 3 fold higher was observed with the indomethacin loaded nanoparticles when compared to the solution and cyclodextrin based formulations [65]. Liu et al., investigated the effect of thiolated chitosan on the precorneal retention and corneal penetration of curcumin (CUR) loaded nanostructured lipid carriers (NLC). The curcumin loaded NLC were prepared by melt emulsification technique and surface modified using thiolated chitosan formed by the covalent modification of chitosan (CS) with N-acetyl-L-cysteine (NAC). The *in vivo* pharmacokinetics of the prepared formulation was evaluated and compared with curcumin eye drops, curcumin loaded NLC and chitosan modified curcumin loaded NLC.

The results indicate that thiolated chitosan significantly increased the retention time and AUC by several folds when compared to all other formulations, thereby increasing the drug permeability and bioavailability [66]. Melatonin loaded PLGA nanoparticles were investigated for a hypotensive (IOP-lowering) effect by Musumeci et al. The nanoparticles were surface modified using PEG. Significant IOP-lowering effect was observed with the PEG-modified melatonin loaded PLGA nanoparticles relative to blank PLGA nanoparticles and the aqueous solution. A lower zeta potential was also observed with surface-modified drug loaded PLGA nanoparticles when compared to the blank-PLGA nanoparticles, which facilitated an improved and extended interactions between the ocular surface and the nanoparticles, resulting in a higher hypotensive effect [67].

1.3.3 Nanomicelles

Nanomicelles are self-assembling colloidal structures with size ranging from 10 to 100 nm. They consist of a central hydrophobic core surrounded by a hydrophilic shell and are generally used as carriers for delivering hydrophobic drug molecules [68]. Nanomicelles can be prepared using surfactants or block copolymers and are easy to sterilize by simple filtration. However, nanomicelles suffer from a few disadvantages including inability to encapsulate hydrophilic molecules and also exhibit premature drug release [20]. Nanomicelles of biotin-12hydroxystearic acid (B-12HS-ACV) and acyclovir were prepared by Vadlapudi and co-workers using vitamin E TPGS and octoxynol-40 by solvent evaporation method. The prepared nanomicellar formulation was evaluated for *in vitro* biocompatibility in human corneal epithelial cells and mRNA expression profiles of various inflammatory cytokines by quantitative real-time PCR (qPCR). The results

indicate that the mixed nanomicelles sustained the release of prodrug and were found to be nontoxic and noninflammatory to the corneal epithelial cells [69]. Guo et al., developed cyclosporine-loaded nanomicelles for anterior segment delivery using polyvinyl caprolactam-polyvinyl-acetate-polyethylene glycol (PVCL-PVA-PEG). The *in vivo* corneal permeation of nanomicelles was determined using rabbits and was compared to commercial oil-based ophthalmic solution. The micellar solution demonstrated excellent tolerance in rabbits. The results also indicated that cyclosporine loaded nanomicelles (0.5 mg/ml) delivered higher amounts of cyclosporine into the cornea when compared to the commercial ophthalmic solution (10 mg/ml) [70]. To improve the bioavailability, surface modified pluronic micelles of metipranolol were prepared by Lin et al., using ethyl acetate as a dispersion agent. The nanomicelles showed sustained release with good pharmacological responses compared to the commercial eye drops. The metipranolol micellar system could also improve patient adherence as it eliminates the need of frequent administration [71].

1.3.4 Nanosuspensions

Nanosuspension can be defined as submicron colloidal dispersions, which consists of hydrophobic drugs, stabilized using polymers or surfactants. These are easy to formulate, less irritant, exhibit increased precorneal residence time and enhanced ocular bioavailability [72]. Flurbiprofen encapsulated in Eudragit RL 100[®] by the solvent displacement technique was studied by Boddeda et al. The formulation displayed a sustained release and was effective for a longer duration of time when compared to marketed eye drops. The *in vivo* animal studies including draize test and

histopathological evaluation revealed that the flurbiprofen nanosuspension was non-irritant and non-toxic. Also, the positive charge on the surface of these nanoparticles can facilitate better adhesion to the negatively charged surface of the cornea [73]. Shi and co-workers encapsulated diclofenac in MPEG-PCL-CS (methoxy poly(ethylene glycol)-poly(ϵ -caprolactone) and chitosan) block polymer and examined its effect on corneal penetration in rabbits. This study concluded that diclofenac/MPEG-PCL-CS nanosuspension showed improved pre-corneal retention and permeation thereby increasing the bioavailability of diclofenac, when compared to marketed eye drops [74]. In another approach, lomefloxacin HCl was formulated as nanosuspension using chitosan by ionic gelation technique. As compared to the drug solution, a three-fold increase in the amount of drug permeated across bovine corneas was observed with the nanosupension. Also, the nanosuspension showed enhanced anti-bacterial activity with ~3.5-fold decrease in minimum inhibitory concentration (MIC) against gram negative bacteria [75].

1.3.5 Contact lenses

Contact lenses are thin curve-shaped discs made up of plastic and are placed in front of the cornea. The lenses adhere to the cornea due to the interfacial tension [76]. They are usually made from polymers including silicone hydrogel and poly (hydroxyethyl methacrylate) (p-HEMA) [77, 78]. This method of administration provides sustained release and thereby improves the patient adherence. Also, it requires smaller doses, limiting the systemic exposure of the drug [79]. Contact lenses loaded with conventional drugs or drug carriers have been developed for ocular delivery of therapeutics. Soft contact lenses of prednisolone-loaded nanoparticles were investigated by Elshaer et al.,

for topical administration. Prednisolone nanoparticles were prepared by solvent-evaporation method using poly (lactic-co-glycolic acid) (PLGA) and then incorporated into contact lenses. The results indicate that clear and transparent nanoparticle-loaded lenses were obtained and were able to prolong the residence time and sustain the drug release when compared to the nanoparticles alone [80]. Vitamin E loaded silicone lenses act as barriers for drug diffusion, resulting in an increased duration of action. This approach was investigated using topical anesthetic drugs including lidocaine, bupivacaine and tetracaine. It was observed that vitamin E loaded lenses provide anesthetic release for up to 7 days. Therefore, the reported method could be highly useful in reducing post-operative pain in patients undergoing corneal procedures such as photorefractive keratectomy [81]. Nanospheres of ciprofloxacin prepared using pullulan and polycaprolactone (PCL) were incorporated into HEMA-based contact lenses. These modified lenses showed sustained anti-microbial activity against *S.aureus* and *P.aeruginosa* [82]. Molecular imprinted contact lens of ketotifen fumarate were prepared using poly (HEMA-co-AA-co-AM-co-NVP-co-PEG200DMA) by Tieppo and co-workers. The prepared lenses demonstrated increased mean residence time which is about 4- and 50-fold higher than non-imprinted lenses and commercial eye drops (Zaditor[®]). Furthermore, the bioavailability of the ketotifen fumarate was also improved with imprinted lenses and was about 9 and 94-fold higher than non-printed lenses and eye drops respectively [83].

1.3.6 Microemulsions

The term “microemulsion” was first introduced in 1943 by Hoar and Schulman. Microemulsions are thermodynamically stable-phase transition systems. They exhibit small particle size (5-200nm) with lower surface tension which facilitates higher drug absorption and permeation [84]. Pharmaceutically, microemulsions are colloidal oil in water (o/w) or water in oil (w/o) nanodispersions, stabilized by a surfactant and cosurfactant. The preparation of microemulsions involves mixing an oil phase containing oil, surfactant, and cosurfactant with an aqueous phase. Microemulsion form spontaneously without the need for significant amount of energy [85].

During the process, the surfactant is adsorbed at the interface of oil and water and this determines the initial curvature of the dispersed phase. The cosurfactant further assists in attaining the required curvature thereby maintaining minimum interfacial tension. The resulting surfactant film exerts a two-dimensional pressure at the surface, which then leads to the bending of the interface with the expansion of film on one side so as to maintain the balance on the other side, until the pressure on both the sides of the interface becomes equal [86]. The side having higher tension is concave and envelops liquid present on that side, creating an internal phase [87]. The elastic nature of the surfactant film is influenced by the type of surfactants, thermodynamic conditions and on the presence of substances such as electrolytes, alcohols and copolymers. The shape and size of the dispersed droplets are mainly determined by the curvature of the film, bending elastic constant and free energy at the interfacial curvature. Thus, a thermodynamically

stable system of either swollen mixed micelles (o/w) or inverse mixed micelles is formed [4].

1.3.6.1 Theories of micro emulsion formation:

Figure 1.1: Schematic of emulsion formation. The oil and water phases are separated by the interface (yellow lines).

There are three main theories related to the formation of microemulsion i.e., mixed film or interfacial, solubilization and thermodynamic theory. According to the interfacial theory, the surfactant system is considered as a duplex film at the interface. The duplex nature alters the properties of oil and water phases and reduces the interfacial tension to a very low value leading to the spontaneous formation of microemulsions. The solubilization theory considers microemulsions as swollen micellar systems with solubilized water or oil phases resulting in the formation of single-phase systems [88]. The thermodynamic theory explaining the formation of microemulsions is the widely accepted one and therefore discussed in detail. Assuming that the Gibbs free energy can be divided into numerous independent contributions, the free energy of the system (G_B) prior to emulsification can be expressed in the form of Eq. 1

$$G_B = G_I + G_E + G_{IE} + G_S \quad \dots\dots\dots \text{Eq1}$$

Where G_I , G_E , G_{IE} are the free energy of internal, external phase and interface respectively. G_S is the interfacial energy between the liquids and the surface of the container. Generally, G_S is considered to be negligible as the solid-liquid interfacial area is too small. G_I and G_E will remain almost the same prior and post emulsification, whereas the G_{IE} is at a minimum value prior to emulsification and can be expressed in the form of Eq. 2 [89].

$$G_{IE} = \gamma_{IE} \cdot A \quad \dots\dots\dots \text{Eq2}$$

Where γ_{IE} and A are the interfacial tension and interfacial area respectively. Once the droplets are formed (post-emulsification), the interfacial area increases and there is a change in entropy (ΔS) which can be expressed in the form of Eq 3.

$$\Delta G_{Emuls.} = \gamma_{IE} \Delta A - T \Delta S \quad \dots\dots\dots \text{Eq3}$$

The formation of droplets, during the emulsification process, increases the ΔS of the system, which eventually makes $T \Delta S$ much higher than $\gamma_{IE} \Delta A$. Therefore, the Gibbs free energy ($\Delta G_{Emuls.}$) will be negative and is responsible for the spontaneous formation and thermodynamic stability of the microemulsions [89].

1.3.6.2 Methods of microemulsion preparation:

- a) **Phase titration/Spontaneous emulsification method:** This method utilizes less energy and allows the spontaneous diffusion of surfactant or solvent molecules into the continuous phase due to the minimum interfacial tension. The preparation of microemulsion involves the construction of a pseudo-ternary phase diagram

composed of oil, water, surfactant & cosurfactant and investigating the formation of single-phase region in the diagram. All the components present in the formulation are mixed in varying proportions ranging from 0 to 100% and investigated for the formation of clear phase. Subsequent optimization is done based on the clearest area in the phase diagram and the most stable composition of microemulsion is then finalized [90, 91].

b) Phase inversion method: This method requires the addition of excess dispersed phase or a change in temperature for the formation of a microemulsion. Radical changes in droplet size are observed with this method, which in turn affects drug release behavior both *in vitro* and *in vivo*. For non-ionic surfactants, a change in temperature from low to high converts oil in water microemulsion to water in oil microemulsion. When the system cools down, it crosses a point of trivial surface tension and zero spontaneous curvature, supporting the formation of oil droplets. The increase in the volume of water also produces a transition in the spontaneous curvature of water in oil (w/o) microemulsion to form an oil in water (o/w) microemulsion [92]. In addition, parameters such as pH and salt concentration may also influence the preparation of microemulsions by phase inversion technique [87].

Another method based on external energy supply is also reported for the preparation of the microemulsions. This technique involves the preparation of a coarse emulsion using a high-speed mixer, then introducing the mixture through a high-pressure homogenizer to obtain a desired size of droplets. In addition, the

dispersion of oil into the aqueous phase can be facilitated by heating the components prior to mixing. Generally, blue colored opalescent microemulsions are formed by this technique [93].

1.3.6.3 Formulation considerations on manufacturing ocular microemulsions

- a) **Selection of lipid phase:** The physicochemical properties of the lipids play a very important role in the development of ocular microemulsions. Oils with maximum solubilizing capacity for the drug are generally chosen for the preparation in order to attain maximum drug loading [94, 95]. The development of microemulsion using high molecular weight oils especially triglycerides is difficult as the long-chain fatty acids cannot penetrate the interfacial film of surfactants and assist in the formation of an optimum curvature. Due to this reason, medium chain length fatty acids with smaller molecular weight are usually preferred [96, 97]. Most commonly used lipids for ocular drug delivery include vegetable oils, esters of fatty acids (ethyl oleate, isopropyl myristate, isopropylpalmitate), monounsaturated fatty acids (oleic acid), saturated fatty acids (capric triglyceride, octanoic acid) [4].
- b) **Selection of surfactant:** Surfactants molecules are typically composed of a polar head region and a non-polar tail. They associate themselves into a variety of shapes including spherical, rod-shaped, hexagonal, reverse micelles and lamellar phases due to various inter and intra molecular interactions and entropy considerations [98]. Critical packaging parameter (CPP) is normally used to

predict the possible structures formed by surfactants. A CPP value greater than one indicates the formation of w/o systems, whereas values lower than one indicates o/w systems [99]. The solubilization of the lipid phase is much higher in microemulsions when compared to most micellar solutions. It may be possible for one surfactant molecule to dissolve 10-30 lipid molecules in oil-water microemulsion or 10-300 water molecules in the case of water-oil microemulsion thereby reducing the interfacial tension to a very minimal value ($<10^{-3}$ mN/m) [86]. This minimal interfacial tensions helps in spontaneous emulsification and also facilitates the formation of flexible film around the droplets, providing an optimal curvature at the interfacial region [92, 100].

Based on the nature of origin, two major types of surfactants are generally available: natural and synthetic. Natural surfactants such as lecithin and related phospholipids are generally more preferred over synthetic agents because of their potential to increase the permeability of the compound across the biological membranes thereby enhancing the intracellular drug concentration [101]. Surfactants are again categorized into either ionic, non-ionic agents or a mixture of both, which determines the stabilizing interactions between the hydrophilic portion of the surfactant and the aqueous phase. Typically, a nonionic agent is stabilized by dipole and hydrogen bond interactions, whereas an ionic agent is stabilized by the electrical double layer. Non-ionic agents are generally more employed in ophthalmic delivery systems because of their enhanced solubilization, lower toxicity, non-irritant nature, and prolonged retention time with enhanced permeability [4]. In addition, these agents show greater stability to

change in ionic strength and pH, which are highly subjected to vary after *in vivo* administration [102].

Generally, surfactants with low HLB (hydrophilic lipophilic balance) favor the formation of w/o microemulsions, whereas surfactants with high HLB (>12) favor o/w microemulsions. An agent with HLB value above 20 often requires cosurfactant to reduce the HLB value to a range which is suitable for the microemulsion formation [92]. Most commonly used surfactants for ocular microemulsions include lecithin and derivatives (soya phosphatidyl choline, sodium cholate), fatty acid esters of sorbitan (Span 20 and 80), polyoxyethylene sorbitan (Tween 20 and 80), and glycerol (cremophor EL) [4].

- c) **Selection of cosurfactant:** The flexibility of the interface is of major importance in the formation of microemulsion. For this purpose, the surfactant is often combined with a cosurfactant, which penetrates into the interfacial film producing a more fluid interface by allowing the free movement of hydrophobic tails of the surfactants at the interface. Adequately low fluidity and surface viscosity of the interfacial film lead to the formation of droplets with a smaller radius of curvature [4]. These molecules aid in the reduction of surface tension which increases the entropy thereby enhancing the thermodynamic stability of the system [103]. Generally, alcohols and glycols of low molecular weight and hydrocarbon chain length ranging from 2-10 are used as cosurfactants for the preparation of microemulsions [91, 97]. Studies indicate that hydrocarbon chain length of alcohols is directly proportional to ocular irritation. Long chain alcohols are

proven to be more irritants than cosurfactants having shorter chains. Most commonly used cosurfactants include alkanols (ethanol, propanol, 1-butanol), alkane-diols (1,2- propane diol, 1, 2- butane diol) and alkane polyols (glycerol, glucitol) [4].

1.3.6.4 Microemulsions in ocular drug delivery: Recent Research

Water in oil microemulsion of moxifloxacin (MXN) was investigated by Bharti and co-workers. Various microemulsions were prepared using different ratios of isopropyl myristate (oil phase), Tween 80 and Span 20 (surfactant system) and acetate buffer (aqueous phase). The developed microemulsion sustained the release of MXN and was stable for 3 months. Most importantly, the *in vivo* antimicrobial efficacy was significantly greater with the microemulsion compared to the conventional MXN solution [104]. El Agamy et al., investigated the safety and efficacy of phase transition microemulsion systems composed of natural oils such as olive oil and castor oil in comparison to ethyl oleate systems. Three systems including microemulsion (ME), liquid crystalline system (LC) and coarse emulsion (EM) were tested and evaluated by monitoring the mydriatic response relative to the tropicamide solution containing polyvinylpyrrolidone (PVP). The studies revealed that ocular irritation in rabbits was least with castor oil system, followed by olive oil with ethyl oleate system being the most irritant. However, the pharmacological response was higher with ethyl oleate and olive oil than the castor oil based system which was comparable to the control [105]. Microemulsion of amphotericin B was prepared by Silveira et al, using titration technique and was evaluated for use in treating ocular infections. The major concern with the conventional amphotericin eye

drops (Fungizone®) is toxicity, leading to poor patient adherence. The results indicate that the developed microemulsion showed higher anti-fungal activity against *Candida* strains and better *in vitro* compatibility with erythrocytes when compared to Fungizone®-[106]. Gan et al., investigated novel microemulsion *in situ* electrolyte-triggered gelling system of cyclosporine to prevent corneal allograft rejection. The microemulsion was prepared using castor oil, solutol HS 15, glycerol and water and later dispersed in Kelcogel® solution to form the gelling system. The developed system was evaluated for *in vitro* drug release, ocular irritation and *in vivo* ocular pharmacokinetics and the evaluations were compared to cyclosporine emulsion. This study concluded that the microemulsion was well tolerated and showed sustained cyclosporine release. A three-fold greater corneal AUC was observed with the microemulsion system compared to emulsion. Moreover, therapeutic concentrations of cyclosporine were maintained even after 32 h of administration, thereby preventing corneal allograft rejection [107]. A novel ocular microemulsion of pilocarpine was prepared using soyabean oil, Span 80, Brij 35P, 1-butanol and water. The developed microemulsion was tested for intraocular pressure (IOP) lowering effect, ocular irritation and histopathological evaluation. The results indicate that the microemulsion was well tolerated in rabbits, showed good stability and produced significant lowering in IOP compared to the commercial collyrium. The histopathological studies did not show any signs of toxicity. Moreover, the microemulsion also showed an improved retention time with prolonged release of pilocarpine relative to commercial collyrium [108]. Ustundag-Okur et al., investigated o/w micro emulsion of ofloxacin (0.3%) prepared using oleic acid (oil phase), Tween 80 (surfactant), ethanol (cosurfactant) and 0.5N NaOH (aqueous phase). The optimized

formulation was further modified using chitosan oligosaccharide lactate (0.75%). The results indicate that the modified microemulsion showed sustained release with greater antibacterial activity against *S.aureus* and *E.coli* followed by the optimized microemulsion and commercial formulation being the least. However, the precorneal residence time and permeation across rabbit corneas were higher with the optimized formulation than the modified microemulsion [9].

1.3.6.5 Recent patents on topical ocular microemulsions for anterior segment drug delivery

US patent number	Publication date	Title
8414904	Apr 9, 2013	Ophthalmic oil-in-water emulsions containing prostaglandins
7655625	Feb 2, 2010	Methods of treating blepharospasm using cyclosporine components
20110178147	Jul 21, 2011	Compositions and methods for controlling pupil dilation
8513353	Aug 20, 2013	Forming copolymer from bicontinuous microemulsion comprising monomers of different hydrophilicity
0108674	May 2, 2013	Ophthalmic compositions for the administration of liposoluble active ingredients

Chapter 2

Significance of research

Conventional preparations such as eye drop solutions, suspensions, ointments represent the majority of the marketed ocular formulations. Topical instillation of these dosage forms is the most preferred route for anterior segment delivery due to its ease of administration. However, these dosage forms show very low ocular bioavailability due to various anatomical and precorneal constraints such as corneal epithelium, tear turnover, nasolacrimal drainage, reflex blinking and induced lacrimation. To improve the bioavailability, several nanocarrier systems such as microemulsions, nanosuspensions, nanomicelles are currently being investigated. As the preparation is intended for ocular use, the ease of sterilization is another important aspect of consideration.

Microemulsions are thermodynamically stable, easy to manufacture and are capable of solubilizing both hydrophilic and lipophilic drugs. They have a low viscosity and are able to deliver the drug in a sustained fashion, thereby improving the overall absorption capacity [85]. Microemulsions have good spreadability, which allows the drugs to spread on the cornea and mix with the precorneal fluids. This improves the contact time of drugs with the corneal epithelium [109]. Also, they can be sterilized easily by filtration for

ocular delivery as eye drops [110]. Therefore, microemulsions were selected as the delivery system of choice.

Microemulsions are prepared by mixing an oil phase containing oil, surfactant and co-surfactant with an aqueous phase. Ricinoleic acid, cremophor-EL and 1-butanol were chosen as oil, surfactant and co-surfactant, respectively. Dexamethasone and tobramycin were chosen as model drugs as they are commonly used in treating ocular inflammation and bacterial infection, respectively. Currently, dexamethasone and tobramycin are marketed in combination as an ointment and as suspension eye drops. Suspension eye drops in general show erratic drug absorption [111], whereas with the ointment dosage form, the strong binding between dexamethasone with the ointment base limits its permeability [112]. These dosage forms are linked to side effects, including transient blurred vision, irritation and redness to the eye [5].

The aim of the study was to develop a drug-loaded microemulsion of dexamethasone (0.1%) and tobramycin (0.3%). The current research is a continuation of our previous study in which we prepared a blank microemulsion composed of ricinoleic acid (2.5%), cremophor EL (9.3%) and 1-butanol (6.2%), and water (82%). Ricinoleic acid is known for its anti-inflammatory and anti-bacterial properties [111, 113]. Therefore, we hypothesize that oil phase containing ricinoleic acid would work synergistically with dexamethasone and tobramycin in reducing anterior segment inflammation and infection. The microemulsion was further characterized and evaluated for its potential in treating inflammation and infection. We believe that this unique system could be a suitable alternative to the marketed formulations. In addition, the microemulsion developed could

overcome the limitations of conventional dosage forms and prolong the residence time, enhance the corneal permeability of both drugs and lower the dosing frequency.

Chapter 3

Development and Evaluation of a Novel Microemulsion of Dexamethasone and Tobramycin for Topical Ocular Administration

3.1 Abstract

Objective: The drug absorption from an ophthalmic suspension dosage form is highly unpredictable. Identical formulations with similar concentrations of active and inactive ingredients tend to exhibit differences in absorption. These differences in absorption could be due to varying physicochemical properties such as pH, particle size, zeta potential, size distribution and viscosity. These properties affect the precorneal residence time, drug release and ocular drug absorption [1, 2]. Drug delivery to the eye using microemulsions has drawn significant attention due to their intrinsic properties and ability to solubilize both hydrophilic and lipophilic drugs. They offer several advantages including thermodynamic stability combined with improved dose uniformity [3], ocular retention, permeation and absorption of drugs [4]. The present study involves the development and evaluation of a novel dexamethasone (0.1%) and tobramycin (0.3%) loaded microemulsion with potential for treating anterior segment eye inflammation and bacterial infection.

Methods: The microemulsion was evaluated for pH, particle size, zeta potential, light transmittance, morphology, and *in vitro* drug release. Sterility of microemulsion was evaluated by direct as well as plate inoculation methods. Anti-inflammatory activity of dexamethasone, bactericidal activity of tobramycin, and cytotoxicity of the microemulsion were evaluated and compared with that of marketed eye drop suspension (Tobradex[®]). Histological evaluation was performed in bovine corneas in order to assess the safety of microemulsion in comparison to Tobradex[®] suspension. In-addition, the stability of the microemulsion was also studied at 4°C, 25°C and 40°C.

Results: The pH of microemulsion was close to the pH of tear fluid. The microemulsion displayed average globule size under 20 nm with light transmittance around 95-100%. Aseptically prepared microemulsion remained sterile for up to 14 days. The cytotoxicity of microemulsion in bovine corneal endothelial cells was comparable to that of Tobradex[®] suspension. Anti-inflammatory activity of dexamethasone and anti-bacterial activity of tobramycin from the microemulsion were significantly higher than those of Tobradex[®] suspension ($p < 0.05$). Histological evaluation showed intact corneal epithelium without any signs of toxicity and the developed microemulsion was found to be stable at 4°C and 25°C for 3 months.

Conclusions: In conclusion, the microemulsion developed could be a suitable alternative to the currently marketed Tobradex[®] suspension for treating anterior segment infection and inflammation. Further *in vivo* studies in animals are warranted to evaluate the clinical utility of the microemulsion.

3.2 Introduction

Drug delivery to the eye is challenging due to its inherent and intricate anatomical and physiological barriers [7]. Conventional delivery systems such as eyedrop solutions, ointments and suspensions account for 90% of marketed ophthalmic formulations [23]. However, conventional ophthalmic formulations demonstrate very low bioavailability due to precorneal clearance mechanisms such as tear turn over, nasolacrimal drainage, reflex blinking and induced lacrimation [9]. Moreover, the corneal epithelium acts as a barrier for hydrophilic drugs, while stroma restricts the passage of hydrophobic drugs [13]. The ocular bioavailability ranges between 1-5% for hydrophobic drugs and less than 0.5% with hydrophilic drugs [114]. Despite these disadvantages, topical instillation remains the most preferred route due to its the ease of administration [8].

Ocular infections are mostly chronic in nature and characterized by inflammation of the cornea, conjunctiva and eyelids. Common ophthalmic infections include conjunctivitis, blepharitis, keratitis, keratoconjunctivitis and sty. The management of anterior segment ocular infections and inflammation requires prompt diagnosis and treatment in order to avoid any serious visual complications [111]. Bacterial infections are commonly treated using a combination of antibiotic and steroidal drugs. The antibiotic reduces the infection, while the corticosteroid treats the inflammation associated with the infection [115]. Dexamethasone is a steroidal anti-inflammatory drug with low aqueous solubility (0.16 mg/ml) and is moderately lipophilic in nature ($\log P = 1.8$) [116]. Dexamethasone inhibits phospholipase A2 activity required for the release of arachidonic acid, thereby preventing the release of prostaglandins, thromboxanes and leukotrienes [117]. Tobramycin is an

aminoglycoside antibiotic used for treating various types of bacterial infections. Tobramycin irreversibly binds to the bacterial 30S and 50S ribosomal subunits, preventing the formation of 70S complex, which eventually inhibits mRNA translation leading to cell death [118]. Currently, dexamethasone and tobramycin are marketed in combination by Alcon Laboratories, Inc, as suspension eye drops (Tobradex[®] and Tobradex ST[®]) and ointment (Tobradex[®]). The usual dose of Tobradex[®] suspension for treating mild to moderate infection is 1 or 2 drops in the affected eye(s) every 4 h, whereas in the case of severe infection the dose is increased to 2 drops every hour until the condition subsides. Similarly, 1.25 cm ribbon of Tobradex[®] ointment is applied to the conjunctival sac 2 to 3 times a day in mild to moderate infection, whereas in the case of severe infection the same dose is applied every 3-4 hours [119].

The administration of drugs by a droptainer bottle (in the case of solutions and suspensions) or ointment tube is highly challenging in both adults and young children due to the lack of acuity and inability to aim effectively [120]. Moreover, the drug absorption from an ophthalmic suspension dosage form is highly varied. This erratic drug absorption is attributed to the clearance of the larger percentage of drug particles from the precorneal area before drug dissolution and absorption can take place. Furthermore, the intrinsic drug dissolution rate fluctuates with the constant in and out flow of the lacrimal fluids [111]. An ointment on the other hand, provides improved ocular retention time. However, the drug binds to the ointment base and limits its penetration into the eye [112]. Moreover, the suspension and ointment formulations interfere with the vision and cause ocular side effects such as irritation and redness of the eye [5]. Therefore, to overcome

these drawbacks and enhance patient acceptance, the current research is focused on exploring a novel strategy for delivering dexamethasone and tobramycin.

An ideal ocular carrier system should improve the precorneal residence time, provide better bioavailability and decrease the dosing frequency [32]. Microemulsions are colloidal oil in water (o/w) or water in oil (w/o) nanodispersions, stabilized by a surfactant and cosurfactant system. They are prepared by mixing an oil phase containing oil, surfactant, and cosurfactant with an aqueous phase [85]. Microemulsions have emerged as promising systems for ocular drug delivery. Microemulsions are advantageous over other delivery systems due to their ease of manufacture & sterilization and ability to deliver both hydrophilic and lipophilic drugs. They are thermodynamically stable, provide sustained release and are able to achieve higher levels of drug in ocular tissues. They also help in reducing the dosing frequency [85]. Ocular microemulsions of several drugs like ofloxacin [9], timolol [121], prednisolone [122], pilocarpine [113] showed sustained release with improved bioavailability.

Ricinoleic acid is known for its anti-inflammatory and anti-bacterial properties [111, 123]. We hypothesize that microemulsion made up of ricinoleic acid as oil phase could work synergistically with dexamethasone and tobramycin in reducing bacterial infection and inflammation. In addition, this unique thermodynamically stable system could prolong the residence time, improve the corneal permeability of both drugs, and decrease the dosing frequency. The objective of this study was to develop and characterize an o/w

microemulsion composed of ricinoleic acid (oil phase), cremophor and 1-butanol (surfactant and cosurfactant) and water (aqueous phase), loaded with 0.1% dexamethasone and 0.3% tobramycin for treating the anterior segment inflammation and bacterial infection.

3.3 Materials and Methods

3.3.1 Materials

Dexamethasone (Lot C163997) and Tobramycin (Lot C164877) were procured from PCCA (Houston, TX). Kolliphor® EL (Lot BCBQ5632V), Lipopolysaccharides from *Escherichia coli* 055:B5 (Lot 105M4134V), N-(1-Naphthyl) ethylenediamine dihydrochloride (Lot SZBF1190V) and Sulfanilamide (Lot SLBN9189V) were purchased from Sigma Aldrich (St. Louis, MO). Ricinoleic acid (Lot XUWOODD) was procured from TCI (Portland, OR). 1-butanol (Lot A0317960) and 2, 4-Dinitrofluorobenzene (Lot A0351447) were purchased from ACROS (Fair Lawn, NJ). MTT powder (Lot#2626103) was procured from Millipore (Billerica, MA). DMEM (Lot# 10092157), 0.25% Trypsin, 2.21mM EDTA 1X (Lot# 25053365) and 1X Trypsin-EDTA solution (Lot# C2030) were purchased from Corning (Manassas, VA). Polysorbate 80 (Lot 120589), Potassium phosphate monobasic (Lot 123304), Sodium hydroxide (Lot 101166), Sodium bicarbonate (Lot#116734) and Hydrochloric acid (Lot#091256) were supplied from Fisher Scientific (Fair Lawn, NJ). Ethanol (Lot 223512) was purchased from Decon Labs, Inc. (King of Prussia, PA). Benzalkonium chloride (Lot Y52651G15) was supplied by Ruger (Irvington, NJ). Ethylenediaminetetraacetic acid disodiumsalt dihydrate (Lot B0136110A) was procured by ACROS (Fair Lawn, NJ). Tryptic soy broth (Soyabean

Casein Digest medium-Bacto™, Lot 2030828) and Mueller-Hinton broth (Lot 108187B), DMSO (Lot# 104549) were purchased from Fisher Scientific (Pittsburgh, PA). Taxo™ blank paper discs (Lot# 232189) were procured from Becton, Dickinson and Company (Sparks, MD). Tobramycin standard 10 10 µg discs (Lot# 1750529) were purchased from Oxoid (Hants, UK). TEM grids were purchased from Ted Pella Inc. (Redding CA). High Performance Liquid Chromatography (HPLC) solvents including acetonitrile (Lot 157773) and methanol (Lot 162124) were supplied by Fisher Scientific (Pittsburgh, PA). Distilled deionized water was used for the preparation of ocular microemulsions.

3.3.2. Construction of pseudoternary phase diagrams

Pseudoternary phase diagrams of oil phase (ricinoleic acid), surfactant (cremophor EL), cosurfactant (1-butanol) and aqueous phase (water) were developed to determine the regions of microemulsion formation. Four phase diagrams were constructed with surfactant to cosurfactant ratios of 1:4, 2:3, 3:2 and 4:1 using a conventional water titration method at room temperature. The final compositions of clear, single-phase formulations were converted to weight percentages and were plotted as points in the ternary diagrams. The area covered by these points representing the regions of microemulsion formation were plotted using TernPlot-Excel Plotting program.

3.3.3. Preparation of blank microemulsion

From the ternary phase diagrams, a composition of ricinoleic acid (2.5% w/w), cremophor EL (9.3% w/w), 1-butanol (6.2% w/w), and deionized water (82% w/w) was selected for further characterization and evaluation. The ratio of surfactant to cosurfactant was kept at 3:2. The microemulsion was prepared by a spontaneous emulsification technique. It involves the mixing of cremophor EL and 1-butanol for a few seconds using a fixed speed vortex mixer (Fisher Scientific, PA) to produce a homogenous mixture. The oil phase (ricinoleic acid) was then added to the surfactant mixture and vortexed to form a self-nanoemulsifying mixture. Finally, increasing amounts of deionized water was gradually added to the mixture with gentle stirring until a clear microemulsion system was obtained.

3.3.4. Preparation of dexamethasone and tobramycin loaded microemulsion

Dexamethasone and tobramycin loaded microemulsion was prepared by adding 0.1% of dexamethasone to self-nanoemulsifying mixture and vortexed until a homogenous mixture was obtained. Tobramycin (0.3%) was dissolved in distilled deionized water and this was gradually added to the self-nanoemulsifying mixture with gentle stirring, which resulted in the formation of clear and transparent microemulsion. In addition, EDTA disodium dihydrate (0.1%) was added to the formulation as an anti-oxidant and benzalkonium chloride (BAK) (0.01%) was added for its preservative property. The composition of the microemulsion is detailed in Table 3.1.

Table 3.1: Composition of the o/w microemulsion

Excipient	Quantity (w/w)
Dexamethasone	0.1%
Tobramycin	0.3%
Ricinoleic acid	2.5%
Cremophor EL	9.3%
1-Butanol	6.2%
Benzalkonium chloride (BAK)	0.01%
Ethylenediaminetetraacetic acid	0.1%
Distilled deionized water	82%

3.3.5. HPLC analysis of Dexamethasone

Dexamethasone was analyzed using HPLC (Waters Alliance e2695 separation module, Milford, MA), equipped with a 2998 PDA detector and a reverse-phase C8 column (5 μ m, 100 $^{\circ}$ A, Luna, Torrance, CA, USA). Mobile phase composed of water and acetonitrile (50:50) was pumped at a flow rate of 1.0 ml/min. The absorbance of dexamethasone was measured at 242 nm and the drug content in the samples was determined by plotting a calibration curve. A stock solution of 1000 μ g/ml of dexamethasone in methanol was prepared and calibration standards ranging from 0.39 - 50 μ g/ml were prepared in the mobile phase. Each calibration standard was analyzed in triplicate and the average peak area was plotted against the amount of dexamethasone to obtain the calibration curve.

3.3.6. UV analysis of tobramycin

Tobramycin was analyzed using a UV-Vis Spectrophotometer (Agilent 8453, UV-Vis Spectroscopy system). Calibration standards (5 ml) ranging from 50-250 μ g/ml were taken in 10 ml glass tubes and to which 1 ml of 2% sodium bicarbonate solution was

added and mixed. Then, 2 ml of freshly prepared 95% ethanolic solution of 2, 4-dinitrofluorobenzene was added (0.25 ml /100 ml) and mixed thoroughly. After 20 min, the solution was neutralized by adding 0.5 ml of 1M HCl. The glass tubes were tapped gently to remove any carbon dioxide bubbles and the absorbance was determined at 415 nm using distilled water as a blank [124]. Each standard was analyzed in triplicates and the absorbance was plotted against the concentration of tobramycin to obtain the calibration curve.

3.3.7. Physiochemical characterization of microemulsion

3.3.7.1 Droplet size

The droplet size was measured using Nicomp 380 ZLS (Particle sizing systems, Santa Barbara, CA). Samples of microemulsion were placed into 6 x 50 mm borosilicate glass tubes (Kimble Chase, Vineland, NJ) and positioned in the path of 100 mW He-Ne laser having a wavelength of 658 nm. The neutral density filter was adjusted until the scattered intensity fluctuated around 300 kHz. The light scattered was collected at 90° and detected using a photodiode array detector. Nicomp software automatically tuned the channel width & base line and Nicomp distribution was considered for Chi-square values greater than 3 [125]. Each sample was analyzed three times and the mean volume-weighted diameter was determined.

3.3.7.2 Zeta potential

Zeta potential was measured using Nicomp 380 in ELS mode. Samples of microemulsion were placed in a plastic cuvette and positioned in the path of the laser. The light scattered was collected at 14.8° and detected using a photomultiplier tube. Each sample was analyzed three times and the zeta potential was determined from the average of the runs.

3.3.7.3 pH

The pH of the samples was measured using Accumet® excel XL 25 pH meter (Fisher Scientific, PA). Before every use, the pH meter was calibrated using standard buffer solutions of pH 4.00, 7.00 and 10.00. Measurements were done in triplicate at 25 ± 2 °C and the data were expressed as mean $\pm$ SD.

3.3.7.4 Clarity

The clarity of the samples was measured using UV-Vis Spectrophotometer at 400 nm. Deionized water was used as a blank for the analysis. Measurements were done in triplicate and the clarity was expressed as percent transmittance.

3.3.7.5. Differential Scanning Calorimetry (DSC)

Thermal and physical state properties of dexamethasone, tobramycin, blank microemulsion and drug-loaded microemulsion were determined using a Differential Scanning Calorimeter (DSC) (822e Mettler Toledo) equipped with a TS0801RO sample robot and TS0800GCI gas flow system. Samples of 5-12 mg were weighed using Mettler MT5 microbalance into a 100 μ l aluminum pan, covered with lid and quickly sealed

using a mechanical crimper. The study was performed at a heating rate of 10°C/min over a range of 10 - 300°C. Nitrogen was used as a standard purging gas at 20 ml/min to prevent any oxidation of the samples. Star-e software V8.10 was used to obtain the DSC scans.

3.3.7.6. Transmission electron microscopy (TEM)

The morphology of the microemulsion was studied using TEM (HITACHI HD-2300 A, Ultra-thin Film Evaluation System by Hitachi High Technologies America, Pleasanton, CA). A small drop of the formulation was placed on a holey carbon 400 mesh copper grid (Ted Pella, Redding, CA) and then negatively stained using 2% phosphotungstic acid solution (Fisher Scientific, Pittsburgh, PA). The copper grids were dried overnight and TEM images were captured using Quartz PCI version 8 software.

3.3.8. In vitro drug release study

The *in vitro* release of dexamethasone from the microemulsion was performed using the dialysis bag method. Dialysis membrane (Spectrum/Por[®]) of molecular weight cut off 12,000-14,000 Da and pore size of 0.45 µm was used for the study. One milliliter of the formulation was placed in the dialysis bag, sealed and then introduced into glass bottles containing 200 ml of phosphate buffer (pH 7.4) and 0.025% (w/v) Tween 80 in order to maintain the sink conditions. The bottles were placed into a shaker bath maintained at 34 ± 0.5°C and 60 rpm. At predetermined time intervals, one-milliliter sample was withdrawn from each bottle and an equal volume of fresh buffer was replaced. The study was conducted in triplicate and samples withdrawn were analyzed using HPLC.

Figure 3.1: Precision™ Reciprocating shaking water bath used for *in vitro* release study

3.3.9. Sterility test

The microemulsion was evaluated for sterility in order to assess whether the preparation was free from microorganisms. Formulation sterility was tested using the plate as well as direct/tube inoculation methods. The study was carried out under aseptic conditions, as per our published protocol [126, 127]. All the glassware used for the study was autoclaved and non-autoclavable materials were thoroughly wiped with isopropyl alcohol. The formulation was sterilized by passing the microemulsion through Millex® sterile nylon syringe filter (0.22 µm). Liquid culture of *Staphylococcus aureus* (Rosenbach ATCC® 6538) was grown in Trypsin soy broth (TSB) at 37°C for 24 h. The concentration of bacteria was determined using Spectronic 20 Genesys spectrophotometer (Spectronic Instruments, USA) at 625 nm. By using sterile water as blank, absorbance of the culture was compared to 0.5 McFarland standard of concentration 1.5×10^8 CFU/ml. The final concentration of the liquid culture was made up to 10^2 CFU/ml, by serial dilution with sterile deionized water. Four sets of controls and a sample were prepared. The compositions are summarized in Table 3.2. All the tubes were tested in duplicates and were incubated at 37°C to speed up the growth of

bacteria. For the plate inoculation method, Mueller Hinton (MH) agar plates were prepared by pouring molten agar into pre-sterilized monoplates. One hundred microliters of sample were withdrawn from the tubes tested for direct inoculation method and was uniformly spread on MH agar plates on day 0, 7, and 14. The plates were tested in duplicates and were incubated at 37°C for 24 h to detect any visual bacterial growth.

Table 3.2: Composition of control and sample tubes for the direct inoculation method

Medium	Negative control	Preservative control	Positive control	Positive sample control	Sterile sample
Trypsin Soya Broth	9 ml of medium + 1 ml of sterile water	9 ml of medium + 1 ml of sterile water containing 0.01% BAK	9 ml of medium + 1 ml of sterile water + 10-100 CFU bacteria	9 ml of medium + 1 ml of sample + 10-100 CFU bacteria	9 ml of medium + 1 ml of sterile sample

3.3.10. Cytotoxicity study

The biocompatibility of the formulation was tested and compared with Tobradex® suspension using bovine corneal endothelial cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Bovine eyes were obtained from Kastel's Slaughterhouse and processing center, Riga, MI and the corneal cells were harvested according to a published protocol [128]. The cells were plated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 100 µg/ml penicillin-streptomycin solutions and incubated at 37°C. After, the cells reached ~80-90% confluency, they were seeded into 96-well plate at a density of 10,000

cells/well. The cells were incubated at 37°C and 5% CO₂ environment for 24 h in order to assist cell adhesion. Cells were then exposed to negative control composed of 200 µl DMEM medium, positive control composed of DMEM with 20% DMSO, DMEM mixed with 0.01%, 0.1%, 1%, 5%, 50%, 100% of microemulsion and DMEM mixed with 0.01%, 0.1%, 1%, 5%, 50%, 100% of Tobradex[®] eyedrop suspension. After 60 min, the medium was aspirated, fresh DMEM medium was replaced with 10% MTT reagent and the cells were incubated in 5% CO₂ at 37°C for 3 h. The yellow medium was then removed and 150 µl of 100% DMSO was added to each well in order to allow dissolution of the formazan salt formed and the viable cells were quantified using a microplate reader at 570 nm.

3.3.11. Anti-inflammatory assay

The anti-inflammatory activity of the drug-loaded microemulsion was compared to the Tobradex[®] suspension, using microglial cells by Griess assay. Microglial cells were plated with DMEM medium supplemented with 10% FBS, streptomycin (100 µg/ml) and penicillin (100 U/ml) and incubated at 37°C and 5% CO₂. After, the cells reached ~80-90% confluency, they were seeded in a polylysine coated 24-well plate at a density of 2 x 10⁵ cells/well and incubated at 37°C and 5% CO₂ for 24 h. Cells were then pretreated with drug-loaded microemulsion and Tobradex[®] suspension at different concentrations of 0.001%, 0.01%, 0.1% for 1 h. Except for negative control, cells were stimulated using LPS (Lipopolysaccharides obtained from *Escherichia coli* 055:B5) at a concentration of 200 ng/ml. After 24 h, cells were subjected to Griess assay for determining the presence of nitrite ions. Equal volume of Griess reagent (0.4% naphthylethylenediamine

dihydrochloride, 2% sulfanilamide in 5% phosphoric acid) and microglial culture supernatant were mixed and incubated for 10 min. The absorbance of the cells was measured using a Synergy H1 microplate reader with Gen5 Data Analysis Software at 540 nm.

3.3.12. Anti-microbial disk diffusion susceptibility test

a) Preparation of Inoculum

The anti-microbial activity of the microemulsion was compared to the Tobradex[®] suspension using *Escherichia coli* by disk diffusion susceptibility test. The bacteria were sub-cultured and tested in their log phase of growth in order to ensure that results obtained were valid. Using a sterile cotton swab, four or five isolated colonies of the bacteria were transferred into a glass tube containing 3ml of saline and was vortexed to obtain a uniform suspension. This suspension was prepared just before inoculating the agar plate with the bacteria. The turbidity of the inoculum tube was adjusted to that of 0.5 McFarland standard by diluting it with sterile saline, if the suspension obtained was too turbid or by the addition of more bacteria if the suspension obtained was too light.

b) Inoculation of MH agar plate

A sterile cotton swab was dipped into the inoculum tube and rotated against the sides of the tube using firm pressure, to ensure that there is no dripping due to excessive fluid. The MH agar plate was then inoculated by streaking six times with the swab covering the entire agar surface. Each time the plate was rotated approximately 60°

to ensure a uniform distribution of the inoculum. The plates were then allowed to dry at room temperature for 3-5 minutes before placing the impregnated discs.

c) Preparation and placement of impregnated discs

Ten microliters of microemulsion and Tobradex® suspension containing 30 µg of tobramycin was carefully transferred onto blank sterile disks. A standard disc of tobramycin (10 µg) was used as a control and to ensure that the medium supported bacterial growth beyond the zone of inhibition. With the use of a sterile forceps, discs containing microemulsion, Tobradex® suspension and tobramycin (standard) were equidistantly placed onto the agar plate and were pressed gently to ensure complete contact with the surface of the agar.

d) Incubation of agar plates and measurement of zones of inhibition

The inoculated plates were incubated at 37°C for 24 h and then the zones of inhibition were measured. All the measurements were taken by viewing the back of the plate against a dark non-reflecting surface illuminated with reflected light. The diameters of the zones were measured using a ruler to the nearest millimeter.

3.3.13. Histological evaluation in bovine cornea

To ensure the safety of the prepared microemulsion for ocular administration, histology was performed using bovine corneas and the evaluation was compared with the Tobradex® suspension. Fresh bovine corneas were obtained from Kastel's Slaughterhouse and processing center, Riga, MI. The bovine corneas were rinsed with phosphate buffer

(pH 7.4) and then treated with one milliliter of the prepared microemulsion and marketed suspension. After 3 h of treatment, the tissues were fixed using 10% formalin solution for 24 h and then stored in 70% ethanol. The formalin fixed tissues were processed using an automatic tissue processor and were transferred to a mold, to which hot paraffin wax (65°C) was added using a paraffin dispenser. The mold was then placed on a cooling surface for 30 minutes until the paraffin was completely hardened. Later, the wax block was removed and sectioned using a microtome at a thickness of 5 μ m. The sections were then placed on microscopic slides, stained using hematoxylin and eosin and were observed under a microscope to detect any morphological changes.

3.3.14. Stability study

Stability testing is a pre-requisite for the approval of any pharmaceutical product as it ensures the product quality, safety and efficacy throughout the shelf life [129]. In order to assess the physical and chemical stability of the prepared formulation, microemulsion samples were stored at different temperatures (4°C, 25°C and 40°C) for a period of 3 months. Samples were withdrawn at regular intervals of 0, 30, 60 and 90 days and evaluated for pH, particle size, clarity/transmittance and drug content.

3.3.15. Statistical analysis

All the data obtained were expressed as mean $\pm$ SD. Analysis of variance (ANOVA) was applied to analyze the significant difference between samples. $p < 0.05$ was considered to be significant in all the cases.

3.4. Results

3.4.1. Construction of pseudoternary phase diagrams

Four phase diagrams were developed and the regions of microemulsion formation were determined. The phase diagrams constructed using 1:4, 2:3, 3:2 and 4:1 (surfactant to cosurfactant) ratios are shown in Fig. 3.2. It was observed that the microemulsion region increased with the ratio of surfactant to cosurfactant ($1:4 < 2:3 < 3:2 < 4:1$). However, the microemulsion region containing 3:2 ratio of surfactant to cosurfactant was considered for further studies due to its lower surfactant concentration. From the microemulsion region in the pseudoternary phase diagram (Fig. 3.2 C), a system was selected for further evaluation and characterization (Table 3.3).

Figure 3.2: Pseudoternary phase diagram of the microemulsion containing cremophor EL and 1-butanol in A) 1:4 ratio B) 2:3 ratio C) 3:2 ratio D) 4:1 ratio.

Table 3.3: Composition of selected microemulsion

Composition	Ingredient	Concentration (w/w)
Oil phase	Ricinoleic acid	2.5%
Surfactant	Cremophor-EL	9.3%
Cosurfactant	1-Butanol	6.2%
Aqueous phase	Water	82.0%

3.4.2. Characterization of microemulsion

3.4.2.1. Droplet size and zeta-potential analysis

In o/w microemulsions, droplet size refers to the size of dispersed oil droplets in a continuous water phase. The average droplet diameter of the undiluted blank and drug-loaded microemulsion was found to be 9.33 ± 0.04 nm and 11.4 ± 0 nm, respectively. The zeta potential of the blank and drug-loaded microemulsion was found to be neutral. Representative images are shown in the Figs. 3.3 and 3.4 respectively.

Figure 3.3: A) Particle size and B) Zetapotential of blank microemulsion

Figure 3.4: A) Particle size and B) Zeta potential of drug-loaded microemulsion.

3.4.2.2. *pH and clarity*

The pH is of major importance as the stability of most of the drugs is largely dependent on the pH of the ocular environment [130]. An unbuffered solution with pH ranging from 4 to 8 is suitable for ocular use. The pH of these solutions is modulated by the buffering capacity of tear fluids to the physiological range upon administration [42]. The pH of the blank and drug loaded formulation was found to be 6.87 ± 0.07 and 6.91 ± 0.03 , respectively. The clarity of the microemulsion was studied to confirm the ability of light transmission through the formulation. Blank and drug-loaded microemulsions were found to be clear with transmittance values of 98.4 ± 0.48 and 98.5 ± 0.47 , respectively.

3.4.2.3. *Differential Scanning Calorimetry (DSC)*

DSC thermograms of pure dexamethasone, tobramycin, blank microemulsion and drug-loaded microemulsion are shown in Fig. 3.5. Dexamethasone exhibited a sharp endothermic peak at an onset temperature of 267.70°C with a peak temperature of 271.93°C , which corresponds to the melting point of dexamethasone [131]. The thermogram confirmed the typical crystalline nature of dexamethasone. Tobramycin exhibited an endothermic peak at an onset temperature of 173.41°C with a peak temperature of 183.30°C , which corresponds to the melting point of tobramycin. An exothermic peak was observed followed by a sharp endothermic peak at 224.39°C . These events correspond to the crystallization and re-melting of tobramycin [132]. Blank and drug-loaded microemulsions exhibited a sharp endothermic peak at an onset temperature

of 97.39°C and 100.19°C, respectively. These correspond to evaporation and loss of water from the samples [133].

Figure 3.5: Differential scanning calorimetry thermograms of (A) dexamethasone (B) tobramycin (C) blank microemulsion (D) drug-loaded microemulsion

3.4.2.4. TEM of microemulsion

TEM images of blank and drug-loaded microemulsions are shown in Fig. 3.6. The observed droplets were roughly spherical in shape and the droplet size in both cases was found to be slightly higher than that determined using DLS.

Figure 3.6: Transmission electron microscopy images of (A) blank microemulsion (B) drug-loaded microemulsion.

3.4.3. HPLC analysis of dexamethasone

Quantitative method for the estimation of dexamethasone using HPLC was successfully developed and validated. The method was validated by determining accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ). The chromatogram of dexamethasone presented a sharp peak with a retention time of 2.9 min (Fig. 3.7 A). A linear calibration curve ($y = 2038.4x - 447.94$) was obtained with a regression coefficient (R^2) of 1 as shown in the Fig. 3.7 B. LOD and LOQ were calculated according to ICH Q2(R1) guidelines [134] and were found to be 0.53 and 1.61 ng, respectively. The percentage recovery of dexamethasone ranged from 97.13-100.24%, signifying high accuracy of the developed method. The method also showed good intra-day precision and suitability with relative standard deviation (RSD) of less than 2%.

Figure 3.7: A) High performance liquid chromatography peak of dexamethasone, and B) Calibration curve of dexamethasone

3.4.4. UV analysis of tobramycin

Quantitative method for the estimation of tobramycin using UV spectrophotometer was successfully developed and validated. A linear calibration curve ($y = 0.0027x + 0.0138$) was obtained in the concentration range of 50-250 $\mu\text{g/ml}$ with a regression coefficient (R^2) of 0.9993 as shown in the Fig. 3.8. LOD and LOQ were found to be 2.26 and 6.85 $\mu\text{g/ml}$, respectively. The percentage recovery of tobramycin ranged from 96.44 - 103.01%. The method also showed good intra-day precision with and suitability with relative standard deviation (RSD) of less than 2%.

Figure 3.8: Calibration curve of tobramycin

3.4.5. Validation of sterility

Except for the positive control, all the test tubes remained clear and did not show any signs of bacterial growth (Fig 3.9 A). The same was observed with the plate inoculation method (Table 4 and Fig 3.9 B). The possible reason for a clear positive sample control could be attributed to the presence of benzalkonium chloride in the formulation [135].

Table 3.4. The presence (+) or absence (-) of bacterial growth on agar plates by plate inoculation method.

Day	Negative Control	Preservative Control	Positive control	Positive sample control	Sample
0	-	-	+	-	-
7	-	-	+	-	-
14	-	-	+	-	-

Figure 3.9: A) Testubes from direct inoculation method and B) Images of MH agar plates showing the presence/absence of bacterial growth (a) negative control, (b) preservative control, (c) positive sample control, (d) positive control, (e) sterile sample, formulation after 14 days of inoculation

3.4.6. *In vitro* drug release study

In vitro release pattern of dexamethasone from the microemulsion is shown in the Fig 3.10. The release pattern was obtained by plotting percentage of cumulative drug release versus time (h). The microemulsion released the drug in a sustained fashion over a period of 8 h. No visual change was observed upon incubation with the PBS (pH 7.4) containing 0.025% (w/v) Tween 80, indicating the stability of the drug-loaded microemulsion in the presence of the release medium.

Figure 3.10: *In vitro* drug release of dexamethasone from microemulsion (n=3). Values are represented as mean $\pm$ SD.

3.4.7. Cytotoxicity study

The cytotoxicity of the microemulsion was compared with the Tobradex[®] suspension using bovine corneal endothelial cells as shown in Fig. 3.11. The percentage of cell viability in the presence of 20% DMSO was 10.88%. With both the formulations, the cell viability was greater than 100% at lower concentrations (0.01%, 0.1%, 1%, and 5%), whereas the cell viability was less than 15% at higher concentrations (50% and 100%).

Figure 3.11: Cell viability of bovine corneal endothelial cells treated with microemulsion and marketed suspension (n=5). Error bars represent standard deviation (SD).

3.4.8. Anti-inflammatory study

The anti-inflammatory activity of the microemulsion and Tobradex[®] suspension determined using Griess assay are shown in the Fig. 3.12. The amount of nitrite ions produced by microglial cells, treated with different concentrations (0.001%, 0.01%, and 0.1%) of microemulsion was lower than that produced by suspension treated cells.

Moreover, the anti-inflammatory activity demonstrated by the drug-loaded microemulsion was significantly ($p<0.05$) higher than Tobradex[®] suspension at 0.1% concentration.

Figure 3.12: Griess assay using microglial cells activated with LPS (n=3). Negative control (cells without LPS treatment), positive control (cells with LPS treatment). Error bars represent standard deviation (SD).

3.4.9. Anti-microbial disk diffusion susceptibility test

The anti-microbial activity of the microemulsion and Tobradex[®] suspension (n=9) is shown in Table 3.5 and Fig. 3.13. The average zone of inhibition observed with the

standard tobramycin disc was 21.8 ± 0.03 mm. The drug-loaded formulation exhibited a higher zone of inhibition (29.33 ± 0.06 mm) than compared to Tobradex[®] suspension (27.4 ± 0.04 mm). Moreover, the anti-bacterial activity demonstrated by the microemulsion was significantly higher ($p < 0.05$) than the Tobradex[®] suspension.

Table 3.5: Anti-bacterial efficacy of microemulsion and Tobradex[®] suspension against *E.coli* (n=9).

Formulation	Zones of inhibition (mm) $\pm$ S.D
Microemulsion	29.33 ± 0.06
Tobradex [®] suspension	27.4 ± 0.04
Standard tobramycin disc (10 μ g)	21.8 ± 0.03

Figure 3.13. Zone of inhibition produced by the developed microemulsion (ME), Tobradex[®] suspension (SUSP) and standard tobramycin 10 µg disk (control).

3.4.10. Histological evaluation in bovine cornea

Images of bovine corneas treated with microemulsion and Tobradex[®] suspension are shown in Fig. 3.14 A and B respectively. Microscopic evaluation showed no signs of toxicity with intact corneal epithelium in both the cases. This confirms the safety of the prepared microemulsion for ocular administration.

Figure 3.14: Representative micrographs of hematoxylin/eosin stained bovine cornea treated with (A) microemulsion and (B) Tobradex[®] suspension.

3.4.11. Stability study

Microemulsion showed good stability when stored at 4°C and 25°C. The results of the study are shown in Table 3.6. At 40°C, the formulation was not stable over the testing period with respect to drug content and clarity (data not shown). The data obtained was analyzed by one-way ANOVA using SPSS software. No significant changes were observed with the samples tested over a period of 3 months.

Table.3.6 Stability data of (a) dexamethasone and (b) tobramycin loaded microemulsion. Values represented as mean±standard deviation.

Temperature	Parameters	0 Day	1 month	2 month	3 month
4 ± 2 °C	Drug content (%) a)	102.2 ± 0.19	99.3 ± 0.04	98.5 ± 0.22	97.5 ± 0.33
	b)	102.2 ± 2.31	98.5 ± 2.78	102 ± 2.24	98.1 ± 0.75
	Particle size (nm)	11.4 ± 0	11.13 ± 0.51	12.4±0.14	12.23 ± 0.63
	pH	7 ± 0.06	7.2 ± 0.02	6.9 ± 0.03	6.9 ± 0.02
	% Transmittance	98.4 ± 0.48	97.8 ± 0.43	96.4 ± 1.61	96.6 ± 0.93
25 ± 2 °C	Drug content (%) a)	102.2 ± 0.19	97.4 ± 0.46	97.3 ± 0.42	98.9 ± 0.46
	b)	102.2 ± 2.31	95.4 ± 3.38	95.4 ± 1.52	100.1 ± 3.25
	Particle size (nm)	11.4 ± 0	10.3 ± 0.21	11 ± 0.28	12.26 ± 0.04
	pH	7 ± 0.06	6.9 ± 0.02	6.9 ± 0.01	6.8 ± 0.02
	% Transmittance	98.4 ± 0.48	97.2 ± 0.74	96.5 ± 0.39	97.2 ± 1.06

3.5. Discussion

We have successfully formulated microemulsion of dexamethasone and tobramycin using ricinoleic acid (2.5%) as an oil phase, cremophor EL (9.3%) as surfactant, 1-butanol (6.2%) as cosurfactant and distilled deionized water (82%) as aqueous phase by spontaneous emulsification method. Ricinoleic acid is a naturally occurring, unsaturated omega-9 fatty acid obtained from mature castor plants. About 90% of the total fatty acid content in castor oil is from ricinoleic acid and is used as one of the lipophilic vehicles in cyclosporine eye drops [136]. Ricinoleic acid is known for its analgesic, anti-inflammatory and anti-bacterial activities upon acute or repeated local application [137-139]. Hence, we have selected ricinoleic acid for the oil phase. Cremophor EL and 1-butanol were chosen as surfactant and cosurfactant respectively, which are non-ionic by nature. Generally non-ionic surfactants cause less irritation to the eye compared to cationic and anionic agents [140]. Cremophor EL is widely used as surfactant in commercial ophthalmic formulations and is shown to be non-irritating and non-toxic to the eye [141]. Even though 1-butanol is not a GRAS excipient, it has been extensively studied for its use in the preparation of microemulsions [142-144]. Recently, Ince et al. investigated the potential of an ocular microemulsion of pilocarpine using soyabean oil, Span 80, Brij 35P, 1-butanol and water. The results of the ocular tolerance and histology in rabbits revealed that the formulation was non-irritant with no signs of toxicity [108]. Hence, we have selected 1-butanol as a cosurfactant for the study.

The formulated ocular microemulsion of dexamethasone and tobramycin was evaluated for pH, particle size, zeta potential, light transmittance/clarity, morphology, drug content,

in vitro drug release and stability. The pH of the blank and drug loaded formulation was found to be 6.87 ± 0.07 and 6.91 ± 0.03 , respectively. These values are close to the physiological range, suggesting the suitability of the microemulsion for ophthalmic use [42]. Ocular preparations should exhibit more than 90% transmittance or absorbance less than 0.1 at 400 nm [145]. Blank and drug-loaded microemulsions were clear with light transmittance around 95-100%. Microemulsions with such clarity can address the problem of transient blurring of vision associated with the marketed eye drop suspension.

Droplet size of microemulsion plays a significant role in predicting the rate and extent of drug absorption [146]. The average droplet diameter of the undiluted blank and drug-loaded microemulsion was found to be 9.33 ± 0.04 nm and 11.4 ± 0 nm, respectively. This small size of the droplets is due to the presence of cosurfactant molecules that penetrate into the interfacial film, lower the fluidity and surface viscosity eventually decreasing the radius of curvature [102]. Slight increase in the droplet size of drug-loaded microemulsion than the blank microemulsion could be attributed to the incorporation of dexamethasone molecules within the core.

The zeta potential of blank and drug-loaded microemulsion was found to be neutral and this could be attributed to the non-ionic nature of the surfactants used in the preparation. DSC was performed to confirm the absence of any un-dissolved drugs in the microemulsion. The characteristic endothermic peaks of dexamethasone and tobramycin were completely absent in the microemulsion indicating that these drugs were either converted to an amorphous form or molecularly dispersed in the formulation [126]. The morphology of the microemulsion was studied using TEM. The images revealed the presence of slightly larger droplets than that observed with DLS. This could be due to the

different experimental conditions used in DLS and TEM. In TEM, the oil droplets spread on to the surface of the grids and this process is facilitated by the low interfacial tension which further results in coalescence and the formation of larger structures as evident from the images [147].

Sterility is a key issue in the preparation of ophthalmic products. In the present study, the microemulsion was passed through Millex® sterile nylon syringe filter (0.22 µm) to obtain a sterile formulation. Later the sterilized microemulsion was validated by direct inoculation as well as plate inoculation methods according to USP guidelines. Except for the positive control, remaining plates/tubes tested were found to be clear with no signs of bacterial growth. The reason for a clear positive sample control plate/tube could be due to the activity of BAK against gram-positive bacteria, such as *S.Aureus* used in the study [148]. The prepared microemulsion remained sterile for 14 days and the test also confirmed that aseptic filtration is sufficient for the sterilization of microemulsions. The *in-vitro* release pattern of dexamethasone was studied by the dialysis bag method. The developed microemulsion showed a complete release of dexamethasone over a period of 8 hours in a sustained manner.

The cytotoxicity of the microemulsion was compared with Tobradex® suspension using MTT assay. MTT assay is a colorimetric test used to assess the viability of cells by measuring cellular metabolic activity through NAD (P) H-dependent oxidoreductase enzymes. The assay is based on the ability of viable cells to reduce tetrazolium dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to purple colored insoluble formazan compound. Higher absorbance reading indicates less toxicity, and therefore more cell viability. Lower concentrations (0.01%, 0.1%, 1%, 5%) did not show any

cytotoxicity whereas higher concentrations (50% and 100%) were toxic with both formulations. As the drug delivery to the anterior segment of the eye is limited by several physiological constraints and anatomical barriers, less than 5-10% of the instilled dose actually reaches the ocular tissues [32]. Therefore, it is highly unlikely that these higher concentrations (50% and 100%) reach the ocular tissues and cause any *in-vivo* toxic effects.

Microglial cells activated with LPS were used to compare the anti-inflammatory activities of microemulsion and Tobradex[®] suspension by Griess assay. The inflammation induced by LPS was reduced by dexamethasone in the formulation. Griess assay was carried out to detect the presence of nitrite compounds. Nitric oxide is an important signaling molecule in the pathogenesis of inflammation. Under abnormal physiological conditions, over generation of nitric oxide acts as a pro-inflammatory mediator that induces inflammation. Nitric oxide is metabolized (oxidization) to a stable, non-volatile nitrite anion. The assay is based on the diazotization reaction between the nitrites present in the sample and sulphanilic acid present in the Griess reagent. The resultant diazonium salt formed reacts with the azo dye agent (Naphthylethylenediamine dihydrochloride) resulting in pink color. The amount of nitrite present was estimated based on the intensity of pink color using a microplate reader. As determined from the nitrite ions produced by the microglial cells, the anti-inflammatory activity exhibited by the drug-loaded microemulsion was significantly higher ($p < 0.05$) than Tobradex[®] suspension at 0.1% concentration. This could be due to the synergistic anti-inflammatory

effects of dexamethasone and ricinoleic acid combined with the improved dose uniformity of the microemulsion.

The Kirby-Bauer disk susceptibility test was performed to compare the anti-microbial efficacy of tobramycin in the microemulsion and Tobradex[®] suspension using *E.coli*. The test measures the sensitivity or resistance of microorganisms to antibiotics on Muller-Hinton agar media using antibiotic-impregnated discs. The absence of growth surrounding the discs reveals the ability of an antibiotic to kill the particular microorganism. In our study, we observed that tobramycin loaded in microemulsion showed higher anti-microbial efficacy ($p < 0.05$) than Tobradex[®] suspension. This could be due to the antibacterial activities of tobramycin & ricinoleic acid present in the formulation. The presence of 1-butanol as well as the dose uniformity of developed formulation could also contribute towards the enhanced anti-bacterial activity. Histological evaluation of bovine corneas treated with the microemulsion and Tobradex[®] suspension showed intact corneal epithelium with no signs of toxicity. Also, the prepared dexamethasone and tobramycin loaded microemulsion was found to be stable at 4 °C and 25°C for over 3 months.

3.6. Conclusion

In this study, we report the development and evaluation of ocular microemulsion of dexamethasone and tobramycin for treating anterior eye bacterial infection and inflammation. Both the blank and drug-loaded microemulsions displayed a droplet size of less than 20 nm and a neutral zeta potential. The pH of microemulsions was close to physiological range with light transmittance around 95-100%. The microemulsion was successfully sterilized using 0.22 μm filter. The cytotoxicity study in bovine corneal endothelial cells showed no signs of toxicity at lower concentrations (0.01% - 5%) when compared with the control. The anti-inflammatory study in microglial cells showed that the activity was significantly greater with microemulsion than Tobradex[®] suspension at 0.1% concentration. The anti-bacterial study revealed that microemulsion had significantly higher efficacy when compared to the marketed suspension. Stability studies indicated that the formulation was stable at 4°C and 25°C for 3 months. In conclusion, o/w microemulsion of dexamethasone and tobramycin was successfully prepared using ricinoleic acid, cremophor EL, 1-butanol and water. This formulation could be a suitable alternative to the Tobradex[®] suspension. Long-term stability of the formulation and efficacy of the formulation in an animal model should be established.

References

1. Saettone, M.F., *Progress and problems in ophthalmic drug delivery*. Business Briefing: Pharmatech, 2002. **1**: p. 167-71.
2. Schoenwald, R. and P. Stewart, *Effect of particle size on ophthalmic bioavailability of dexamethasone suspensions in rabbits*. Journal of pharmaceutical sciences, 1980. **69**(4): p. 391-394.
3. Stringer, W. and R. Bryant, *Dose uniformity of topical corticosteroid preparations: difluprednate ophthalmic emulsion 0.05% versus branded and generic prednisolone acetate ophthalmic suspension 1%*. Clin Ophthalmol, 2010. **4**: p. 1119-1124.
4. Hegde, R.R., A. Verma, and A. Ghosh, *Microemulsion: new insights into the ocular drug delivery*. ISRN pharmaceuticals, 2013. **2013**.
5. Patel, A., et al., *Ocular drug delivery systems: an overview*. World journal of pharmacology, 2013. **2**(2): p. 47.
6. Pascolini, D. and S.P. Mariotti, *Global estimates of visual impairment: 2010*. British Journal of Ophthalmology, 2011: p. bjophthalmol-2011-300539.
7. Gaudana, R., et al., *Ocular drug delivery*. The AAPS journal, 2010. **12**(3): p. 348-360.

8. Gökçe, E.H., et al., *Cyclosporine A-loaded solid lipid nanoparticles: ocular tolerance and in vivo drug release in rabbit eyes*. Current eye research, 2009. **34**(11): p. 996-1003.
9. Üstündag-Okur, N., et al., *Novel ofloxacin-loaded microemulsion formulations for ocular delivery*. Journal of Ocular Pharmacology and Therapeutics, 2014. **30**(4): p. 319-332.
10. Ahmed, I., *The noncorneal route in ocular drug delivery*. DRUGS AND THE PHARMACEUTICAL SCIENCES, 2003. **130**: p. 335-364.
11. Gipson, I.K. and P. Argüeso, *Role of mucins in the function of the corneal and conjunctival epithelia*. International review of cytology, 2003. **231**: p. 1-49.
12. Holly, F. and M. Lemp, *Tear physiology and dry eyes*. Survey of ophthalmology, 1977. **22**(2): p. 69-87.
13. Cholkar, K., et al., *Novel strategies for anterior segment ocular drug delivery*. Journal of Ocular Pharmacology and Therapeutics, 2013. **29**(2): p. 106-123.
14. Maurice, D. and S. Mishima, *Ocular pharmacokinetics*, in *Pharmacology of the Eye*. 1984, Springer. p. 19-116.
15. Hornof, M., E. Toropainen, and A. Urtti, *Cell culture models of the ocular barriers*. European journal of pharmaceutics and biopharmaceutics, 2005. **60**(2): p. 207-225.
16. Huang, A., S. Tseng, and K. Kenyon, *Paracellular permeability of corneal and conjunctival epithelia*. Investigative ophthalmology & visual science, 1989. **30**(4): p. 684-689.

17. Srinivasan, B., F. Jakobiec, and T. Iwamoto, *Conjunctiva*, in *Ocular anatomy, embryology and teratology*. 1982, Harper & Row, Philadelphia. p. 733-760.
18. Horibe, Y., et al., *Polar solute transport across the pigmented rabbit conjunctiva: size dependence and the influence of 8-bromo cyclic adenosine monophosphate*. *Pharmaceutical research*, 1997. **14**(9): p. 1246-1251.
19. Kim, Y.C., et al., *Ocular delivery of macromolecules*. *Journal of Controlled Release*, 2014. **190**: p. 172-181.
20. VADlApuDi, A.D. and K. CholKAr, *Ocular Drug Delivery*.
21. Gaudana, R., et al., *Recent perspectives in ocular drug delivery*. *Pharmaceutical research*, 2009. **26**(5): p. 1197-1216.
22. Karla, P.K., et al., *Molecular expression and functional evidence of a drug efflux pump (BCRP) in human corneal epithelial cells*. *Current eye research*, 2009. **34**(1): p. 1-9.
23. Le Bourlais, C., et al., *Ophthalmic drug delivery systems—recent advances*. *Progress in retinal and eye research*, 1998. **17**(1): p. 33-58.
24. Saettone, M.F., B. Giannaccini, and D. Monti, *Ophthalmic emulsions and suspensions*. *Journal of Toxicology: Cutaneous and Ocular Toxicology*, 2001. **20**(2-3): p. 183-201.
25. Vulovic, N., et al., *Some studies on the preservation of indometacin suspensions intended for ophthalmic use*. *Die Pharmazie*, 1990. **45**(9): p. 678-679.
26. Meseguer, G., et al., *Gamma scintigraphic comparison of eyedrops containing pilocarpine in healthy volunteers*. *Journal of ocular pharmacology and therapeutics*, 1996. **12**(4): p. 481-488.

27. GEBHARDT, B.M., E.D. VARNELL, and H.E. KAUFMAN, *Cyclosporine in collagen particles: corneal penetration and suppression of allograft rejection*. Journal of ocular pharmacology and therapeutics, 1995. **11**(4): p. 509-517.
28. Sasaki, H., et al., *Pharmacokinetic prediction of the ocular absorption of an instilled drug with ophthalmic viscous vehicle*. Biological and Pharmaceutical Bulletin, 2000. **23**(11): p. 1352-1356.
29. Saettone, M.F., et al., *Evaluation of ocular permeation enhancers: in vitro effects on corneal transport of four β -blockers, and in vitro/in vivo toxic activity*. International journal of pharmaceutics, 1996. **142**(1): p. 103-113.
30. Van Der Bijl, P., A.D. van Eyk, and D. Meyer, *Effects of three penetration enhancers on transcorneal permeation of cyclosporine*. Cornea, 2001. **20**(5): p. 505-508.
31. Burgalassi, S., et al., *Cytotoxicity of potential ocular permeation enhancers evaluated on rabbit and human corneal epithelial cell lines*. Toxicology letters, 2001. **122**(1): p. 1-8.
32. Mitra, A.K., *Treatise on Ocular Drug Delivery*. Vol. 1. 2013: Bentham Science Publishers.
33. Marsh, R. and D. Maurice, *The influence of non-ionic detergents and other surfactants on human corneal permeability*. Experimental eye research, 1971. **11**(1): p. 43-48.
34. Newton, C., B. Gebhardt, and H. Kaufman, *Topically applied cyclosporine in azone prolongs corneal allograft survival*. Investigative ophthalmology & visual science, 1988. **29**(2): p. 208-215.

35. Sasaki, H., et al., *Modification of ocular permeability of peptide drugs by absorption promoters*. Biological and Pharmaceutical Bulletin, 2000. **23**(12): p. 1524-1527.
36. Keister, J., et al., *Limits on optimizing ocular drug delivery*. Journal of pharmaceutical sciences, 1991. **80**(1): p. 50-53.
37. Del Valle, E.M., *Cyclodextrins and their uses: a review*. Process biochemistry, 2004. **39**(9): p. 1033-1046.
38. Kurkov, S.V. and T. Loftsson, *Cyclodextrins*. International journal of pharmaceutics, 2013. **453**(1): p. 167-180.
39. Loftsson, T. and D. Duchêne, *Cyclodextrins and their pharmaceutical applications*. International journal of pharmaceutics, 2007. **329**(1): p. 1-11.
40. Zhang, J., et al., *Ocular pharmacokinetics of topically-applied ketoconazole solution containing hydroxypropyl beta-cyclodextrin to rabbits*. Journal of Ocular Pharmacology and Therapeutics, 2008. **24**(5): p. 501-506.
41. Mannermaa, E., K.-S. Vellonen, and A. Urtti, *Drug transport in corneal epithelium and blood–retina barrier: emerging role of transporters in ocular pharmacokinetics*. Advanced drug delivery reviews, 2006. **58**(11): p. 1136-1163.
42. Remington, J.P., D.B. Troy, and P. Beringer, *Remington: The science and practice of pharmacy*. Vol. 1. 2006: Lippincott Williams & Wilkins.
43. Kinoshita, S., et al., *Rebamipide (OPC-12759) in the treatment of dry eye: a randomized, double-masked, multicenter, placebo-controlled phase II study*. Ophthalmology, 2012. **119**(12): p. 2471-2478.

44. Sasaki, H., et al., *Enhancement of ocular drug penetration*. Critical Reviews™ in Therapeutic Drug Carrier Systems, 1999. **16**(1).
45. Shell, J.W., *Pharmacokinetics of topically applied ophthalmic drugs*. Survey of ophthalmology, 1982. **26**(4): p. 207-218.
46. Eguchi, H., et al., *The inhibitory effect of vancomycin ointment on the manifestation of MRSA keratitis in rabbits*. Journal of infection and chemotherapy, 2009. **15**(5): p. 279-283.
47. Gray, C., *Systemic toxicity with topical ophthalmic medications in children*. Paediatric and Perinatal Drug Therapy, 2006. **7**(1): p. 23-29.
48. Guinedi, A.S., et al., *Preparation and evaluation of reverse-phase evaporation and multilamellar niosomes as ophthalmic carriers of acetazolamide*. International journal of pharmaceutics, 2005. **306**(1): p. 71-82.
49. Mahmoud, S.S., et al., *Liposomal phospholipid preparations of chloramphenicol for ophthalmic applications*. Journal of pharmaceutical sciences, 2008. **97**(7): p. 2691-2701.
50. Pal Kaur, I. and M. Kanwar, *Ocular preparations: the formulation approach*. Drug development and industrial pharmacy, 2002. **28**(5): p. 473-493.
51. Agarwal, R., et al., *Liposomes in topical ophthalmic drug delivery: an update*. Drug delivery, 2016. **23**(4): p. 1075-1091.
52. Dharma, S.K., P.H. Fishman, and G.A. Peyman, *A preliminary study of corneal penetration of 125I-labelled idoxuridine liposome*. Acta ophthalmologica, 1986. **64**(3): p. 298-301.

53. Elorza, B., et al., *Comparison of particle size and encapsulation parameters of three liposomal preparations*. Journal of microencapsulation, 1993. **10**(2): p. 237-248.
54. Taha, E.I., et al., *Design of liposomal colloidal systems for ocular delivery of ciprofloxacin*. Saudi Pharmaceutical Journal, 2014. **22**(3): p. 231-239.
55. Kouchak, M., R. Bahmandar, and N. Bavarsad, *Ocular Dorzolamide Nanoliposomes for Prolonged IOP Reduction: in-vitro and in-vivo Evaluation in Rabbits*. Iranian Journal of Pharmaceutical Research, 2016. **15**(1): p. 205-212.
56. Biju, S., et al., *Vesicular systems: an overview*. Indian Journal of Pharmaceutical Sciences, 2006.
57. Abdelkader, H., et al., *Design and evaluation of controlled-release niosomes and discomes for naltrexone hydrochloride ocular delivery*. Journal of pharmaceutical sciences, 2011. **100**(5): p. 1833-1846.
58. Vyas, S., et al., *Discoidal niosome based controlled ocular delivery of timolol maleate*. Die Pharmazie, 1998. **53**(7): p. 466-469.
59. Piplani, P., et al., *Development and evaluation of an ocular niosomal delivery system for some newly synthesized Beta blockers*. Asian Journal of Biomedical and Pharmaceutical Sciences, 2015. **5**(40): p. 8.
60. Gaafar, P.M., et al., *Preparation, characterization and evaluation of novel elastic nano-sized niosomes (ethoniosomes) for ocular delivery of prednisolone*. Journal of liposome research, 2014. **24**(3): p. 204-215.
61. Kaur, I.P., et al., *Vesicular systems in ocular drug delivery: an overview*. International journal of pharmaceutics, 2004. **269**(1): p. 1-14.

62. Sahoo, S.K., F. Dilnawaz, and S. Krishnakumar, *Nanotechnology in ocular drug delivery*. Drug discovery today, 2008. **13**(3): p. 144-151.
63. Date, A.A., et al., *Development and evaluation of a thermosensitive vaginal gel containing raltegravir+ efavirenz loaded nanoparticles for HIV prophylaxis*. Antiviral research, 2012. **96**(3): p. 430-436.
64. Bhatta, R., et al., *Mucoadhesive nanoparticles for prolonged ocular delivery of natamycin: in vitro and pharmacokinetics studies*. International journal of pharmaceutics, 2012. **432**(1): p. 105-112.
65. Hippalgaonkar, K., et al., *Indomethacin-loaded solid lipid nanoparticles for ocular delivery: development, characterization, and in vitro evaluation*. Journal of Ocular Pharmacology and Therapeutics, 2013. **29**(2): p. 216-228.
66. Liu, D., et al., *Potential advantages of a novel chitosan-N-acetylcysteine surface modified nanostructured lipid carrier on the performance of ophthalmic delivery of curcumin*. Scientific Reports, 2016. **6**.
67. Musumeci, T., et al., *Polymeric nanoparticles augment the ocular hypotensive effect of melatonin in rabbits*. International journal of pharmaceutics, 2013. **440**(2): p. 135-140.
68. Vadlapudi, A.D. and A.K. Mitra, *Nanomicelles: an emerging platform for drug delivery to the eye*. Therapeutic delivery, 2013. **4**(1): p. 1-3.
69. Vadlapudi, A.D., et al., *Aqueous nanomicellar formulation for topical delivery of biotinylated lipid prodrug of acyclovir: formulation development and ocular biocompatibility*. Journal of Ocular Pharmacology and Therapeutics, 2014. **30**(1): p. 49-58.

70. Guo, C., et al., *Nanomicelle formulation for topical delivery of cyclosporine A into the cornea: in vitro mechanism and in vivo permeation evaluation*. Scientific reports, 2015. **5**.
71. Lin, H.R. and P.C. Chang, *Novel pluronic-chitosan micelle as an ocular delivery system*. Journal of Biomedical Materials Research Part B: Applied Biomaterials, 2013. **101**(5): p. 689-699.
72. Patravale, V. and R. Kulkarni, *Nanosuspensions: a promising drug delivery strategy*. Journal of pharmacy and pharmacology, 2004. **56**(7): p. 827-840.
73. Boddeda, B., et al., *Design and ocular tolerance of flurbiprofen loaded nanosuspension*. Pharmaceutical Nanotechnology, 2015. **3**(1): p. 56-67.
74. Shi, S., et al., *Chitosan grafted methoxy poly (ethylene glycol)-poly (ϵ -caprolactone) nanosuspension for ocular delivery of hydrophobic diclofenac*. Scientific reports, 2015. **5**.
75. Abdelrahman, A.A., et al., *Modeling, optimization, and in vitro corneal permeation of chitosan-lomefloxacin HCl nanosuspension intended for ophthalmic delivery*. Journal of Pharmaceutical Innovation, 2015. **10**(3): p. 254-268.
76. Gupta, H. and M. Aqil, *Contact lenses in ocular therapeutics*. Drug discovery today, 2012. **17**(9): p. 522-527.
77. Bengani, L.C. and A. Chauhan, *Extended delivery of an anionic drug by contact lens loaded with a cationic surfactant*. Biomaterials, 2013. **34**(11): p. 2814-2821.
78. Kim, J., A. Conway, and A. Chauhan, *Extended delivery of ophthalmic drugs by silicone hydrogel contact lenses*. Biomaterials, 2008. **29**(14): p. 2259-2269.

79. Alvarez-Lorenzo, C., H. Hiratani, and A. Concheiro, *Contact lenses for drug delivery*. American Journal of Drug Delivery, 2006. **4**(3): p. 131-151.
80. ElShaer, A., et al., *Nanoparticle-laden contact lens for controlled ocular delivery of prednisolone: formulation optimization using statistical experimental design*. Pharmaceutics, 2016. **8**(2): p. 14.
81. Peng, C.-C., M.T. Burke, and A. Chauhan, *Transport of topical anesthetics in vitamin E loaded silicone hydrogel contact lenses*. Langmuir, 2011. **28**(2): p. 1478-1487.
82. Garhwal, R., et al., *Sustained ocular delivery of ciprofloxacin using nanospheres and conventional contact lens materials*. Investigative ophthalmology & visual science, 2012. **53**(3): p. 1341-1352.
83. Tieppo, A., et al., *Sustained in vivo release from imprinted therapeutic contact lenses*. Journal of controlled release, 2012. **157**(3): p. 391-397.
84. Hoar, T. and J. Schulman, *Transparent water-in-oil dispersions: the oleopathic hydro-micelle*. Nature, 1943. **152**: p. 102-103.
85. Vandamme, T.F., *Microemulsions as ocular drug delivery systems: recent developments and future challenges*. Progress in retinal and eye research, 2002. **21**(1): p. 15-34.
86. Chen, S., et al., *Curvature as a determinant of microstructure and microemulsions*. The Journal of Physical Chemistry, 1986. **90**(5): p. 842-847.
87. Dixit, G.R. and V. Mathur, *MICROEMULSIONS: PLATFORM FOR IMPROVEMENT OF SOLUBILITY AND DISSOLUTION OF POORLY*

- SOLUBLE DRUGS*. Asian Journal of Pharmaceutical and Clinical Research, 2015. **8**(5): p. 7-17.
88. Garti, N. and D.J. McClements, *Encapsulation technologies and delivery systems for food ingredients and nutraceuticals*. 2012: Elsevier.
 89. Sharma, M. and D. Shah. *Introduction to macro-and microemulsions*. in *ACS symposium series*. 1983. Oxford University Press.
 90. Shinoda, K. and B. Lindman, *Organized surfactant systems: microemulsions*. Langmuir, 1987. **3**(2): p. 135-149.
 91. Saint Ruth, H., et al., *Phase studies and particle size analysis of oil-in-water phospholipid microemulsions*. International journal of pharmaceutics, 1995. **116**(2): p. 253-261.
 92. Talegaonkar, S., et al., *Microemulsions: a novel approach to enhanced drug delivery*. Recent patents on drug delivery & formulation, 2008. **2**(3): p. 238-257.
 93. Fawzia, H., E.-M. Mona, and M. Shaheer, *Ocular drug deliver and the importance of microemulsion as a potential delivery system*.
 94. Malcolmson, C. and M.J. Lawrence, *A comparison of the incorporation of model steroids into non-ionic micellar and microemulsion systems*. Journal of pharmacy and pharmacology, 1993. **45**(2): p. 141-143.
 95. Warisnoicharoen, W., A. Lansley, and M. Lawrence, *Nonionic oil-in-water microemulsions: the effect of oil type on phase behaviour*. International journal of pharmaceutics, 2000. **198**(1): p. 7-27.

96. Lawrence, M.J., *Surfactant systems: microemulsions and vesicles as vehicles for drug delivery*. European journal of drug metabolism and pharmacokinetics, 1994. **19**(3): p. 257-269.
97. Aboofazeli, R., et al., *Investigations into the formation and characterization of phospholipid microemulsions. IV. Pseudo-ternary phase diagrams of systems containing water-lecithin-alcohol and oil; the influence of oil*. International journal of pharmaceutics, 1995. **125**(1): p. 107-116.
98. Sahu, G., et al., *Advancements in Microemulsion Based Drug Delivery Systems for Better Therapeutic Effects*. Int J Pharm Sci Dev Res 1 (1): 008, 2015. **15**(008).
99. Kronberg, B., K. Holmberg, and B. Lindman, *Surface chemistry of surfactants and polymers*. 2014: John Wiley & Sons.
100. Kahlweit, M., et al., *Preparing nontoxic microemulsions*. Langmuir, 1995. **11**(11): p. 4185-4187.
101. Gupta, S., *Biocompatible microemulsion systems for drug encapsulation and delivery*. Curr Sci, 2011. **101**(2): p. 174-188.
102. Tenjarla, S., *Microemulsions: an overview and pharmaceutical applications*. Critical Reviews™ in Therapeutic Drug Carrier Systems, 1999. **16**(5).
103. Mehta, S. and G. Kaur, *Microemulsions: thermodynamic and dynamic properties*. 2011: INTECH Open Access Publisher.
104. Bharti, S.K. and K. Kesavan, *Phase-transition W/O Microemulsions for Ocular Delivery: Evaluation of Antibacterial Activity in the Treatment of Bacterial Keratitis*. Ocular immunology and inflammation, 2016: p. 1-12.

105. El Agamy, H.I. and G.M. El Maghraby, *Natural and synthetic oil phase transition microemulsions for ocular delivery of tropicamide: efficacy and safety*. 2015.
106. da Silveira, W., et al., *Development and Characterization of a Microemulsion System Containing Amphotericin B with Potential Ocular Applications*. Current neuropharmacology, 2015.
107. Gan, L., et al., *Novel microemulsion in situ electrolyte-triggered gelling system for ophthalmic delivery of lipophilic cyclosporine A: in vitro and in vivo results*. International journal of pharmaceutics, 2009. **365**(1): p. 143-149.
108. Ince, I., et al., *A novel pilocarpine microemulsion as an ocular delivery system: in vitro and in vivo studies*. Journal of Clinical & Experimental Ophthalmology, 2015. **2015**.
109. Fialho, S.L. and D. Silva-Cunha, *New vehicle based on a microemulsion for topical ocular administration of dexamethasone*. Clinical & experimental ophthalmology, 2004. **32**(6): p. 626-632.
110. Haße, A. and S. Keipert, *Development and characterization of microemulsions for ocular application*. European journal of pharmaceutics and biopharmaceutics, 1997. **43**(2): p. 179-183.
111. Boddu, S.H., S.P. Bonam, and R. Jung, *Development and characterization of a ricinoleic acid poloxamer gel system for transdermal eyelid delivery*. Drug development and industrial pharmacy, 2015. **41**(4): p. 605-612.
112. Robin, J.S. and P.P. Ellis, *Ophthalmic ointments*. Survey of ophthalmology, 1978. **22**(5): p. 335-340.

113. Naveh, N., S. Muchtar, and S. Benita, *Pilocarpine incorporated into a submicron emulsion vehicle causes an unexpectedly prolonged ocular hypotensive effect in rabbits*. Journal of Ocular Pharmacology and Therapeutics, 1994. **10**(3): p. 509-520.
114. Zhang, W., M.R. Prausnitz, and A. Edwards, *Model of transient drug diffusion across cornea*. Journal of controlled release, 2004. **99**(2): p. 241-258.
115. Agarwal, S., A. Agarwal, and D.J. Apple, *Textbook of ophthalmology*. Vol. 1. 2002: Jaypee Brothers Publishers.
116. Kesavan, K., et al., *Positively charged microemulsions of dexamethasone: comparative effects of two cosurfactants on ocular drug delivery and bioavailability*. Therapeutic delivery, 2013. **4**(11): p. 1385-1395.
117. Vane, J. and R. Botting, *Inflammation and the mechanism of action of anti-inflammatory drugs*. The FASEB journal, 1987. **1**(2): p. 89-96.
118. Babić, F., V. Venturi, and G. Maravić-Vlahoviček, *Tobramycin at subinhibitory concentration inhibits the RhII/R quorum sensing system in a Pseudomonas aeruginosa environmental isolate*. BMC infectious diseases, 2010. **10**(1): p. 1.
119. Inc, A.C., *Tobradex® Product Monograph* 1990, Alcon Canada Inc.
120. Winfield, A., et al., *A study of the causes of non-compliance by patients prescribed eyedrops*. British Journal of Ophthalmology, 1990. **74**(8): p. 477-480.
121. Hegde, R.R., et al., *Physicochemical and pharmacological investigation of water/oil microemulsion of non-selective beta blocker for treatment of glaucoma*. Current eye research, 2014. **39**(2): p. 155-163.

122. El Maghraby, G.M. and A.A. Bosela, *Investigation of self-microemulsifying and microemulsion systems for protection of prednisolone from gamma radiation*. Pharmaceutical development and technology, 2011. **16**(3): p. 237-242.
123. Bonam, S.P., *Preparation and evaluation of pluronic lecithin organogel containing ricinoleic acid for transdermal drug delivery*. 2013, University of Toledo.
124. Ryan, J.A., *Colorimetric determination of gentamicin, kanamycin, tobramycin, and amikacin aminoglycosides with 2, 4-dinitrofluorobenzene*. Journal of pharmaceutical sciences, 1984. **73**(9): p. 1301-1302.
125. Lee, C.-M., *Nanoparticle mobility in viscoelastic media*. 2008: ProQuest.
126. Nesamony, J., et al., *Development and characterization of nanostructured mists with potential for actively targeting poorly water-soluble compounds into the lungs*. Pharmaceutical research, 2013. **30**(10): p. 2625-2639.
127. Patel, S., et al., *Development and evaluation of dexamethasone nanomicelles with potential for treating posterior uveitis after topical application*. Journal of Ocular Pharmacology and Therapeutics, 2015. **31**(4): p. 215-227.
128. MacCallum, D.K., et al., *Bovine corneal endothelium in vitro: elaboration and organization of a basement membrane*. Experimental cell research, 1982. **139**(1): p. 1-13.
129. Bajaj, S., D. Singla, and N. Sakhuja, *Stability Testing of Pharmaceutical Products*. 2012.
130. Florence, A.T. and J. Siepmann, *Modern Pharmaceutics: Applications and Advances*. Vol. 2. 2008: Informa Health Care.

131. Jansook, P., et al., *yCD/HPyCD mixtures as solubilizer: solid-state characterization and sample dexamethasone eye drop suspension*. Journal of Pharmacy & Pharmaceutical Sciences, 2010. **13**(3): p. 336-350.
132. Dash, A.K. and R. Suryanarayanan, *Solid-state properties of tobramycin*. Pharmaceutical research, 1991. **8**(9): p. 1159-1165.
133. Menczel, J.D. and R.B. Prime, *Thermal analysis of polymers: fundamentals and applications*. 2014: John Wiley & Sons.
134. Guideline, I.H.T., *Validation of analytical procedures: text and methodology*. Q2 (R1), 2005. **1**.
135. Lucero, J.C., *Enhancement of benzalkonium chloride preservative activity in formulations containing an incompatible drug*. 1996, Google Patents.
136. Said, T., et al., *Benefits and side effects of different vegetable oil vectors on apoptosis, oxidative stress, and P2X7 cell death receptor activation*. Investigative ophthalmology & visual science, 2007. **48**(11): p. 5000-5006.
137. Vieira, C., et al., *Effect of ricinoleic acid in acute and subchronic experimental models of inflammation*. Mediators of inflammation, 2000. **9**(5): p. 223-228.
138. Vieira, C., et al., *Pro-and anti-inflammatory actions of ricinoleic acid: similarities and differences with capsaicin*. Naunyn-Schmiedeberg's archives of pharmacology, 2001. **364**(2): p. 87-95.
139. Novak, A.F., G.C. Clark, and H.P. Dupuy, *Antimicrobial activity of some ricinoleic acid oleic acid derivatives*. Journal of the American Oil Chemists Society, 1961. **38**(6): p. 321-324.

140. Furrer, P., et al., *Application of in vivo confocal microscopy to the objective evaluation of ocular irritation induced by surfactants*. International journal of pharmaceutics, 2000. **207**(1): p. 89-98.
141. Knagenhjelm, S., et al., *Toxicological evaluation of cyclosporine eyedrops*. Acta Ophthalmologica Scandinavica, 1999. **77**(2): p. 200-203.
142. Boonme, P., et al., *Characterization of microemulsion structures in the pseudoternary phase diagram of isopropyl palmitate/water/Brij 97: 1-butanol*. Aaps Pharmscitech, 2006. **7**(2): p. E99-E104.
143. Ansari, R.R., et al., *Microemulsion characterization by the use of a noninvasive backscatter fiber optic probe*. Applied optics, 1993. **32**(21): p. 3822-3827.
144. Gallarate, M., et al., *Preparation and evaluation in vitro of solutions and o/w microemulsions containing levobunolol as ion-pair*. International journal of pharmaceutics, 1993. **100**(1-3): p. 219-225.
145. Cholkar, K., et al., *Nanomicellar topical aqueous drop formulation of rapamycin for back-of-the-eye delivery*. AAPS PharmSciTech, 2015. **16**(3): p. 610-622.
146. Carrigan, P.J. and T.R. Bates, *Biopharmaceutics of drugs administered in lipid-containing dosage forms I: Gi absorption of griseofulvin from an oil-in-water emulsion in the rat*. Journal of pharmaceutical sciences, 1973. **62**(9): p. 1476-1479.
147. Nesamony, J., et al., *Nebulized oil-in-water nanoemulsion mists for pulmonary delivery: development, physico-chemical characterization and in vitro evaluation*. Drug development and industrial pharmacy, 2014. **40**(9): p. 1253-1263.

148. Fazlara, A. and M. Ekhtelat, *The disinfectant effects of benzalkonium chloride on some important foodborne pathogens*. American-Eurasian Journal of Agricultural & Environmental Science, 2012. **12**(1): p. 23-29.