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Formulation Strategies to Address Physiological and Anatomical Constraints for Improved Topical Ocular Drug Delivery

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FORMULATION STRATEGIES TO ADDRESS PHYSIOLOGICAL AND ANATOMICAL CONSTRAINTS FOR IMPROVED TOPICAL OCULAR DRUG DELIVERY

A Dissertation
presented in partial fulfillment of requirements
for the degree of Doctor of Philosophy
in Pharmaceutical Sciences with an emphasis in Pharmaceutics and Drug Delivery
The University of Mississippi

By
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ABSTRACT

Delivery of therapeutic molecules to the eye has been a complicated task owing to its anatomical and physiological barriers. This dissertation delineates several formulation strategies to circumvent dynamic and static constraints hampering the ocular drug delivery.

Advanced vesicular systems, bilosomes and transfersomes, were prepared to improve the ocular pharmacotherapy of natamycin (NT). NT bilosomes and transfersomes were further loaded into in situ gel systems which transformed immediately to hydrogel, in the simulated tear fluid, with good viscoelastic and mucoadhesive properties. *In vitro* corneal transport studies confirmed the superior permeability characteristics of NT from the optimized bilosome and transfersome in situ gel formulations, over control marketed (5 % w/v NT) suspension. In the ocular distribution studies, considerably higher mean dose normalized drug levels in the ocular tissues from bilosome and transfersome in situ gel formulations for 6h, compared to that from the control suspension, demonstrated the effectiveness of ion-sensitive *in situ* hydrogels of bilosomes and transfersomes as potential carrier systems for improved and prolonged topical ocular drug delivery.

The magnitude of P-glycoprotein (P-gp) and multidrug resistance protein (MRP1) were investigated by comparative ocular distribution studies in the P-gp and MRP1 gene knock out (KO) and wild type (WT) rats. The blood ocular penetration of paclitaxel (PTX), a P-gp substrate and, methotrexate (MTX), an MRP 1 substrate, were significantly higher in the KO rats compared to WT rats. These results suggested that the penetration of anticancer substrate drugs across the BOB is restricted by efflux transporter proteins. Further, the concurrent intravenous administration of specific P-gp and MRP 1 inhibitors, along with PTX or MTX, in the WT rats resulted in improved
blood ocular penetration of substrate anticancer drugs by effective inhibition of efflux protein activity in the BOB. The electroretinography (ERG) studies demonstrated that the inhibitor and substrate interactions did not induce any toxic effects on retina functions. However, at higher concentration of PTX and MTX showed toxic effects on the retina functions.

Feasibility of coadministration of topical ocular inserts of P-gp inhibitors and intravenous anticancer substrate drug, PTX, was investigated in rats to examine the potential of this strategy to improve blood ocular penetration of PTX. Ocular films of P-gp inhibitors were fabricated by melt cast method and the transocular membrane permeability of the P-gp inhibitors, elacridar (EQ) and tariquidar (TQ), was investigated in the isolated rabbit cornea. Both EQ and TQ showed transcorneal permeability but no penetration through Sclera-choroidal retinal pigmented epithelium (RPE) tissue. The ocular distribution studies in rats showed improved blood ocular penetration of PTX in the rats co-administered with EQ and TQ. These results demonstrated that the topically administered P-gp inhibitor effectively inhibits the P-gp activity and thereby improves ocular chemotherapy. ERG and Ultrastructure analyses of RPE confirmed that the PTX-EQ/TQ interactions were compatible with retina.

TA protransfersome gel formulations were successfully prepared with at least two-fold higher drug load, compared to the marketed topical dosage form, which allows higher, localized, concentration gradients. Microscopic studies confirmed complete drug dissolution in the lipid phase and rapid formation of transfersomes on hydration. Significantly higher trans-eyelid TA permeation with the protransfersome gel formulation – demonstrated their potential in enhancing drug delivery to the ocular surface and deeper tissues via the eyelid. Experiments in dead rabbits confirmed that TA
efficiently penetrated into the eyelid and formed a depot leading to increasing concentration-time profiles in all ocular tissues tested. The overall results suggest that trans-eyelid protransfersome gel formulations can provide a platform for the sustained delivery of therapeutic agents to both the surface of the eye as well as the anterior and posterior segment ocular tissues.
DEDICATION

For all their blessings, prayers, love, care, encouragement, patience and moral support, this dissertation is dedicated to my parents Shashekala Yadav Janga, Madhusudhan Yadav Janga and to my brother Akilesh Yadav Janga.
LIST OF ABBREVIATIONS

NT - Natamycin
PTX - Paclitaxel
MTX - Methotrexate
EQ - Elacridar
TQ- Tariquidar
DTX - Docetaxel
LOP - Loperamide
Ph- Phenacetin
TA- Triamcinolone Acetonide
P-gp – P-glycoprotein
MRP- Multidrug resistant Protein
MDR 1a – Multidrug Resistant 1a gene
G- Gellan Gum
X- Xanthan gum
FS- Eudragit® FS 100
PEO N10 - Polyethylene oxide N10
βCD - Beta cyclodextrin
HPβCD - Hydroxy propyl beta cyclodextrin
RMβCD - Randomly methylated beta cyclodextrin
SLN’s - Solid lipid nanoparticles
NLC’s - Nano structured lipid carriers
DR - Diabetic retinopathy
RPE - Retinal pigmented epithelium
BOB – Blood Ocular Barriers
BAB – Blood Aqueous Barriers
BRB – Blood Retinal Barriers
EE - Entrapment efficiency
LC/MS- Liquid Chromatography and Mass Spectrophotometry
IPBS - Isotonic phosphate buffered saline
DPBS- Dulbecco's Phosphate Buffered Saline.
DSC - Differential scanning calorimetry
FTIR - Fourier transform infrared spectroscopy
HPLC-UV - High performance liquid chromatography- Ultra violet
mM - Millimolar
μg- Microgram
DMSO - Dimethyl sulfoxide
AH - Aqueous humor
VH - Vitreous humor
IC - Iris ciliary bodies
RC - Retina-choroid
ACKNOWLEDGMENTS

PhD degree could have been an unachievable dream for me without my advisor Dr. Soumyajit Majumdar. His constant intellectual support, expertise, and professional guidance helped me to widen my research from various perspectives. I bow down for his valuable advises, insightful comments, encouragement, patience, understanding and support throughout my graduate studies. He was very kind and supportive during my tough times. I offer my sincerest gratitude and admire his help and attention towards my academic growth. One simply could not wish for a better or friendlier supervisor. Thank you very much for being my advisor!

I deem it my privilege in expressing fidelity to my dissertation committee members Dr. Michael A. Repka, Dr. Samir A. Ross, and Dr. Narasimha Murthy for their guidance, suggestions, invaluable time and help during the evaluation of this dissertation. I extend my thanks to Dr. Seong Bong Jo, Dr. Mahavir B. Chougule, Dr. Chalet Tan and Dr. Christy M. Wyandt for making the experience at Ole Miss worthwhile, Ms. Deborah King for her help, patience and affection. I also thank the support extended by Ms. Penni Bolton during animal experiments.

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wish to acknowledge my very special friends Srinivas, Vijay, Prit, Sameer, Dr. Narendra, Dr. Srinath and Dr. Bandari they have made my grad school very joyful and memorable.

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Thank you, Mom and Dad, for everything!
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CHAPTER 1
INTRODUCTION

1.1. Introduction

Eye, the most important and sensitive organ, regulates the visual sensory activities in the humans. The architecture of the eye is extremely complex with the intraocular tissues aligned in two segments, namely, the anterior segment and the posterior segment (Fig 1.1). Conjunctiva, cornea, aqueous humor (AH), iris, ciliary body, trabecular meshwork, schlemm’s canal, pupil, and lens are situated in the anterior segment. Most of these tissues control the aqueous humor dynamics which maintains the intraocular pressure. Sclera, choroid, retina, vitreous humor (VH) and optic nerve constitutes posterior segment. The rods and cones in the retina play a critical role in

Figure 1.1: Anatomy of the human eye
processing the visual information in the central nervous system. Any damage, either by disease or injury, to the eye may lead to the temporary or permanent vision problems

1.1.1. Ocular Protective Barriers

The anatomical and physiological barriers that safeguard the eyes from toxins or xenobiotics from external environment or systemic circulation are presented in table 1.1.

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<td>6. Conjunctiva ultrastructure</td>
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1.1.1.1. Physiological barriers

The physiological barriers lower precorneal residence time of the toxins or xenobiotics, from the external environment (topical), through increased lacrimation, dilution by tear, reflux blinking and nasolacrimal drainage. Moreover, the precorneal metabolism results in precorneal loss and, thus, prevents the entry of toxic materials into the ocular compartments (1, 2).

1.1.1.2. Anatomical Barriers

Anatomical barriers restrict the penetration of toxins from the external environment and protect the deeper ocular tissues. The conjunctiva vasculature, unique structural features of the cornea,
with the hydrophilic stroma sandwiched between the hydrophobic epithelium and the endothelium, and sclera limits the permeability of the xenobiotics (2). The presence of complex blood ocular barriers (BOB) comprising; blood-aqueous barrier (BAB), and the blood-retinal barrier (BRB), with tight-junction proteins and the efflux proteins (P-gp/MRP 1), protect the anterior and posterior segment ocular tissues by preventing the paracellular and/or transcellular transportation of endobiotics and xenobiotics from the systemic circulation (3, 4).

1.1.2. **Eye Ailments**

In 2011, as per the World Health Organization (WHO) report, approximately 285 million global population is suffering from eye ailments. Additionally, National Eye Institute (NEI) reported more than 30 million individuals in the United States suffering from ocular diseases (5, 6). Age-related macular degeneration (AMD), diabetic retinopathy (DR), uveitis, retinoblastoma, dry eyes, conjunctivitis, keratomycosis, blepharitis, postoperative endophthalmitis, corneal ulcers, bacterial keratitis, mycotic keratitis, choroidal neovascularization (CNV) and retinitis pigmentosa were the most commonly encountered eye diseases worldwide (7-10). These diseases involve infection, inflammation and/or oxidative stress (alone or in combination) of anterior and posterior segment ocular tissues. It is predicted that these numbers would be doubled by 2050 (2,11,12). Alone in USA, approximately $40 billion/year expenditure is incurred in the treatment of ocular disorders, and, more than a half of the treatment regimen includes anti-infective and steroidal anti-inflammatory agents (11,12).

Currently, conventional, topical ophthalmic formulations are utilized for the treatment of external eye surface and anterior ocular tissue infections/inflammation (1,2). A host of physiological and anatomical protective barriers shorten the precorneal residence and restricts the ocular penetration of therapeutic agents from these topical formulations (1,2,13). Thus, frequent
administration (3 - 6 times/day) is often needed to maintain therapeutic drug levels (1,2,13), which leads to noncompliance issues. Moreover, the topical route of administration has not been effective in the treatment of conditions affecting the back-of-the eye, for which intravitreal injections/systemic administration are the best options (13,14). Poor patient compliance and injection associated risks, however, are major challenges for intravitreal injections (13,15). While, drugs from systemic circulation need to overcome the blood ocular barriers for which higher dose administration is required, that could result in systemic toxicity (14). Hence, there is an urgent need for robust noninvasive drug delivery platforms that can improve and prolong therapeutic drug levels in the anterior and posterior ocular segments.

1.2. Specific Objectives:

The major objective of this project is to develop novel topical ophthalmic formulations of Natamycin (NT), an anti-fungal agent, Triamcinalone acetonide (TA), a steroidal anti-inflammatory agent, and P-gp/Mrp 1 inhibitors. NT and TA are used in the treatment of ocular fungal infections and inflammations. P-gp/Mrp 1 inhibitors are utilized in the inhibition of the P-gp/Mrp1 activity in the BOB to improve blood ocular penetration of chemotherapeutics. The selected molecules were formulated and tested for in vitro and in vivo performance to demonstrate the potential of advanced vesicular in situ hydrogels and melt-cast films for improved and prolonged ocular pharmacotherapy.

1.3. Specific Aims

1. Development and evaluation of the ion sensitive in situ hydrogels of NT loaded vesicular systems for improved and prolonged ocular delivery.

2. Screening, development and evaluation of P-gp/Mrp1 inhibitor loaded topical melt cast films for improved blood ocular penetration of substrate chemotherapeutic drugs.

2.2. Development and evaluation of specific inhibitor loaded topical ophthalmic melt-cast films for the inhibition of P-gp/Mrp1 activity in the blood ocular barriers to improve blood ocular penetration of substrate chemotherapeutic drugs.

3 Development, quality control, and *in vivo* evaluation of protransfersomes gel formulation for improved and prolonged ocular drug delivery through eyelid application.
CHAPTER 2
ION SENSITIVE IN SITU HYDROGELS OF ADVANCED NATAMYCIN LOADED VESICULAR SYSTEMS FOR IMPROVED AND PROLONGED OCULAR PHARMACOTHERAPY

2.1. Introduction

Despite advancement in the field of pharmaceutical technology, effective topical ocular drug delivery is still a complicated task for formulation scientists due to the complex anatomical, physiological and formulation constraints (16-18). Beside various protective ocular barriers, short pre-corneal residence and poor corneal permeability are the key factors contributing to low ocular bioavailability (< 5%) of the therapeutics instilled as conventional ophthalmic dosage forms (14, 16-19). The increased lacrimation, reflux blinking and nasolacrimal drainage accounts for the rapid elimination of the drugs from the cul-de-sac (17,18). Various formulations like ocular ointments, hydrogels, polymeric or ion-exchange resin based topical ocular inserts and in situ gel systems have been reported to improve the residence of the drugs in the pre-corneal space (20-23). In situ hydrogels have better patient compliance unlike ocular ointments and inserts which causes blurred vision and grittiness influenced distressed blinking (22,24,25). The in situ gel systems are solutions which undergo phase transition to form viscoelastic hydrogel in the ocular milieu in response to the temperature, pH, electrolytes or ions and photo radiation (22,24,25). Due to the abundance of electrolytes in tear fluid, the ion or electrolyte sensitive in situ hydrogel formation is triggered at lower polymer concentrations with respect to that induced by other stimuli (26,27).
The distinct anatomical features of the cornea, with hydrophilic stroma sandwiched between lipophilic epithelium and endothelium, restricts the penetration of hydrophilic and lipophilic drugs into the deeper ocular tissues [16-19, 28]. Numerous lipid/polymeric nano-formulations such as solid lipid nanoparticles (SLN), nanostructured lipid carriers (NLC), polymeric nanoparticles (PN), micelles and phospholipid or non-ionic surfactant based vesicles have shown success in enhancing the transcorneal drug permeability (29-34). The combination of in situ gel systems and nanocarriers offers improved tear/corneal surface contact time and corneal permeability of the drugs (35). Several pH and/or temperature triggered in situ gel forming systems of SLN, polymeric nanoparticles, nanoemulsions, liposomes and niosomes have been successfully investigated as suitable ocular drug delivery platforms (35-39).

In recent years, advanced vesicular systems, bilosomes and transfersomes, have emerged as potential carriers for improved oral, nasal and/or transdermal delivery of small and large molecules due to their ultra-deformable and flexible properties (40-42). The inclusion of the amphiphilic bile salt (bilosomes) or hydrophilic edge activator (transfersomes) in the vesicle bilayer allows bilosomes and transfersomes to penetrate through the intercellular lipid layers of intact biological membranes, such as skin, nasal mucosa and intestine (41-44]). Considering these advantages, a few studies investigated the suitability of bilosomes and transfersomes for improved ex vivo corneal permeability of a lipophilic molecule (42,44). To the best of our knowledge, bilosomes and transfersomes ion sensitive in situ hydrogel forming systems have not been investigated till date.

In this study, we report the fabrication and evaluation of ion sensitive in situ hydrogels of natamycin (NT)-loaded bilosomes (NB) and transfersomes (FN) for improved ocular pharmacotherapy. NT, a macrolide polyene antifungal agent, is the only commercially available
ophthalmic product (Natacyn® (NT 5% w/v suspension)) approved by US-FDA for treating fungal keratitis and other fungal infections (45). Due to poor aqueous solubility, limited corneal permeability and low precorneal residence time of NT, current therapy requires multiple dosing (a drop every 1-2h) for several weeks (4-6 weeks) to attain and/or maintain therapeutic concentrations at the desired site (45,46). Hence, there is a need to develop a delivery system, with improved NT corneal permeability and prolonged drug levels in the ocular sites, for improved patient compliance. A few literature reports described improved NT levels in the tear and prolonged in vitro corneal drug permeability from mucoadhesive nanoparticles and niosomes thermosensitive in situ gels, respectively [39,47]. However, there are no reports on the ocular disposition of NT or any other drug from topically instilled bilosomes and transfersomes in situ gels.

Thus, for the first time, the ocular disposition of ion sensitive in situ hydrogel forming system of NB and FN are evaluated in the New Zealand male albino rabbits.

2.2. Materials and Methods

NT was procured from Cayman Chemical (Ann Arbor, MI, USA). Sodium taurocholate (ST) was obtained from Biosynth International, Inc. (Itasca, IL, USA). Phospholipon 90H (PL) was procured from Lipoid, LLC (Newark, NJ, USA), Kolliphor TPGS (D-alpha tocopheryl polyethylene glycol 1000 succinate) (T) was obtained from BASF Corporation (NJ, USA), (Sorbitan monostearate (Span 60 (S60)) and MTT reagent (Methylthiazolyldiphenyl-tetrazolium bromide) were received from Sigma Aldrich (St. Louis, MO, USA. Cholesterol (CH) was procured from Alfa Aesar (Ward Hill, MA, USA). Gelan Gum (G) and Xanthum gum (X) were purchased from MP Biomedicals, LLC (Santa Ana, CA, USA). DMEM/F-12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12) was procured from Mediatech, Inc. (A Corning Subsidiary Manassas, VA). Glycerin, Amicon® Ultracentrifugal filter devices with regenerated cellulose membrane (molecular weight
cut off 100 kDa), high performance liquid chromatography (HPLC) - grade solvents, and other chemicals (analytical grade) were supplied by Fisher Scientific (Hampton, NH, USA). Whole eyes of male albino New Zealand rabbits were obtained from Pel-Freez Biologicals (Rogers, AR). Male albino New Zealand rabbits were procured from Harlan Labs (Indianapolis, IN).

2.2.1. Quantification of natamycin

The NT content in all the samples was determined with HPLC-UV (ultraviolet) method, as reported earlier, with minor modifications (48). In brief, a mobile phase of acetonitrile and 0.02 M potassium dihydrogen phosphate (30:70 % v/v) mixture (pH 5.5) was pumped through a phenomenex Luna® C₁₈(2) 100 Å column (4.6 mm x 250 mm; 5µ particle size), at a flow rate of 1.0 mL/min, by Water 600 controller pump in HPLC system. A 20 µL sample was injected by Waters 717 plus Autosampler into the mobile phase and the column eluent was monitored at λ_{\text{max}} (detection wavelength) of 304 nm using Waters 2487 Dual λ Absorbance detector. The absorbance peaks were recorded with the help of Agilent 3395 Integrator.

2.2.2. Preparation of natamycin bilosomes and in situ gel formulations

The film hydration technique was employed to prepare the natamycin bilosomes (NB) (37,49). Briefly, in 20 mL of organic solvent mixture of chloroform, ethanol and methanol (7:1:2), accurately weighed quantities of lipid mixture (210 or 230 µM of S60 and CH taken in 2:1, 1:1 and 1:2 molar ratios) and drug were dissolved in a round bottom (RB) flask. The RB flask was then mounted onto a rotary flash evaporator (Laborota 4000, Heidolph, Germany) operated at 200 rpm for 30 min at 45 ± 5°C to evaporate the organic solvent, under vacuum, leaving a thin film of lipid on the wall of the flask. The lipid film was dried in vacuum oven at 40°C for 2h and then hydrated with the deionized water (8-10 mL) containing ST (20 or 40 µM) and glycerin, maintained at 60 ± 5°C, to form NB with vigorous shaking. Further, the NB was sonicated with
probe sonicator (Vibra-Cell™ Ultrasonic, Sonics and Material INC., CT, USA) for 5 min with pulse on for 20 secs and off for 40 secs at 30 % amplitude.

The NB in situ gel NBG or NBX formulations were obtained by adding 8 mL of NB 2 to 2 mL of G or X dissolved in deionized water (60 ± 5°C). The formulations were allowed to cool to room temperature and stored in screw-capped glass vials until further evaluation. The composition of the NB and NBG or NBX formulations are presented in Table 2.1.

2.2.3. Preparation of natamycin transfersomes and in situ gel formulations

The NT transfersomes formulations (FN) were prepared by the film hydration technique (42,49). Accurately weighed quantities of PL, S60, TPGS and NT were dissolved in 20 mL of organic solvent mixture (chloroform, ethanol and methanol (7:1:2)), in a round bottom (RB) flask (Table 2.2). The RB flask was then mounted onto a rotary flash evaporator (Laborota 4000, Heidolph, Germany) operated at 200 rpm for 30 min at 45 ± 5°C to evaporate the organic solvent, under vacuum, leaving a thin residual lipid film on the flask wall. The lipid film was dried in vacuum oven at 40°C for 2h and then hydrated with the deionized water (8-10 mL) containing glycerin, maintained at 60 ± 5°C, to form transfersomes with vigorous shaking. Further, the FN was sonicated with probe sonicator (Vibra-Cell™ Ultrasonic, Sonics and Material INC., CT, USA) for 5 min with pulse on for 20 secs and off for 40 secs at 30 % amplitude.

The FN in situ gel formulations (FNG) were obtained by adding 8 mL of FN 2 to 2 mL of G dissolved in deionized water (60 ± 5°C). The formulations were allowed to cool to room temperature and stored in screw-capped glass vials until further evaluation. The composition of the FN and FNG formulations are presented in Table 2.2.
2.2.4. Particle size, zeta potential and polydispersity index measurement

The mean particle size (PS) and polydispersity index (PDI) of the NB, NBG, NBX, FN and FNG formulations were determined by dynamic light scattering using Zetasizer Nano ZS Zen3600 (Malvern Instruments, Inc. UK). Samples were suitably diluted with filtered double distilled water (0.2 μm nylon filter) and analysis was performed at 25°C and 173° angle of detection using a helium-neon laser of 633 nm, in disposable clear cells. Zeta potential (ZP) measurements were also carried out at 25°C using the laser Doppler velocimetry function of the same instrument.

2.2.5. Drug Content and Entrapment Efficiency

The drug content was determined by extracting the NT from the formulations using methanol and 190-proof alcohol (70:30 %v/v) then centrifuged (AccuSpin 17R centrifuge, Fisher Scientific, USA) at 13,000 rpm for 20 min. The supernatant was separated, appropriately diluted and quantified for the drug content by HPLC.

The percentage drug entrapment efficiency (% EE) was determined by estimating the free NT concentration in the dispersion medium using ultra-filtration technique. In brief, 500 μL of each formulation was added to the sample reservoir of the Amicon Ultra-centrifugal filter device (Molecular weight cutoff of 100 kilo Daltons) (Fisher Scientific, USA) and centrifuged at 5,000 rpm for 10 min (AccuSpin 17R centrifuge, Fisher Scientific, USA). The NT content in the aqueous filtrate was evaluated by HPLC. The % EE was calculated from the Eq. (1). The experiment was carried out in triplicate.

\[
\%EE = \left[ \frac{W_i - W_f}{W_i} \right] \times 100
\]  

(1)

Where \( W_i \) = total drug content, and \( W_f \) = amount of free drug in aqueous phase.
2.2.6. *In vitro* gelation behavior of NB and FN *in situ* gels

The gelling capacity of bilosomes (NBG or NBX) and transfersomes (FNG) *in situ* gel formulations was visually evaluated by determining the gel formation time (GT) and residence time (GRT) of intact gel without dissolution or breaking (39,50). Briefly, 50 µL of NBG or NBX and FNG formulations was dropped into a separate glass vials with 2 mL of freshly prepared simulated tear fluid (STF) and maintained at 34 °C in a shaking water bath (Precision™, Fisher Scientific, USA) operated at 100 rpm for 12h. The time taken for the transformation of sol-to-gel by NBG or NBX and FNG formulations was recorded as the gel formation time (GT). Each vial was visually inspected every hour and the time required for the gel to dissolve or break was recorded as the intact gel residence time (GRT).

2.2.7. Rheological studies

The viscosity of the NB 2, NBG, NBX, FN 2 and FNG formulations were measured on Brookfield cone and plate viscometer (LVDV-II+ Pro Viscometer, Middleboro, USA). Briefly, 500 µL of each formulation was placed in the cup plate, and the gap between cone and plate was adjusted. The samples in the cup were maintained at 25°C using a circulating water bath. A CP-40 Cone/Spindle was operated at 0.1, 1 and 10 rpm and the viscosity was recorded from Rheocalc software. To determine viscosity of the hydrogels, 500 µL of mixture of bilosomes *in situ* gel or transfersomes *in situ* gel formulations and STF (50:7) was placed in cup plate and similar procedure was repeated to record viscosity (n=3) (37).

2.2.8. Texture analysis

The adhesive strength of hydrogels obtained from NBX, NBG and FNG formulations were evaluated with TA.XT2i texture analyzer (Texture Technologies Corp. NY. USA) set to the compression mode (51). The hydrogels were placed in soft matter holder (TA-275) positioned
below the acrylic cylindrical probe (1-in. diameter (TA-3)) which was then lowered into the product (1mm distance from the surface) at a test-speed of 0.5 mm/s with 3 g trigger force. An acquisition rate of 500 points/sec was selected and the texture analysis was conducted at room temperature (n=3).

2.2.9. Transmission electron microscopy

The morphology of the vesicles in the NB 2, NBG 2, FN 2 and FNG 3 formulations were evaluated by transmission electron microscopy (TEM) (JEOL JEM1200EX II electron microscope) as per the published protocol with minor modifications (52). Briefly, the formulations were centrifuged for 1h at 25,000 rpm and 15°C (SORVALL, WX Ultra Series Centrifuge, USA) to separate the bilosomes and transfersomes pellets which were then resuspended in 5ml deionized water and recentrifuged (as mentioned earlier). This step is repeated 3 times to wash out any soluble impurities. The resuspended bilosomes and transfersomes were centrifuged for 5min at 2000 rpm (15°C) (Eppendorf AG centrifuge, Hamburg, Germany), to separate the aggregates/insoluble impurities, and the clean vesicular suspensions were then diluted 1:100 with deionized water. 2 μL of the diluted suspension was placed on 400 mesh copper grids covered with Formvar film (Electron Microscopy Sciences EMS, Hatfield, PA, USA). The grids were allowed to dry overnight in a desiccator and then examined by TEM.

2.2.10. Fourier transform infrared spectroscopy (FTIR)

The infrared spectra of drug, sodium taurocholate, phospholipon 90H, TPGS, cholesterol, span 60 NBG 2 and FNG 3 samples were recorded using Cary 660 series FTIR (Agilent Technologies, USA) to understand the chemical compatibility between the drug and excipients.

2.2.11. Stability studies
The NB 2, NBG 2, FN 2 and FNG 3 formulations were taken in glass vials, wrapped in aluminum foil and stored at 4°C and 25°C for 60 days. Their physical and chemical stability, on storage, were evaluated by withdrawing the samples at definite time intervals (0, 30 and 60 days) and analyzing the particle size, PDI, zeta potential, % EE and drug content as described earlier.

2.2.12. In vitro cytotoxicity

In vitro cytotoxicity of NB 2, NBG 2, FN 2, FN 5 and FNG 3 formulations were evaluated by the methyl thiazolyl tetrazolium (MTT) assay. Normal saline and triton x 100 served as negative and positive controls for the experiment respectively. The assay was carried out in 96-well plates (Costar 3596, Corning Inc., Corning, NY). Briefly, 100 μl of the human corneal limbal epithelial (HCLE) cells were plated at a concentration of 15x10^4 cell/mL, and incubated in an incubator at 37°C with 5% CO₂ for 24h in culture medium (DMEM/F-12 50:50, with 10% calf serum, and 1% penicillin/streptomycin). Then, 100 μl of the diluted formulations, negative control (normal saline), NB 2, NBG 2, FN 2, FN 5, FNG 3 and positive control (triton X 100)) (i.e., 50 μl formulation + 50 μl of DMEM/F-12 50:50 Medium) was added to each well. After 24h incubation, the formulations were removed, and the cells were washed twice with the culture medium to remove all traces of the formulations. 100 μl of MTT reagent (1 mg/ml in culture medium without serum) was added to each well and the plates were incubated at 37°C for 4h. During the incubation, the active enzymes (mitochondrial reductase) of the viable cells converted the yellow MTT dye into purple formazan crystals. After incubation, the medium was removed and 100 μl DMSO was added to each well to dissolve the formazan crystals and the intensity of color was assessed at 570 nm with µ-Quant universal microplate spectrophotometer (Bio-Tek Instruments, Inc. Winooski, VT).
2.2.13. *In vitro* corneal permeation studies

*In vitro* transcorneal permeation studies were performed in the corneas separated from rabbit whole eyes shipped overnight in Hanks balanced salt solution (over wet ice) from Pel-Freez Biologicals. They were used immediately upon receipt. To confirm the secure mounting between the donor and receiver chambers of diffusion cell during the study period, corneas were excised with some scleral portion. They were placed on Valia-Chien cells (PermeGear, Inc®), maintained at 34°C, with the endothelial side facing receiver chamber while the epithelial side facing the donor chamber. 200 µL of control (Natacyn®), NB 2, NBG 2, FN 2, FN 5 and FNG 3 formulations were added to the individual donor chambers. 50 µL of DPBS was placed in the donor chamber holding 200 µL of NBG 2 or FNG 3 formulation to ensure the hydrogel formation. The Natacyn® suspension was diluted to obtain 0.1% w/v NT, equivalent to that in NB 2 and NBG 2 formulations. The receiver chamber was filled with 5 mL of DPBS (pH 7.4) with 2.5% w/v of RMβCD medium. At predetermined time points, an aliquot of 600 µL was withdrawn from the receiver chamber and replenished with equal volume of fresh medium to maintain the constant volume, until 3h. Samples were stored at −20°C until further analysis by HPLC.

2.2.14. Data analysis

The transcorneal permeation parameters across rabbit cornea such as the cumulative amount of drug permeated ($M_a$), steady state flux ($J$) and the apparent permeability coefficient ($P_{app}$) were calculated as per the earlier reports (53). The cumulative amount of NT permeated across the cornea was determined using the equation Eq. (2).

\[
M_n = V_r C_{r(n)} + \sum_{x=1}^{x=n} V_s(x-1) C_{r(x-1)}
\]  

(2)

Where: $n$ indicates sampling time point ($n = 1, 2, 3, \ldots, 8$ corresponding to 15, 30, 45, \ldots, 180 min respectively); $V_r$ and $V_s$ represents the volume of the medium in the receiver chamber (mL) and
the volume of the sample collected at the \(n^{th}\) time point (mL); and \(C_r(n)\) specifies the concentration of the drug in the receiver chamber medium at \(n^{th}\) time point (µg/mL).

The rate of NT transported through the rabbit cornea was computed from the slope of the cumulative amount of NT permeated vs. time (\(t\)) plot. The steady-state flux (SSF) (\(J\)) of natamycin was calculated according to the following equation (Eq. (3)) (30):

\[
Flux(J) = \frac{(dM/dt)}{A}
\]  
(3)

Where \(M\) and \(A\) designates the cumulative amount of drug transported and the surface area of the cornea utilized in the experiment (0.636 cm\(^2\)).

The transcorneal permeability of NT was determined from the ratio of steady state flux (\(J\)) and amount of drug taken in the donor chamber (\(C_d\)) (Eq. (4)) (30,53).

\[
P_{app} = \frac{Flux(J)}{C_d*60}
\]  
(4)

Further, the enhancement ratio (ER) was determined from the ratio of flux (\(J\)) of natamycin from bilosomes (NB 2) or bilosome in situ gel (NBG 2) or transfersomes (FN 2 or FN 5) or transfersomes in situ gel (FNG 3) formulation and control formulation.

2.2.15. Corneal Histology studies

The corneal histological evaluation was performed on the corneas utilized in the in vitro transcorneal permeation studies. At the end of the study, corneas were fixed with 4% paraformaldehyde for 24 h, embedded in paraffin wax and were sliced into 5-µm cross sections using a microtome (American Optical® 820 Rotary Microtome). The tissue sections were mounted on a slide and dried overnight in an oven. The slide was washed with xylene to remove the paraffin and the tissue was hydrated by washing with alcohol and water. The tissue was stained with nuclear dye Gill III hematoxylin (StatLab medical) by rinsing for 10 min and later counterstained with
eosin. Finally, these slides were washed in running water, alcohol, and xylene. A cover slip was placed on the tissue section, examined under Nikon Eclipse 800 microscope and images were captured using PictureFrame 3.0 software (Optronics) to observe morphological changes in cornea, if any.

2.2.16. Ocular distribution study

The ocular distribution study was carried out in conscious male New Zealand albino rabbits. Prior to the study, the animals were allowed free access to food and water. The experiment was performed according to the approved protocols of the University of Mississippi Institutional Animal Care and Use committee and Association for Research in Vision and Ophthalmology (ARVO) statement. The rabbits were divided into 5 groups based on the formulation they were treated with and represented as control (Natacyn®), NB 2, NBG 2, FN 2 and FNG 3 groups. 50 µL of Natacyn® or NB 2 or NBG 2 or FN 2 or FNG 3 formulation was topically administered in the cul-de-sac of the right eye of each rabbit in the corresponding group. At the end of 2 h and 6 h post treatment, rabbits (n=4) were anesthetized with intramuscular injection of ketamine and xylazine combination and euthanized with an excess amount of pentobarbital injected through a marginal ear vein. The treated eye was washed thoroughly with ice cold IPBS and enucleated immediately. The ocular tissues were carefully separated, weighed and stored at -80 °C until analysis.

2.2.17. NT extraction from ocular tissue matrix

Protein precipitation technique was employed for the extraction of NT from the ocular tissue matrix. Briefly, tissues such as cornea, sclera, iris ciliary (IC), retina-choroid (RC) were taken in separate Eppendorf tubes and cut into small pieces followed by the addition of 1 mL ice cold
methanol with 0.1% w/v formic acid to precipitate tissue proteins. The proteins in the aqueous humor (AH) (200 µL) and vitreous humor (VH) (500 µL) were precipitated by adding 200 µL and 500 µL of ice-cold acetonitrile with 0.1% w/v formic acid respectively. All the Eppendorf tubes were vortexed for 30 seconds, allowed to stand at room temperature for 15 min and kept under sonication in bath sonicator (Fisher Scientific, USA) for 10 min to extract the drug into solvent. These samples were centrifuged at 13,000 rpm for 30 min, the supernatant was collected in the separate vials and stored at -80 °C until the analysis. The NT content in the supernatant solutions were estimated by injecting an aliquot of 100 µL into the HPLC operated with the chromatographic conditions described earlier (48). The column eluent was monitored at λ_{max} (detection wavelength) of 304 nm using Waters 2487 Dual λ absorbance detector, and the sensitivity was set at 0.05 AUFS at room temperature. The NT was quantified using standard calibration plots constructed with the concentration range of interest in various ocular tissues. The limit of detection and quantification in various ocular tissues were 10 and 20 ng/mL, respectively. The standard curves in all the ocular tissues were linear with a coefficient of determination (r²) ≥ 0.95.

2.2.18. Statistical analysis

The data obtained were subjected to one-way analysis of variance (ANOVA) and the statistical significance of difference between formulations was calculated by Tukey’s post hoc HSD (version 5.00; GraphPad Software, San Diego California). A ‘p’ value less than 0.05 was set to represent the statistically significant difference.

2.3. Results and Discussion

NB and FN formulations were successfully fabricated by thin-film hydration technique coupled with probe sonication. Since the selection of vesicle forming components is critical to obtain the stable vesicles with high drug entrapment, S60 and PL were selected as vesicular components in
bilosomes and transfersomes, respectively (41-44). Earlier reports suggested the formation of small size niosomes, a nonionic surfactant vesicle, with S60 and stable liposomes with the phospholipon 90H (PL), due to the absence of unsaturated bonds in the fatty acid tails which leads to oxidation of phospholipid (42,54).

2.3.1. Physicochemical properties of NB

The physicochemical properties of bilosomes such as particle size (PS), polydispersity index (PDI), zeta potential (ZP) and drug entrapment (% EE) are dependent on the proportion of S60-to-CH and the bile salt (ST) (45,55). Hence, in the present study, the influence of cholesterol and the bile salt on these parameters were investigated by varying the S60-to-CH molar ratio and ST quantity, respectively, keeping overall lipid content 250 µM. The NT content in all the NB

| Table 2.1: Composition and physicochemical properties of natamycin bilosomes (mean ± SD; n=3). |
|-----------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Component/Evaluation parameter    | NB 1            | NB 2            | NB 3            | NB 4            | NB 5            | NB 6            |
| Molar ratio (Span 60:Cholesterol) | 2:1             | 1:1             | 1:2             | 2:1             | 1:1             | 1:2             |
| Span 60 (mg)                      | 66              | 50              | 33              | 60              | 45              | 30              |
| Cholesterol (mg)                  | 30              | 44              | 59              | 27              | 41              | 54              |
| ST (mg)                           | 10              | 10              | 10              | 21              | 21              | 21              |
| PS (nm)                           | 330.1 ± 21.6    | 235.0 ± 05.2    | 287.9 ± 12.1    | 380.4 ± 27.9    | 303.3 ± 16.0    | 348.2 ± 13.1    |
| PDI                               | 0.30 ± 0.10     | 0.20 ± 0.01     | 0.23 ± 0.13     | 0.43 ± 0.23     | 0.28 ± 0.09     | 0.31 ± 0.03     |
| ZP (mv)                           | -45.4 ± 4.5     | -47.0 ± 1.5     | -51.1 ± 3.3     | -67.2 ± 2.6     | -69.0 ± 1.5     | -66.3 ± 9.1     |
| % EE                              | 78.6 ± 4.1      | 93.7 ± 4.3      | 84.1 ± 2.6      | 70.1 ± 3.6      | 81.4 ± 2.5      | 74.1 ± 3.5      |
| Drug content (%)                  | 96.7 ± 1.1      | 96.2 ± 2.0      | 96.1 ± 2.5      | 97.9 ± 4.1      | 90.1 ± 7.2      | 95.6 ± 1.2      |
| pH                                | 6.2 ± 0.9       | 6.4 ± 0.3       | 6.3 ± 0.1       | 6.5 ± 0.2       | 7.1 ± 0.2       | 6.6 ± 0.3       |

- NB and ST indicates natamycin loaded bilosomes and sodium taurocholate, respectively.
- PS, ZP, PDI and % EE represents particle size, zeta potential, polydispersity index and % entrapment efficiency respectively.
- Total 250 µM of lipid mixture (including sodium taurocholate) was used in all preparations.
- 10 mL of each formulation contains 10 mg of natamycin and 225 mg of glycerin.
formulations (pH 6.2-7.1) was in the range of 90.1 ± 7.2% to 97.9 ± 4.1% (Table 2.1). The NB formulations showed PS ranging from 235.03 ± 05.29 nm to 380.43 ± 27.94 nm with a PDI of 0.20 ± 0.01-0.43 ± 0.23, ZP of -45.41 ± 4.54 mV - -69.03 ± 1.57 mV and % EE of 70.12 ± 3.66 % – 93.75 ± 4.32 % (Table 2.1).

2.3.2.1. Effect of cholesterol

The PS and % EE of NT in bilosomes appeared to be dependent on the CH composition. It was noticed that the PS of bilosomes decreased as the CH content increased in the formulations (NB 1 to NB 2 or NB 4 to NB 5) (Table 2.1). Generally, CH interacts with the hydrophobic tails of the lipids in the vesicle bilayer and controls the packing and rigidity of the vesicles (56). Thus, the decrease in PS can be attributed to the increased interactions of CH and S60 leading to close packing of the bilayers in the bilosomes. The % EE increased proportionately with the CH concentration in the formulations NB 1 to NB 2 or NB 4 to NB 5 suggesting effective intercalation of NT within the bilayer with increased hydrophobic interactions and reduced leakage of drug from rigid bilosomes (Table 2.1) (56,57). Interestingly, further increase in the CH concentration in the formulations (NB 3 or NB 6) resulted in higher PS and lower % EE (Table 2.1). This can be attributed to the perturbation of bilayer structure by the accommodation of excess cholesterol and thereby leading to the expulsion of drug from the vesicles (55,57).

2.3.2.2. Effect of bile salt

Consistent with the earlier reports, the PS, PDI, ZP and % EE of the bilosomes were influenced by the amount of bile salt (40,45,58). With a steroid like structure, ST, a salt of taurocholic acid (bile acid), resides in the bilayer of the bilosomes imparting negative ZP due to its anionic nature (44,58). The PS, PDI and ZP of the formulations with 40 μM (21 mg) ST (NB 4 – NB 6) were higher compared to the bilosomes with 20 μM (10 mg) ST (NB 1- NB 3), at similar molar ratio of
S60 and CH (Table 2.1). These results corroborate with earlier literature reports which demonstrated the increased PS and PDI because of the repulsion between the bilayers of the vesicles due to the greater negative charge (ZP) at higher concentration of ST (59). Moreover, the accommodation of extra ST in the bilayers can increase the bulkiness of bilosomes resulting in increased particle size and the excess of amount of ST forms micelles in the dispersion medium thereby resulting in increased PDI (60,61). Strikingly, the %EE of NT in the bilosomes, with same molar ratio of S60 and CH, decreased with the increased ST quantity (Table 2.1). This drop in %EE, at higher concentration of ST, is attributed to the leakage of the entrapped drug from the bilosomes owing to the fluidization of vesicle bilayer by bile salt micelles (44).

The NB 2 formulation showed uniformly distributed bilosomes (235.0 ± 05.2 nm) in the colloidal dispersion (PDI: 0.20 ± 0.01) with negative ZP (-47.0 ± 1.5 mV), and higher % EE (93.7 ± 4.3 %). Additionally, TEM image revealed layered nanoparticles confirming the vesicular structure of the bilosomes in the NB 2 formulation (Fig. 2.1A). Hence, this formulation was optimized and used in further investigations and for the preparation of in situ gel formulations.
2.3.2. Physicochemical properties of FN

The impact of phospholipid and the TPGS on natamycin transfersomes PS, PDI, ZP and % EE were investigated by varying the PL-to-S60 molar ratio and T quantity, respectively, keeping overall lipid content 250 µM. The NT content in all the FN formulations (pH 6.1 -6.9) was in the range of 94.7 ± 7.2% to 97.9 ± 4.1% (Table 2.2).

The FN formulations showed PS ranging from 137.3 ± 3.0 nm to 192.1 ± 8.6 nm with a PDI of 0.20 ± 0.01-0.41 ± 0.1, ZP of -13.2 ± 2.6 mV - -20.9 ± 3.7 mV and % EE of 69.4 ± 2.4 % – 91.4 ± 2.7 % (Table 2.2).

It was noticed that the PS, PDI and % EE of NT in transfersomes were influenced by the molar ratio of PL:S60. The FN obtained from equimolar composition of PL and S60 (FN 2 and FN 5)

Table 2.2: Composition and physicochemical properties of natamycin transfersomes (mean ± SD; n=3).

<table>
<thead>
<tr>
<th>Component/Evaluation parameter</th>
<th>Formulation</th>
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<tbody>
<tr>
<td></td>
<td>FN 1</td>
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<tr>
<td>Molar ratio (Phospholipon 90H:Span60)</td>
<td>2:1</td>
</tr>
<tr>
<td>Phospholipon 90H (mg)</td>
<td>91</td>
</tr>
<tr>
<td>Span60 (mg)</td>
<td>52</td>
</tr>
<tr>
<td>T (mg)</td>
<td>10</td>
</tr>
<tr>
<td>PS (nm)</td>
<td>192.1 ± 8.6</td>
</tr>
<tr>
<td>PDI</td>
<td>0.33 ± 0.12</td>
</tr>
<tr>
<td>ZP (mv)</td>
<td>-14.4 ± 2.4</td>
</tr>
<tr>
<td>% EE</td>
<td>74.1 ± 4.2</td>
</tr>
<tr>
<td>Drug content (%)</td>
<td>94.7 ± 2.2</td>
</tr>
<tr>
<td>pH</td>
<td>6.3 ± 0.4</td>
</tr>
</tbody>
</table>

- FN and T indicates natamycin loaded transfersomes formulation and TPGS, respectively.
- PS, ZP, PDI and % EE represents particle size, zeta potential, polydispersity index and % entrapment efficiency respectively.
- Total 250 µM of lipid mixture was used in all preparations.
- 10 mL of each formulation contains 10 mg of natamycin and 225 mg of glycerin.
were small compared to those resulted from 2:1 and 1:2 molar proportion (Table 2.2). This could be attributed to the stronger hydrophobic interactions between the nonionic surfactant (S60) and fatty acid chains of phospholipid (PL) in the vesicle bilayer which determines the critical packing of the vesicles (39). The higher % EE of NT in transfersomes with equimolar proportion of PL and S60 (FN 2 or FN 5) suggested the effective intercalation of NT within the bilayer with increased hydrophobic interactions leading to closely packed transfersomes with no or low drug leakage (Table 2.2) (39,40). The higher PS and lower % EE of transfersomes obtained from 2:1 and 1:2 molar proportion of P and S60 could be due to either low hydrophobic interactions between vesicular components leading to poor packing of the bilayers or the perturbation of bilayer structure by the accommodation of excess of P and thereby leading to the expulsion of drug from the vesicles (39,40). Interestingly, the proportion of edge activator (T) in the structure of transfersomes influenced the PDI and % EE, but not PS and ZP, of these vesicles (Table 2.2). At any given molar proportion of PL:S60, the EE and PDI of the transfersomes increased with increase in the amount edge activator (T) (table 2.2). This higher EE could be due to the formation drug entrapped micelles by the excess amount of T that leaked into the dispersion medium from bilayer of the transfersomes and, thereby resulted in increased PDI (43,44).

The FN 2 (145.2 ± 2.9 nm) and FN 5 (137.3 ± 3.0 nm) formulations showed uniformly distributed transfersomes in the colloidal dispersion (PDI: 0.20 ± 0.01) with higher % EE ((FN 2-84.8 ± 4.8 %)- FN 5 (91.4 ± 2.7 %)). Additionally, TEM image revealed layered nanoparticles confirming the vesicular structure of the transfersomes in the FN 2 formulation (Fig. 2.1C).

2.3.3. Evaluation of NB and FN in situ gel formulations

The NB in situ hydrogel systems (NBG/NBX) were derived by introducing the NB 2 formulations into the solution comprising gellan gum/xanthum gum, anionic polysaccharide polymers, as ion
sensitive gelling agents. Similarly, the FNG formulations were prepared by incorporating FN 2 preparation into the solution with gellan gum. The physiochemical properties of bilosomes in the in situ gel formulations (NBG and NBX) were similar to NB 2 preparation, and the properties of transfersomes in the FNG formulations were no different to the FN 2 preparation, confirming no interactions of gelling agents with vesicular systems. Several literature reports have successfully demonstrated the formulation of nanoparticles or nanoemulsion loaded in situ gels using these gelling agents as they crosslink in the presence of electrolytes to form hydrogel matrix with higher pore size facilitating the movement of nanoparticles through the gel (26,35-39). The tested concentrations of gelling agents were below the limits specified in the inactive ingredients database from US FDA (62). Owing to the thickening property of gellan gum and xanthum gum, it is essential to measure the viscosity of the in situ gel preparations as it determines the free flow of the formulation from the container ensuring the ease of administration. A viscosity of 25-50 cP is most common for ophthalmic solutions (63). The formation, residence time, viscoelastic behavior and adhesive property of the hydrogels are critical parameters in deciding the pre-corneal residence and shear thinning pseudoplastic behavior for comfort in the eye while blinking (27,35,37). However, these parameters depend on the kind of gelling agent. Hence, this study focused on the influence of the two most commonly used ion sensitive gelling agents on the critical properties of in situ gel formulations.

2.3.3.1. Impact of type of gelling agent

The evaluation parameters of the bilosomes in situ gel formulations (NBG and NBX) and transfersomes in situ gel systems (FNG) are represented in the Table 2.3 and Table 2.4, respectively. The viscosity of the formulations (NBG or NBX) increased with the concentration of gelling agents confirming their thickening property (Table 2.3). At any given concentration of
gelling agent, the viscosity of the NBG formulations were lower compared to that observed in NBX formulations (Table 2.3). These results were in accordance with the previous reports explaining the higher viscosity of the xanthan gum due to the formation of helical structures via hydrogen bonding resulting in the immobilization of water (64). Additionally, the increased intermolecular accumulation of polymer chains because of the high molecular weight of xanthan gum (~ 2000K Daltons) could be the reason for high viscosity of NBX systems (65). Conversely, the lower viscosity of the gellan gum formulations (NBG) can be attributed to the formation of weak double helices associated with the van-der-Waal forces (37). The NBG formulations immediately (< 5 sec) underwent in situ phase transition and formed hydrogels while the NBX formulations slowly (≥ 20 sec) formed in situ hydrogels in the STF suggesting the slow or poor interaction of electrolytes with the xanthan gum compared to the gellan gum (37) (Table 2.3).

<table>
<thead>
<tr>
<th>Component/Evaluation parameter</th>
<th>NB 2</th>
<th>NBG 1</th>
<th>NBG 2</th>
<th>NBG 3</th>
<th>NBX 1</th>
<th>NBX 2</th>
<th>NBX 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molar ratio (Span 60:Cholesterol)</td>
<td>1:1</td>
<td>1:1</td>
<td>1:1</td>
<td>1:1</td>
<td>1:1</td>
<td>1:1</td>
<td>1:1</td>
</tr>
<tr>
<td>Span 60 (mg)</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Cholesterol (mg)</td>
<td>44</td>
<td>44</td>
<td>44</td>
<td>44</td>
<td>44</td>
<td>44</td>
<td>44</td>
</tr>
<tr>
<td>ST (mg)</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>G (mg)</td>
<td>--</td>
<td>20</td>
<td>30</td>
<td>40</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>X (mg)</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>20</td>
<td>30</td>
<td>40</td>
</tr>
<tr>
<td>Viscosity (cP) (Formulation)</td>
<td>17.1 ± 2.7</td>
<td>24.7 ± 1.5</td>
<td>37.5 ± 2.4</td>
<td>68.3 ± 3.2</td>
<td>39.5 ± 4.1</td>
<td>87.8 ± 2.5</td>
<td>124.2 ± 4.7</td>
</tr>
<tr>
<td>GT (sec)</td>
<td>--</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
<td>&gt; 20</td>
<td>&gt; 20</td>
<td>15-20</td>
</tr>
<tr>
<td>GRT (h)</td>
<td>--</td>
<td>3-4</td>
<td>6-8</td>
<td>&gt;12</td>
<td>&lt;1</td>
<td>1-2</td>
<td>5</td>
</tr>
<tr>
<td>Viscosity (cP) (hydrogel)</td>
<td>--</td>
<td>104.5 ± 1.5</td>
<td>514.9 ± 3.8</td>
<td>1285.6 ± 6.8</td>
<td>128.9 ± 6.5</td>
<td>220.3 ± 5.7</td>
<td>410.6 ± 4.2</td>
</tr>
<tr>
<td>Work of Adhesion (g. sec)</td>
<td>--</td>
<td>0.80 ± 0.01</td>
<td>1.39 ± 0.06</td>
<td>1.31 ± 0.07</td>
<td>0.68 ± 0.02</td>
<td>0.71 ± 0.01</td>
<td>1.14 ± 0.10</td>
</tr>
</tbody>
</table>

- NB, NBG, NBX, ST, GT and GRT represents natamycin loaded bilosomes, natamycin bilosome in situ gels with gellan gum, natamycin bilosome in situ gels with xanthan gum, gel formation time and intact gel residence time and sodium taurocholate, respectively.
- Total 250 µM of lipid mixture (including sodium taurocholate) was used in all preparations.
- 10 mL of each formulation contains 10 mg of natamycin and 225 mg of glycerin.
intact gel residence time and the viscosity of the hydrogels increased with the increasing proportion of the gelling agent (Table 2.3). However, at a given concentration of the gelling agent, these properties were higher for the hydrogels formed from NBG formulations compared to that from NBX preparations (Table 2.3). Similar findings have been reported in the literature where higher viscosities/stiffness of the gellan gum based in situ gels was due to the cross-linking of the polymer chain by the mono/divalent cation mediated aggregates of double helices (66). In contrast, the lower viscosity/intactness of the hydrogel formed from the NBX formulations can be attributed to the presence of the trisaccharide side chain in the branched structure of xanthan gum which avoids the cation triggered helix formation leading to low or no cross linking of the polymer (26). The adhesiveness of the in situ hydrogel is an important factor that determines its contact with the corneal or scleral surface (38). In this study, the work of adhesion of hydrogels was measured to understand their adhesive characteristics. The work of adhesion increased with respect to the concentration of gel forming agent in NBX but similar trend in NBG formulations were seen up

<table>
<thead>
<tr>
<th>Component/Evaluation parameter</th>
<th>FN 2</th>
<th>FNG 1</th>
<th>FNG 2</th>
<th>FNG 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molar ratio (Phospholipid 90H:Span60)</td>
<td>1:1</td>
<td>1:1</td>
<td>1:1</td>
<td>1:1</td>
</tr>
<tr>
<td>Phospholipid 90H (mg)</td>
<td>122</td>
<td>122</td>
<td>122</td>
<td>122</td>
</tr>
<tr>
<td>Span60 (mg)</td>
<td>36</td>
<td>36</td>
<td>36</td>
<td>36</td>
</tr>
<tr>
<td>T (mg)</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>G (mg)</td>
<td>--</td>
<td>10</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>Viscosity (cP) (Formulation)</td>
<td>16.8 ± 3.1</td>
<td>18.7 ± 1.5</td>
<td>27.5 ± 1.4</td>
<td>39.6 ± 2.6</td>
</tr>
<tr>
<td>GT (sec)</td>
<td>--</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>GRT (h)</td>
<td>--</td>
<td>&lt; 1</td>
<td>3-4</td>
<td>6-7</td>
</tr>
<tr>
<td>Viscosity (cP) (hydrogel)</td>
<td>--</td>
<td>94.1 ± 2.5</td>
<td>206.9 ± 4.1</td>
<td>651.3 ± 1.7</td>
</tr>
<tr>
<td>Work of Adhesion (g. sec)</td>
<td>--</td>
<td>0.56 ± 0.05</td>
<td>0.94 ± 0.02</td>
<td>1.33 ± 0.04</td>
</tr>
</tbody>
</table>

- FN, FNG, T, GT and GRT represents natamycin loaded transfersomes, natamycin transfersomes in situ gels with gellan gum, TPGS, gel formation time and intact gel residence time, respectively.
- Total 250 µM of lipid mixture was used in all preparations.
- 10 mL of each formulation contains 10 mg of natamycin and 225 mg of glycerin.
to the gellan gum concentration of 0.3% w/v. This might be due to the formation of stiff gel with poor shear thinning behavior, at higher proportion of gellan gum (0.4 %), which could lead to discomfort in the eye (37). At a given concentration of gelling agent, it was noticed that the work of adhesion of hydrogels from NBG formulations was high over that from NBX formulations (Table 2.3). These findings agree with the measured viscosities of the hydrogels and could be attributed to the structure related differences in cross-linking of the polymer chains of gellan and xanthan gum (37,64). These results exemplified the superior in situ gelling features of gellan gum, over xanthan gum. Thus, gellan gum based FN in situ gel systems were further studied. As observed with the NBG formulations, the FNG formulations showed increasing in situ gelling behavior (GT), intact gel residence time (GRT), rheological properties (viscosity of FNG formulations and their corresponding in situ hydrogels), and adhesive properties with increase in the composition of gellan gum (Table 2.4).

Thus, NBG 2 and FNG 3 formulations which showed acceptable viscosity (37.5 ± 2.4 cp and 39.6 ± 2.6 cp, respectively), immediate transition into a hydrogel in the STF which remained intact for > 6h with superior adhesive property were selected as suitable NB and FN in situ gel systems and investigated further. Layered nanoparticles seen in the STF with hydrogel suggested that the in situ gels system did not alter morphometric properties of bilosomes and or transfersomes (Fig. 2.1 B and C).
2.3.4. Fourier transmission infrared spectroscopy (FTIR)

The FTIR spectra of NT, NBG 2 and FNG 3 formulations and other excipients are portrayed in Figure 2.2. The existence of signature functional group stretching of NT at 3260 cm\(^{-1}\) (NH\(^{+3}\) deformation), 1713 cm\(^{-1}\) (-C=O), 1567 cm\(^{-1}\) (-CH=CH-) and 1266 cm\(^{-1}\) (-C-O-C-) in the FTIR spectra of NBG 2 and FNG 3 formulations suggested no drug-excipient chemical interactions. However, the slight shift in the IR bands of NT in NBG 2 and FNG 3 spectra could be due to the transformation in NT physical state (Fig 2.2).

2.3.5. In vitro cytotoxicity

The cytotoxicity of NB 2, FN 2, FN 5, NBG 2 and FNG 3 formulations in HCLE cells is presented in Figure 2.3. About 100.5 ± 4.5, 110.7 ± 5.6 and 93.0 ± 6.7 % cell viability was noticed for NB
2, NBG 2 and FNG 3 groups, respectively, which were not statistically different from the negative control group (saline) (Fig. 2.3). The results noted from FN 2 and FN 5 formulations were significantly different from that observed in negative control group (saline). The positive control group treated with triton X 100 (1 %), a nonionic surfactant, showed 6.9 ± 2.5 % cell viability (Fig. 2.3). Hence, the drop in % cell viability of the groups exposed to FN formulations (FN 2 and FN 5) could be related with the overall nonionic surfactant (NS) composition (T and S60) in formulations. The group treated with FN 2 (0.45 % w/v of NS) showed considerably higher % cell viability (65.7 ± 4.8%) compared to FN 5 (0.86 %w/v of NS) (17.7 ± 5.4%) (Fig. 2.3). Hence, it is presumed that the higher NS concentration might have attributed to lower % cell viability. However, no reports in the literature has demonstrated the corneal toxicity of the combination of S60 and T. The higher % cell viability of FNG 3 group suggested that the in situ gels of FN 2 formulations are well tolerated by corneal epithelial cells (Fig. 2.3). Hence, the cytotoxicity results of NB 2 and NBG 2 formulations suggest that the optimized bilosomes and its in situ gel

Fig. 2.3: Cytotoxicity profile of NB 2, FN 2, FN 5, NBG 2 and FNG 3 formulations in HCLE cells (mean ± SEM; n=6).
formulations are well tolerated by corneal epithelial cells and can be explored as a suitable vehicle for topical ocular drug delivery. Similar literature reports discussed no ocular cytotoxicity with the vesicular systems containing nonionic surfactants (S60) and/or bile salts (44,55). In addition, a study outlined no corneal cytotoxicity with the liposomes containing the sodium taurocholate, whereas formulations containing sodium deoxycholate were toxic to HCE cells (58). In the cytotoxicity studies, the HCLE cells were exposed to the formulations for 24h. But, most of the topical ophthalmic formulations will be in contact with cornea for short time due to rapid clearance from the precorneal space. Thus, to further understand the toxic effects of bilosomes and transfersomes on cornea anatomy, histology studies were performed on the corneas exposed to the formulations in corneal transport studies.

### 2.3.6. Corneal transport studies

The saturation solubility of NT was in the following order: DPBS (2.5 % w/v RMβCD) > DPBS ≥ IPBS > water. Hence, to maintain sink, DPBS (2.5 % w/v RMβCD) was utilized as the release
medium in the *in vitro* corneal transport studies. The transcorneal permeability parameters of NT from control, NB 2, FN 2, FN 5, NBG 2 and FNG 3 formulations are detailed in Figure 2.4. A significant improvement in the transcorneal flux of drug was observed from NB 2, FN 2, FN 5, NBG 2 and FNG 3 over that from control suspension (p<0.05) (Fig. 2.4A). An ER of 9.01 and 6.37 transcorneal flux of NT from NB 2/FN 2 and NBG 2/FNG 3 formulations, respectively, indicated their potential to improve the corneal transport of NT. Further, significantly higher apparent permeability coefficient ($P_{\text{app}}$) of NT from NB 2, FN 2, NBG 2 and FNG 3, compared to control, could be attributed to the improved permeability of these advanced vesicles (p<0.05) (Fig. 2.4B)). These findings verify the previous literature reports, which described the penetration of bilosomes or transfersomes through intact cornea owing to the fluidization of corneal lipids (44,58,42,43). In addition, the superior flexibility of these vesicles with bile salt (ST) and edge activator (T) enables them to permeate across the intact biological membranes (40-43). The lower transcorneal flux and $P_{\text{app}}$ of NT from NBG 2, compared to NB 2, were consistent with the earlier reports which suggested the slower release of drug loaded nanoparticles or vesicles from the *in situ* hydrogel matrix (p<0.05) (Fig. 2.4) (36,37).

Interestingly, an ER of ~16 fold transcorneal flux of NT from the FN 5 formulation was seen with apparent permeability coefficient ($P_{\text{app}}$) $3.9 \times 10^{-5}$ Cm/s (Fig. 2.4). These results were significantly superior over those observed from any other formulations (p<0.05). A sudden increase in the % cumulative drug release, from FN 5 formulation, was noticed after 1h timepoint (data not shown). These findings suggest that there might be a damage to the corneal structure.

### 2.3.7. Corneal histology

Corneal histology studies were carried out to access the corneal tissue architecture after the treatment with NB 2, FN 2, FN 5, NBG 2 and FNG 3 formulations. The histology of corneas
exposed to DPBS, marketed suspension, NB 2 FN 2, FN 5, NBG 2 and FNG 3 in the *in vitro* transcorneal permeation studies are portrayed in Figure 2.5. Normal anatomy of an intact cornea represents epithelial layer (EP) and stroma layer (SL) separated by Bowman’s membrane (BM), and descemet’s membrane that separates the stroma from endothelial layer. Any observations like tear or loss, degeneration or disappearance of epithelial layer, separation of Bowman’s membrane from epithelial and stroma layers, vacuoles in the stroma layer confirms the damage of corneal structure. In this study, the corneas exposed to NB 2, FN 2, NBG 2 and FNG 3 formulations did not show any such signs, suggesting no alterations in the corneal tissue architecture during the *in vitro* transcorneal permeability studies (Fig. 2.5). Moreover, the corneas exposed to NB 2, FN 2, NBG 2 and FNG 3 formulations were not structurally different from those exposed to control marketed suspension and DPBS (Fig. 2.5) suggesting the corneal compatibility of these advanced
vesicular systems and their corresponding ion sensitive in situ gel formulations. Similar findings were reported in the literature which indicated safety of ST and/or nonionic surfactant based vesicles to ocular sites (37,55,58,42,43).

The loss of epithelial layer on the cornea exposed to FN 5 formulation confirmed that the transfersomes, prepared with 0.8 % w/v of NS (T and S60), is toxic to the corneal structure and, hence, showed superior transcorneal permeability characteristics (Fig. 2.5F). These results corroborate with the observations in the in vitro cytotoxicity studies (Fig. 2.3). Hence, NB 2, FN 2, NBG 2 and FNG 3 formulations were selected for the in vivo performance evaluation.

2.3.8. Ocular disposition studies
Since the performance of pharmaceutical product depends on the drug and other excipients in it, undiluted Natacyn suspension (5 % w/v natamycin) was used, to exclude the underestimation of the product efficacy, as a control formulation in the ocular distribution studies. The amount of drug observed in the ocular tissues of the rabbits at the end of 2h and 6h post treatment with control, NB 2, FN 2, NBG 2 and FNG 3 formulations are given in table 2.5. To understand the permeability and prolong delivery of NT from control, NB 2, FN 2, NBG 2 and FNG 3 formulations, the mean dose normalized drug levels in the rabbit ocular tissues were calculated and presented in Figure 2.6.

At the end of 2h, the concentration of NT observed in the cornea and AH of rabbits administered with control at 50-fold high dose were disproportionately higher by ~13-18 and ~3-6-fold, respectively, compared to that treated with NB 2, FN 2, NBG 2 and FNG 3 formulations (Table 2.5). Hence, the mean dose normalized NT concentration in cornea and AH of rabbits treated with NB 2, FN 2, NBG 2 and FNG 3 formulations were significantly higher, over that seen in the control group, indicating the improved penetration of
Table 2.5: The amount of natamycin in the ocular tissues of rabbits at the end of 2h and 6h post-topical administration with control, NB 2, FN 2, NBG 2 and FNG 2 formulations (mean ± SEM; n=4).

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Dose (µg)</th>
<th>Time point (h)</th>
<th>NT levels in ocular tissues (ng/g or ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>Cornea</td>
</tr>
<tr>
<td>Control</td>
<td>2500</td>
<td></td>
<td>4390.4 ± 894.2</td>
</tr>
<tr>
<td>NB 2</td>
<td>50</td>
<td></td>
<td>333.6 ± 59.4</td>
</tr>
<tr>
<td>FN 2</td>
<td>50</td>
<td>2</td>
<td>539.8 ± 106.0</td>
</tr>
<tr>
<td>NBG 2</td>
<td>50</td>
<td>2</td>
<td>238.1 ± 10.2</td>
</tr>
<tr>
<td>FNG 2</td>
<td>50</td>
<td>2</td>
<td>376.3 ± 35.2</td>
</tr>
<tr>
<td>Control</td>
<td>2500</td>
<td>6</td>
<td>290.1 ± 56.9</td>
</tr>
<tr>
<td>NB 2</td>
<td>50</td>
<td>6</td>
<td>ND</td>
</tr>
<tr>
<td>FN 2</td>
<td>50</td>
<td>6</td>
<td>ND</td>
</tr>
<tr>
<td>NBG 2</td>
<td>50</td>
<td>6</td>
<td>212.3 ± 44.2</td>
</tr>
<tr>
<td>FNG 2</td>
<td>50</td>
<td>6</td>
<td>243.6 ± 22.6</td>
</tr>
</tbody>
</table>

- AH, IC, RC and VH indicates aqueous humor, iris ciliary body, retina choroid and vitreous humor, respectively.
- NT, NB, NBG, FN and FNG- represents natamycin, natamycin bilosomes, natamycin bilosome in situ gel formulation, natamycin transfersomes and natamycin transfersomes in situ gel formulation, respectively.
Fig. 2.6: Mean dose normalized natamycin levels in rabbit ocular tissues from control, optimized natamycin bilosomes (NB 2), bilosomes in situ gel (NBG 2), transfersomes (FN 2) and transfersomes in situ gel formulations, at A) 2h and B) 6h time point (mean ± SEM; n=4). *- significantly different at p<0.05. ND-not detected. *-significantly different from control (p<0.05); ¶- significantly different from NBG 2 group (p<0.05); ǂ-significantly different from corresponding in situ gel formulation group (p<0.05); ¥-significantly from any formulations group (p<0.05).
NB’s and FN’s across the cornea (p<0.05) (Fig. 2.6A). These results support the findings from corneal transport studies and could be attributed to the intercalation of bilosomes or transfersomes with corneal lipids, and their amphiphilic nature, due to the presence of bile salt (ST) or edge activator (T), driving them to hydrophilic AH (37,67). In contrast, the lipophilic character might have led the drug, in the cul-de-sac from control suspension (higher amount), to partition into corneal epithelial cells resulting in high corneal drug levels, however, the poor aqueous solubility could have limited the penetration through hydrophilic stroma layer in cornea leading to low dose normalized AH drug concentration. Unlike seen in the cornea, sclera is 10-fold more permeable to hydrophilic drugs, but it still is impervious to lipophilic molecules (68,69). Thus, the actual amount of NT in rabbit sclera from control group treated with 50-fold high drug dose was disproportionately higher by ~6-7 and ~10-12 fold higher over that noted in NB 2, FN 2, NBG 2 and FNG 3 group, respectively, confirming the limited scleral permeation of NT from marketed suspension (Table 2.5). The significantly higher mean dose normalized NT level in sclera of rabbits treated with NB 2, FN 2, NBG 2 and FNG 3 formulations, compared to that noticed in the control group, exemplifies the ability of bilosomes and transfersomes as the potential vehicles to reduce the scleral barrier characteristics (p<0.05) (Fig. 2.6A). The amount of NT in the IC of rabbits from all the treatment groups could be attributed to the permeation of drug into the ciliary body through cornea-scleral limbus junction (29,30) (Table 2.5). The mean dose normalized IC NT levels were significantly higher in the rabbits treated with NB 2, FN 2, NBG 2 and FNG 3 formulations, compared to that treated with control suspension, which could be attributed to the transcytosis/endocytosis of these advanced vesicles, through corneal/scleral epithelial cells into the IC (p<0.05) (Fig. 2.6A) (37,58). As expected, the mean dose normalized drug levels in cornea, AH, IC and sclera of rabbits administered with NB 2 and FN 2 formulation were significantly
higher compared to that treated with their corresponding in situ gel systems which could be due to the slow release of bilosomes and/or transfersomes from the in situ hydrogel matrix (44,47) (p<0.05) (Fig. 2.6A). The drug levels in the RC and the VH of rabbits from all the treatment groups, except FN 2 group, were below the detection limit (Table 2.5 & Fig. 2.6). Interestingly, the mean dose normalized drug levels in IC, sclera and RC of the rabbits treated with FN 2 formulation were significantly different from that observed in the rabbits in any other groups which could be attributed to the flexibility of the transfersomes, imparted by the edge activator (TPGS) that helps distribute them into scleral surface and thereby, diffuse into RC (42,43).

At the end of 6h, no detectable drug levels were noticed in any of the ocular tissues, except sclera, in the rabbits treated with NB 2 and FN 2 indicating the clearance of the formulation from the precorneal space (Table 2.5 & Fig. 2.6B). Surprisingly, the cornea, the IC and the sclera of rabbits from the control group showed some drug levels that could be attributed to depo formation of drug in the cornea and sclera (Fig. 2.6B). The undetectable drug levels in the AH, the RC and the VH in these rabbits, however, confirmed no or low drug in the cul-de-sac and poor transcorneal/transscleral penetration of NT (Fig. 2.6B). These results agree with a study which reported poor NT levels (< 0.8 µg/mL) in the rabbit tear at 5h post topical administration of marketed suspension (47). In contrast, significantly higher mean dose normalized drug levels in all the ocular tissues, except VH, in the rabbits topically administered with NBG 2 and FNG 3 formulations confirmed improved pre-corneal residence time of the in situ hydrogel of NT bilosomes and transfersomes (Fig. 2.6B). As reported earlier, in situ hydrogels bond with glycoprotein moieties of the epithelial cells through hydrophobic interactions/van der Waals forces/ionic/hydrogen bonding and facilitates higher mucoadhesion with the biological membranes ensuring longer residence time (70). These results confirmed the ability of ion-
sensitive *in situ* hydrogels of these advanced vesicles, bilosomes and transfersomes, as potential platforms for improved and prolong ocular drug delivery.

### 2.3.9. Stability Studies

Table 2.6: Physical stability of optimized natamycin bilosomes and bilosome *in situ* gel formulations stored at 4°C and 25°C for 60 days (mean ± SD; n=3).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Stored at</th>
<th>Time (days)</th>
<th>PS (nm)</th>
<th>ZP (mv)</th>
<th>PDI</th>
<th>% EE</th>
<th>Drug content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NB 2</strong></td>
<td>Initial</td>
<td></td>
<td>235.0 ± 5.3</td>
<td>-47.0 ± 1.6</td>
<td>0.20 ± 0.01</td>
<td>93.8 ± 4.3</td>
<td>99.2 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>4°C</td>
<td>30</td>
<td>239.3 ± 4.4</td>
<td>-44.6 ± 3.9</td>
<td>0.21 ± 0.01</td>
<td>91.5 ± 1.2</td>
<td>98.4 ± 1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>243.6 ± 5.9</td>
<td>-45.7 ± 2.0</td>
<td>0.21 ± 0.01</td>
<td>91.4 ± 4.6</td>
<td>98.5 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>25°C</td>
<td>30</td>
<td>240.7 ± 3.7</td>
<td>-44.6 ± 3.2</td>
<td>0.21 ± 0.02</td>
<td>92.4 ± 2.2</td>
<td>98.4 ± 1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>264.2 ± 10.9*</td>
<td>-44.2 ± 4.7</td>
<td>0.28 ± 0.04*</td>
<td>81.9 ± 1.9*</td>
<td>97.9 ± 2.0</td>
</tr>
<tr>
<td><strong>NBG 2</strong></td>
<td>Initial</td>
<td></td>
<td>234.3 ± 6.1</td>
<td>-48.0 ± 1.5</td>
<td>0.22 ± 0.07</td>
<td>90.5 ± 1.2</td>
<td>98.9 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>4°C</td>
<td>30</td>
<td>236.7 ± 6.9</td>
<td>-46.8 ± 0.4</td>
<td>0.21 ± 0.02</td>
<td>90.5 ± 0.3</td>
<td>98.4 ± 1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>244.4 ± 8.4</td>
<td>-46.7 ± 0.9</td>
<td>0.22 ± 0.01</td>
<td>90.4 ± 1.2</td>
<td>98.3 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>25°C</td>
<td>30</td>
<td>240.4 ± 3.4</td>
<td>-47.5 ± 4.2</td>
<td>0.22 ± 0.01</td>
<td>89.7 ± 2.3</td>
<td>98.0 ± 1.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>245.4 ± 10.4</td>
<td>-46.7 ± 6.2</td>
<td>0.29 ± 0.16</td>
<td>81.9 ± 1.2</td>
<td>97.9 ± 0.5</td>
</tr>
</tbody>
</table>

- NB, NBG, PS, ZP, PDI and % EE represents natamycin bilosomes, natamycin bilosome *in situ* gel formulation, particle size, zeta potential, polydispersity index and % entrapment efficiency respectively.

- *- indicates significant difference (p<0.05) between the NB 2 or NBG 2 at 25°C for 60 days and fresh NB 2 or NBG 2 at initial conditions (day 0 at room temperature).

The physical stability of the optimized formulations at 4°C and 25°C are specified in Table 2.6 and Table 2.7. The PS, PDI, ZP and % EE of NB 2 and FN 2 formulations stored at 25°C were not statistically different for 30 days but changed significantly by 60 days, with respect to those observed initially (p<0.05) (Table 2.6 and Table 2.7). This could be due to the aggregation of these vesicles with the leakage of drug. Surprisingly, the physicochemical properties of the NBG 2 and
FNG 3 formulations did not differ upon storage at 25°C for 60 days which could be attributed to the viscosity limited migration and aggregation of bilosomes and/or transfersomes (Table 2.6 and Table 2.7). No variations in the PS, PDI, ZP and % EE of all the optimized formulations stored at 4°C for 60 days suggests that these preparations can be stored at refrigerated conditions (4°C) (Table 2.6 and Table 2.7).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Stored at</th>
<th>Time (days)</th>
<th>PS (nm)</th>
<th>ZP (mv)</th>
<th>PDI</th>
<th>% EE</th>
<th>Drug content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FN 2</td>
<td>Initial</td>
<td></td>
<td>145.3 ± 2.9</td>
<td>-20.0 ± 1.5</td>
<td>0.20 ± 0.01</td>
<td>84.8 ± 4.8</td>
<td>95.2 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>4°C</td>
<td>30</td>
<td>146.2 ± 3.4</td>
<td>-19.4 ± 3.9</td>
<td>0.23 ± 0.02</td>
<td>82.4 ± 1.6</td>
<td>97.3 ± 2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>148.4 ± 4.2</td>
<td>-18.6 ± 1.3</td>
<td>0.22 ± 0.02</td>
<td>80.4 ± 3.6</td>
<td>94.5 ± 3.3</td>
</tr>
<tr>
<td></td>
<td>25°C</td>
<td>30</td>
<td>149.0 ± 3.7</td>
<td>-16.4 ± 2.4</td>
<td>0.25 ± 0.01</td>
<td>83.1 ± 3.2</td>
<td>96.4 ± 2.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>164.5 ± 10.9*</td>
<td>-12.2 ± 1.7*</td>
<td>0.38 ± 0.02*</td>
<td>81.9 ± 1.9*</td>
<td>97.9 ± 2.0</td>
</tr>
<tr>
<td>FNG 3</td>
<td>Initial</td>
<td></td>
<td>148.9 ± 2.1</td>
<td>-18.1 ± 3.5</td>
<td>0.22 ± 0.01</td>
<td>87.5 ± 3.2</td>
<td>98.9 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>4°C</td>
<td>30</td>
<td>149.7 ± 2.3</td>
<td>-17.8 ± 1.4</td>
<td>0.21 ± 0.01</td>
<td>86.5 ± 1.3</td>
<td>97.1 ± 2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>150.2 ± 4.4</td>
<td>-16.7 ± 2.4</td>
<td>0.27 ± 0.07</td>
<td>86.4 ± 2.2</td>
<td>98.1 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>5°C</td>
<td>30</td>
<td>151.4 ± 4.1</td>
<td>-17.5 ± 2.2</td>
<td>0.25 ± 0.01</td>
<td>84.3 ± 3.7</td>
<td>96.2 ± 3.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>157.2 ± 9.3</td>
<td>-16.7 ± 6.2</td>
<td>0.29 ± 0.11</td>
<td>82.9 ± 4.2</td>
<td>98.1 ± 6.5</td>
</tr>
</tbody>
</table>

- FN, FNG, PS, ZP, PDI and % EE represents natamycin transfersomes, natamycin transfersomes in situ gel formulation, particle size, zeta potential, polydispersity index and % entrapment efficiency respectively.
- * indicates significant difference (p<0.05) between the FN 2 or FNG 3 at 25°C for 60 days and fresh FN 2 or FNG 3 at initial conditions (day 0 at room temperature).

2.4. Conclusion

NT bilosomes (NB), corresponding in situ gel systems, NT transfersomes and corresponding in situ gel systems were successfully fabricated and evaluated. The NB and FN formulation with equimolar ratio of vesicular components and low amount of bile salt (ST) or edge activator (T) resulted in uniformly distributed small size vesicles with higher drug % EE. The optimized NB
and FN loaded \textit{in situ} gel (NBG and FNG) systems with 0.3 \% w/v gellan gum rapidly transformed into hydrogel in the STF, which displayed good viscoelastic and adhesive properties. The cytotoxicity and corneal histology evaluations confirmed compatibility and safety of the bilosomes formulations on ocular tissues. These studies also revealed that the transfersomes prepared with 0.45 \% w/v nonionic surfactants (NS) were safe and compatible for ocular drug delivery but, the transfersomes obtained with 0.8 \% w/v NC induced corneal toxicity causing damage to the structure of the cornea. The \textit{in vitro} corneal transport studies confirmed the superior permeability characteristics of NT from the optimized bilosomes and transfersomes (0.45\% w/v NS) formulations, over control suspension. Furthermore, considerably higher mean dose normalized drug levels in the ocular tissues from NBG and FNG formulations for 6h, compared to that from the control suspension, demonstrated the effectiveness of ion-sensitive \textit{in situ} hydrogels of bilosomes and transfersomes as the potential carrier systems for the improved and prolonged topical ocular drug delivery. Nevertheless, the ocular distribution studies of NT from NBs and FNs with higher drug loads is necessary to confirm the efficiency of these systems as suitable vehicle for enhanced ocular drug delivery platforms.
CHAPTER 3
EFFLUX PROTEINS ON THE BLOOD OCULAR BARRIERS REGULATE OCULAR DELIVERY OF CHEMOTHERAPEUTIC AGENTS: IMPACT OF SPECIFIC INHIBITORS

3.1. Introduction

The delivery of therapeutics into the deeper ocular tissues via the systemic route remains a challenge due to the presence of complex blood ocular barriers (BOB). The BOB comprises of the blood-aqueous barrier (BAB), formed by the epithelial cells of the iris-ciliary bodies, and the blood-retinal barrier (BRB), made up of the endothelial cells of the retinal blood vessels and the retinal pigmented epithelium (RPE) (3,4,71-73). The tight-junction proteins and the efflux proteins present in the BAB and BRB protect the anterior and posterior segment ocular tissues by preventing the paracellular and/or transcellular transportation of endobiotics and xenobiotics (3,4,74,75).

In the recent past, numerous researchers have investigated the impact of efflux transporters on the permeability of hydrophobic anticancer and other drugs across various blood-organ barriers (71-73). In this regard, the most common efflux proteins are P-glycoprotein (P-gp) and multidrug resistance protein (Mrp), major efflux proteins belonging to the ATP-binding cassette (ABC) transporters sub-family. These proteins are widely expressed in many vital organs such as the brain, liver, lung and kidney, protecting them through the efflux of chemical and biological toxic substances. The broad substrate specificity of these efflux proteins leads to a severe reduction in the blood to organ penetration of chemotherapeutic agents (76-79).
Several investigators have reported the expression of P-gp and Mrp1 or Mrp2 on the ciliary epithelial cells in the BAB, and on the endothelial cells of the retinal blood vessels and the RPE in the BRB, in humans and in various animal models (80-84). However, the specific impact of P-gp/Mrp1 in the blood-organ penetration of lipophilic drugs remains uncertain due to the presence of multiple influx and efflux transporters in the blood-organ membrane barriers (80,85). The co-existence of several influx transporters, like organic cation/carnitine transporters (Oct), organic anionic transporters (Oat), organic anionic transporter polypeptides (Oatp) and multidrug resistance associated efflux proteins in the BOB confounds delineation of the contribution of P-gp and Mrp1 in hindering the blood-ocular transport of drugs (85-87).

The application of multidrug resistance Mdr1a gene, responsible for P-gp expression, and Mrp1 gene knock-out (KO) mice, and recently rat, models have gained importance in delineating the P-gp and/or Mrp1 mediated efflux, and its effect on pharmacokinetics of substrates drugs (88,89). Numerous reports have discussed the use of transgenic mice models lacking the Mdr1a gene (P-gp KO) or Mrp1 gene as a powerful tool in unlocking the complexity and the role of efflux proteins on the penetration of anticancer drugs across the blood brain barrier (BBB) (88,89). The utilization of Mdr1a gene and Mrp1 gene KO mice and/or rats, to determine the impact of efflux proteins in the BOB, however, has remained largely unexplored.

Several investigations reported the possibility of inhibiting P-gp and Mrp1 activity to enhance penetration of substrate molecules into the target organs (90,91). Drugs like verapamil, quinidine, cyclosporine etc., emerged as the first-generation P-gp inhibitors, modulating efflux activity at the blood-organ interface (92,93). Nonetheless, the toxic and antagonistic effects associated with their pharmacological activity and their ability to modulate the kinetics of substrate drugs, limited their use as specific inhibitors (94,95). Interestingly, the second-generation P-gp
inhibitors with diminished pharmacological activities were more potent and compatible in reducing the barrier characteristics (90,95,96). The third-generation P-gp inhibitors, like elacridar (EQ) and tariquidar (TQ), were developed with no pharmacological activity and with higher affinity to P-gp (at nanomolar concentrations) (90,97,98). EQ and TQ alleviate breast cancer resistance protein (BCRP) mediated efflux transport of the substrate molecules (97,98). Both EQ and TQ have been extensively explored for modulating the P-gp transporter at the blood brain barrier (BBB) thereby, improving the blood to brain penetration of substrate anticancer molecules (76,97-99). Similarly, MK 571 (MK) and probenecid (PB) were exclusively investigated as potent Mrp1 inhibitors (100,101). Beside modulating the Mrp1 activity, PB also inhibits the activity of several other transporters such as Mrp 2-5, Oat, Oct, Oatp etc. (102).

Inhibition of P-gp, but not Mrp1 efflux protein, in the BOB has been studied by several investigators. The effect of either EQ or TQ on the BRB has been investigated on humans, rabbits and in vitro cell models, but not in rats (103-105). Previously, the contribution of topically administered first-generation P-gp inhibitors in improving the blood-ocular penetration of systemically and intravitreally administered substrates has been successfully investigated in New Zealand albino rabbits (106,107). However, the effectiveness of the third-generation P-gp inhibitors (EQ and TQ) and Mrp1 inhibitors (MK and PR) in regulating the activity of the respective efflux proteins in the BOB in Sprague-Dawley (SD) rats, has not been investigated till date.

In view of this, the current study, for the first time, aimed to examine 1) the impact of P-gp and Mrp1 barrier properties by comparing the transport of substrate anticancer drugs, paclitaxel (PTX) and methotrexate (MTX), across the BOB of Mdr1a and Mrp1 KO and wild type (WT) rats and, 2) the impact of third-generation P-gp inhibitors and Mrp1 inhibitors on the blood-ocular
transport of these substrate anticancer drugs. Earlier literature reports demonstrated that the blood-brain transport of PTX was restricted by P-gp and that the transmembrane penetration of MTX was influenced by Mrp1 activity (88,89,108). Hence, these substrate anticancer drugs were selected for the present study. Electroretinography (ERG) studies were undertaken to evaluate the effect of the substrate/inhibitors on the functional activity of the retinal cells.

3.2. Materials and Methods

3.2.1. Animals.
Six to eight-week-old male WT, Mdr1a gene KO and Mrp1 gene KO Sprague-Dawley (SD) rats were purchased from Horizon Discovery (Saint Louis, MO). The animals had free access to food and water. All animal experiments were performed according to the approved protocols of the University of Mississippi Institutional Animal Care and Use committee, the University of Tennessee Health Science Center Institutional Animal Care and Use committee and the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research.

3.2.2. Chemical Reagents
PTX, MTX, Cremophor EL ® and glycofurol was procured from Sigma Aldrich (St. Louis, MO). EQ, TQ, MK and PR were acquired from Alfa Aesar (Haverhill, MA). Propylene glycol (PG) (1,2-Propanediol), Polyethylene glycol 400 (PEG-400), Polyethylene glycol 300 (PEG-300), Dimethyl Sulfoxide (DMSO) were purchased from Fisher Scientific (St. Louis, MO). All other high purity chemicals and HPLC grade solvents were also obtained from Fisher Scientific (St. Louis, MO).

3.2.3. P-gp protein expression by western blotting
Western blot was performed to understand the expression of P-gp in the BAB and BRB of the WT and P-gp KO SD rats. The WT and P-gp KO rats were euthanized by injecting an excess dose of
ketamine and xylazine combination through the I.P. route (n=6). The eyes were enucleated immediately and the ocular tissue (IC and RC) samples were collected. The tissues were harvested, and tissue lysates were prepared using 2X lysis buffer (Cell Signaling Technology, USA). Protein concentrations in the tissue lysates were estimated by Bradford assay (Bio-Rad USA). Equal amounts (20 µg) of proteins, from the various tissue lysates, were loaded into the wells of 10% SDS-PAGE mini-gels (Bio-Rad, USA), electrophoresed and the proteins were then transferred to nylon membranes (Cell Signaling Technology, USA) as per the western blot protocol. Membranes were then blocked in 5% non-fat dried milk in 1X TBST [20 mM Tris–HCl (pH 7.5), 137 mM NaCl and 0.1% (v/v) Tween 20] for 1h at room temperature. Blots were probed overnight with primary antibody MDR1/ABCB1 (Cat # 13978 Cell Signaling Technology) following the manufacturers protocol. The membranes were washed with 1X TBST and probed with anti-rabbit secondary antibody conjugated to HRP (Cat # 7074 Cell Signaling Technology) for 1 hr. For equal loading, samples were probed with beta actin antibody (Cell Signaling Technology, USA). The blots were developed with signal flame enhanced chemiluminescence (ECL) TM kit (Cell Signaling Technology, USA). The images were captured, processed and analyzed on ChemiDoCTM MP imaging system (Bio-Rad, USA). ImageJ was used to compute the area of the protein band in the blot. Further, relative P-gp protein intensity was calculated from the ratio of P-gp band area and the corresponding β-actin band area.

3.2.4. Preparation and evaluation of substrate and inhibitor intravenous solutions

PTX intravenous solution (3 mg/mL) was prepared by dissolving an appropriate quantity in a mixture of ethanol (30 %v/v) and cremophor EL (30 % v/v) and final volume was adjusted with saline (40 % v/v). MTX solution (8 mg/mL) was obtained by dissolving accurately weighed amount drug in 0.1N NAOH (20 % v/v), pH adjusted to 7.0 with 0.1N HCl (5 % v/v) and final
volume was made up with water for injection (75 % v/v). EQ solution (10 mg/mL), for intravenous administration, was obtained by dissolving an accurately weighed amount of EQ in DMSO (5 % v/v) and then the final volume was made up with glycofurol (5% v/v), PEG 300 (20% v/v), ethanol (30% v/v) and saline (40 % v/v). A clear solution of 2.5 mg/mL of TQ was prepared by dissolving an accurately weighed quantity of TQ in DMSO (5% v/v), glycofurol (10% v/v) PEG 400 (30% v/v), propylene glycol (25% v/v), and the required volume adjusted by the addition of saline. 2.5 mg/mL of MK intravenous solution was prepared by dissolving accurately weighed amount in DMSO (5 % v/v), glycofurol (10% v/v), ethanol (20 % v/v), PG (30 % v/v) and final volume was made up with saline (35 % v/v). PB intravenous solution (25 mg/mL) was formulated by dissolving appropriate amount of PB in DMSO (5 % v/v), 1N NAOH (20% v/v), ethanol (10 % v/v), PG (25 % v/v) and saline (40% v/v) was added to make up to the required volume. All these intravenous solutions were tested for pH, assay, precipitation in phosphate buffered saline (pH 7.4), stability at room temperature and for loss during filtration across 0.22µm nylon filter (Millipore, USA).

3.2.5. Electroretinography

The evaluation of substrate (PTX or MTX) or inhibitor (EQ/TQ/MK/PR) incompatibility with the retinal tissues was investigated by employing electroretinography (ERG) recordings. The analysis was performed on WT (n=33), P-gp KO (n=12) and Mrp1 KO (n=6) SD rats from the same cohort utilized in ocular distribution studies, as per the standard protocols.

Substrates only: WT and P-gp KO rats, 12 of each, were intravenously administered 1 mg/kg or 3 mg/kg of PTX and the ERG recording was collected before administration as well as at the end of 2h and 24h (n=3 of each WT and P-gp KO rats/dose/time point) post-drug administration. Three of each WT and Mrp1 KO rats were administered 8 mg/kg of MTX while another three of each WT and Mrp1 KO rats were administered 4 mg/kg of MTX. The ERG recording was collected.
before the administration of MTX as well as at the end of 2 h, post-drug administration. Since, significant changes in ERG patterns were noticed at end of 2h post 8 mg/kg dose treatment, suggesting the retinal toxicity, no ERG measurements were recorded for 24h.

**Inhibitors only**: Fifteen WT rats were divided into 5 groups with 3 rats in each group (n=3). Each study group received only one treatment of intravenous EQ (15 mg/kg) or TQ (15 mg/kg) or TQ (5 mg/kg) or MK (5 mg/kg) or PR (25 mg/kg). The ERG recordings were measured before and 2h after inhibitor administration.

Briefly, dark-adapted rats (overnight prior to commencement) were anesthetized with an I.P. injection of ketamine and xylazine combination. Gold ring electrodes of 2-3 mm diameter (Roland Consult, Stasche & Finger GmbH), were positioned on both corneas after anesthetizing them with topical 0.5% proparacaine hydrochloride followed by topical application of 1% tropicamide for pupil dilation. The stainless-steel surgical needles were inserted subcutaneously into the forehead and back leg to serve as reference and ground electrodes respectively. The dark-adapted ERG responses were recorded from both the eyes simultaneously, exposing them to flashes of various intensities (from −4.0 Log units to 2.88 Log units) delivered by a Ganzfeld light source. The amplitudes of a- wave (relevant to photoreceptor functioning by light accommodation) and b-wave (relevant to bipolar and/or Müller cell functioning) of WT, P-gp KO and Mrp1KO rats obtained before dosing were considered as baseline to compare and find the differences in the amplitudes of a- and b- waves recorded by the end of 2 h and/or 1 day after dosing. The deviations in the intensities of the amplitudes were reported as percentage changes of amplitude.

**3.2.6. Ocular distribution**

The first objective was to estimate the impact of P-gp and Mrp1 on the ocular penetration of PTX and MTX, respectively. Ocular distribution studies were thus undertaken in WT, P-gp KO and
Mrp1 KO rats. Since there was no indication in the literature that the expression of the transporters on the ocular tissues was gender dependent, male SD rats only were selected for the study. A total of 96 rats were used in these studies (48 WT, 24 P-gp KO, and 24 Mrp1 KO). Twelve of the WT and 12 of the P-gp KO rats were administered 1 mg/kg PTX, while the other 24 (12 WT and 12 P-gp KO) were injected 3 mg/kg dose, through the tail vein. Similarly, twelve of each WT and Mrp1 KO rats were administered 4 mg/kg MTX, while the other 24 (12 WT and 12 Mrp1 KO) were injected 8 mg/kg dose, through the tail vein. At the end of 60 and 120 min, post dosing, WT and P-gp KO or Mrp1 KO rats from each group (n=6 at each time point) were anesthetized with an intraperitoneal (I.P.) administration of a combination of ketamine (35 mg/kg) and xylazine (3.5 mg/kg). Blood was collected via cardiac puncture. The animals were then euthanized using an excess combination dose of ketamine and xylazine through the I.P. route and the eyes were enucleated immediately. Aqueous humor (AH) and vitreous humor (VH) were collected from both eyes of each rat and was pooled. The blood samples collected were immediately centrifuged at 10,000 rpm for 5 min and the plasma was separated. Plasma, AH and VH samples were stored at -80 °C until analysis.

The second objective was to estimate the ability of P-gp inhibitors (EQ and TQ) and Mrp1 inhibitors (MK and PR) to modify the P-gp and Mrp1 activity in the BOB, as evidenced by changes in the blood-ocular penetration of PTX and MTX. Ocular distribution studies were carried out in WT SD rats. The rats were placed in 12 treatment groups (n=6), so that each group receive only one treatment: PTX (1 mg/kg) + EQ (5 mg/kg); PTX (1 mg/kg) + EQ (15 mg/kg); PTX (1 mg/kg) + TQ (2.5 mg/kg); PTX (1 mg/kg) + TQ (5 mg/kg); PTX (3 mg/kg) + EQ (5 mg/kg); PTX (3 mg/kg) + EQ (15 mg/kg); PTX (3 mg/kg) + TQ (2.5 mg/kg); PTX (3 mg/kg) + TQ (5 mg/kg); MTX (4 mg/kg) + MK (2.5 mg/kg); MTX (4 mg/kg) + MK (5 mg/kg); MTX (4 mg/kg) + PR (15
mg/kg); and MTX (4 mg/kg) + PR (25 mg/kg), through the tail vein. Animals were injected with P-gp (EQ or TQ) or Mrp 1 inhibitor (MK or PR) 30 min prior to the PTX or MTX administration. At the end of 60 min post PTX or MTX dosing, the rats were anesthetized by an intraperitoneal (I.P.) administration of a combination of ketamine (35 mg/kg) and xylazine (3.5 mg/kg) and the blood was collected via cardiac puncture. The animals were then euthanized with an excess dose of ketamine and xylazine combination injection through the I.P. route. Immediately after euthanization, the eyes were enucleated. Aqueous humor (AH) and vitreous humor (VH) were collected from both eyes and pooled. The blood samples were processed similarly as mentioned earlier and plasma was collected. All these tissue samples were kept at -80 °C until further analysis were performed.

3.2.7. Sample Preparation

To 80 µL plasma, 20 µL AH and 40 µL VH tissue samples, 10 µL, 2.5 µL, and 5 µL, respectively, of 1 µg/mL docetaxel (DTX) (internal standard for PTX(IS)) or 0.5 µg/mL of phenacetin (IS for MTX) solution was added. Then ice-cold acetonitrile (2 parts) was added to the samples (1 part) to extract drug and internal standard from the biological matrices with complete precipitation of proteins. Furthermore, the samples were vortexed and then centrifuged at 13000 rpm for 15 min and the clear supernatant solution was collected. The eluents in the supernatant solution (2 µL) were quantified using the following LC/MS method. The extraction efficiency (EE), limit of detection (LOD) and linearity in plasma (10-100 ng/mL), AH (0.5-40 ng/mL) and VH (0.5-20 ng/mL) was assessed.

3.2.8. Quantification of PTX and MTX

The quantity of PTX and MTX in the plasma, the AH and the VH samples were determined with the ACQUITY UPLC® I-Class System (Waters Corporation, Milford, MA, USA) connected to
Waters Xevo TQ-S triple quadrupole tandem mass spectrometer with an electrospray ionization (ESI). Data acquisition and processing were controlled by Waters Xevo TQ-S quantitative analysis TargetLynx software and MassLynx mass spectrometry software, respectively. Separation of PTX was achieved on an Acquity UPLC™ Cortecs C18 (2.1x100 mm; 2.7 µm) column with operations were accomplished using a C18 column (Acquity UPLC® BEH C18 100 mm×2.1 m, 1.7µm particle size). The mobile phase comprised of water (A) and acetonitrile (B), both containing 0.1 % formic acid, at a flow rate of 0.7 mL/min, which were applied in the following gradient elution: 0 min, 55 % A: 45% B, in next 2.5 min to 50% B, and next 0.2 min; to 100 % B, and then to 55% A for next 2.5 min. Each run was followed by a 3 min wash with 100 % acetonitrile, and an equilibration period of 1 min. The column temperature was set at 50 °C. Two microliters of sample was injected into the column and the effluent was directed into the ESI probe (source) operated using following parameters: source temperature 60°C, desolvation temperature 600°C, capillary voltage 2.0 kV, cone voltage 30 V, nebulizer pressure-7 bar, desolvation gas flow 1200 L/h N\textsubscript{2} and cone gas flow 150 L/h N\textsubscript{2}. The collision energies ranging from 20 to 64 eV were optimized, for individual analytes. Argon served as the collision gas. All the instrument operations were controlled by MassLynx software (version 4.1, Waters, Milford, MA, USA). Mass spectra of each sample was acquired in positive mode. The precursor to daughter ions transitions (quantifier ion to qualifier ions transitions) of PTX (m/z 854.31 → m/z 286.14, 122.08, 195.06) and docetaxel (DTX) (m/z 808.37 → m/z 527.23, 327.15, 105.06) was monitored in multiple reaction monitoring (MRM) mode. The compounds were confirmed in each spectrum by the three daughter ions, each for PTX and DTX.

Similarly, the MTX was also analyzed using the same LC/MS system, column mobile phase, at a flow rate 0.8 mL/min, with slight changes in gradient elution as following; till 0.2 min,
98 % A: 2% B, in next 2.5 min to 100% B and maintain for 1 min then to 98% A:2% B, in next 1 min. Each run was followed by a 1 min wash with 100 % acetonitrile, and an equilibration period of 3 min. The column and sample temperatures were set at 50 °C and 10 °C, respectively. Two microliters of sample was injected into the column and the effluent was directed into the ESI probe (source) operated using following parameters: source temperature 60°C, desolvation temperature 600°C, capillary voltage 2.0 kV, cone voltage 10 V, nebulizer pressure-7 bar, desolvation gas flow 1200 L/h N₂ and cone gas flow 200 L/h N₂. The collision energies ranging from 18 to 34 eV were optimized, for individual analytes. Mass spectra of each sample was acquired in positive mode. The precursor to daughter ions transitions (quantifier ion to qualifier ions transitions) of MTX (m/z 455.08 → m/z 308.16, 175.08, 134.08) and phenacetin (PH) (m/z 180.06 → m/z 110.06, 92.90, 65.08) was monitored in multiple reaction monitoring (MRM) mode. The compounds were confirmed in each spectrum by the three daughter ions, each for MTX and PH.

3.2.9. Data Interpretation

The variations in the plasma concentration of substrate drug (PTX or MTX) could be attributed to the differences in their AH and the VH levels, which could confound the impact of P-gp or Mrp1 on the permeation process. Hence, ocular tissue-to-plasma ratio of substrate drug concentration is a more important parameter to determine the impact of P-gp or Mrp1 activity and inhibition in the BOB.

Thus, drug (PTX or MTX) distribution into the AH and VH from plasma was calculated according to equations 1 & 2 respectively,

\[
D_{AH} = \frac{\text{Concentration of PTX or MTX in AH (ng/mL)}}{\text{Concentration of PTX or MTX in Plasma (ng/mL)}}
\]  \hspace{1cm} (Eq. 1)

\[
D_{VH} = \frac{\text{Concentration of PTX or MTX in VH (ng/mL)}}{\text{Concentration of PTX or MTX in Plasma (ng/mL)}}
\]  \hspace{1cm} (Eq. 2)
Where, $D_{AH}$ and $D_{VH}$ represents the distribution ratios delineating the penetration of PTX or MTX from plasma into AH and VH, respectively.

### 3.2.10. Statistical analysis

The data obtained from ocular distribution and ERG studies were subjected to one-way analysis of variance (ANOVA) and Tukey’s post hoc HSD (GraphPad Software, San Diego California, USA), to estimate the statistical significance. The $p < 0.05$ were considered statistically significant.

### 3.3. Results and discussions

All intravenous solutions were clear with pH of 6.7 -7.0 and showed an assay of 94.8-103.0% (Table 3.1). No significant loss of drug (< 1 %) was noted during filter validation (passing these solutions through 0.22 µm nylon filter). Moreover, no precipitation was seen after introducing these solutions into phosphate buffer saline (pH 7.4), ruling out the possibility of precipitation at the injection site (Table 3.1).

<table>
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<tr>
<th>Evaluation parameters</th>
<th>Formulations</th>
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<tr>
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<td>PTX</td>
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<td>% Assay</td>
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<td>pH</td>
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<td>Loss on Filtration (%)</td>
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<td>On-site precipitation</td>
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<td>Stability @ RT</td>
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All these formulations were stored at room temperature for 1-4 weeks and were found to be stable with ≤ 2% change in the assay during this period (Table 3.1). These intravenous solutions were prepared as mentioned in earlier literature reports, with slight modifications (103-105,108). The excipient concentrations utilized in the formulations were within the limits noted in the inactive ingredients (IIG) database of US Food and Drug Administration (US-FDA) and/or below reported intravenous LD50 values in rats, to ensure safety and biocompatibility of these parenteral solutions (109,110).

The LC/MS method adopted for the analysis of PTX and MTX was sensitive with a limit of detection (LOD) of 0.1 ng/mL, and a limit of quantitation (LOQ) of 0.5 ng/mL in the ocular tissues and was linear over the range of concentrations tested with $r^2$ values of 0.993, 0.990 and 0.994 in plasma, AH and VH, respectively. >85 % of PTX and ~92 % of MTX were extracted from all tissues by protein precipitation technique using ice cold acetonitrile. The bioanalytical method was

![Figure 3.1](https://via.placeholder.com/150)

**Fig. 3.1:** A) Western-blot displaying the P-gp protein and β-actin bands in the IC and RC samples obtained from WT and P-gp KO SD rats. B) The relative P-gp protein intensity was determined from the ratio of area obtained from P-gp protein band and β-actin band using ImageJ.
specific to PTX and/or MTX, specific IS’s as well, without any interference. The % recovery of these substrate drugs from the samples stored at 40 °C and freshly obtained samples were 93.3 % and 91.3%, respectively, suggesting low or no drug loss during the storage of the samples.

### 3.3.1. P-gp protein expression in ocular tissues

Literature reports describe the utility of the western blotting, reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry techniques in determining the P-gp protein expression in the ocular tissues (111). In the current study, the western blot results portrayed a protein band with MW ~170 kDa in the IC and RC samples obtained from the WT, confirming the existence of the P-gp protein in the BAB and the BRB, respectively (Fig. 3.1A). The higher relative P-gp protein intensity in the IC compared to that in the RC demonstrated higher P-gp expression in the IC than in the RC, suggesting greater MDR 1a efflux activity in the BAB over that in the BRB (Fig. 3.1B). A few literature reports also demonstrated the variations in the P-gp mediated efflux of substrates at the BAB and the BRB, in rat models, confirming the differential expression of P-gp in the BAB and the BRB (112, 113). In contrast, no band at 170 kDa MW mark in the IC and RC tissues collected from P-gp KO rats confirmed the absence of P-gp expression in their BAB and the BRB. Thus, these results suggest the applicability of P-gp KO rats as a suitable model to delineate impact of P-gp mediated efflux of anticancer drugs in the BOB.

### 3.3.2. Electroretinography

Electroretinography (ERG) was performed to understand substrate-inhibitors interactions with the retina thereby affecting the functioning of photoreceptors cells and inner retinal cells. The ERG measures the functioning of photoreceptor cells and bipolar/Müller cells as a-wave and b-wave, respectively. Changes in the a-wave recording suggest light or chemical induced alterations in the functioning of photoreceptors cells in the outer segment of the retina, while the variations in the
b-wave recordings suggest damage to the inner retina (114-116). Hence, the % change in the amplitude of a- and b-wave were examined to understand the cytocompatibility of substrate and/or inhibitor interactions with retina of WT, P-gp KO and Mrp1 KO SD rats.

The percentage changes of a- and b- wave amplitudes obtained from the ERG studies in WT and

![Image](image-url)

Fig. 3.2: Electroretinography results depicting the % change from the baseline in the amplitude of a- and b-wave recorded A) at 2h and 1day post PTX (two doses) in WT and P-gp KO rats, and B) at 2h post administration with MTX (two doses) in WT and Mrp1 KO rats, and C) at 2h post P-gp (EQ and TQ) or Mrp1 inhibitor (MK and PR) in WT rats (mean ± SEM, n=3). * - significantly different at p<0.05.

P-gp KO rats, pre- and post-dosing of PTX (two doses), is presented in Figure 3.2A. The a-wave amplitude in P-gp KO rats, 2h and 1day post treatment with 3 mg/kg of PTX, changed significantly with respect to baseline (p<0.05) (Fig. 3.2A). Similarly, at 1day post treatment with 3 mg/kg PTX, the b-wave amplitude, in P-gp KO rats, changed significantly from the baseline suggesting the
damage of inner retinal layer by PTX, at this dose (p<0.05) (Fig. 3.2A). These results verify several reports which demonstrated PTX induced retinal degeneration, at higher dose (3-6 mg/kg) (117,118). No change was observed in the a- and b-wave amplitude in both WT and P-gp KO rats administered with low dose (1 mg/kg) of PTX (Fig. 3.2A), confirming the lack of structural and functional alterations in the retina.

The WT rats treated with MTX high dose (8 mg/kg) showed a significant % change in the a-wave amplitude, from the baseline, 2h post treatment, but no change in the b- wave was noticed. The ERG wave pattern recorded in the Mrp1 KO rats, 2h post MTX treatment, were not different from the baseline recording. These results suggested that MTX at higher dose could cause a toxic interaction with retina (Fig. 3.2B). ERG recordings measured in WT and Mrp1 KO rats administered with low dose (4 mg/kg) of MTX were not statistically different from the baseline recording and suggested that MTX at low dose (4 mg/kg) is retina compatible (Fig. 3.2B).

All the inhibitors (EQ, TQ, MK and PR) were tested for their retinal compatibility, in WT rats, and, safe doses were selected for the ocular distribution studies. The % change in the amplitude of a- and b-wave recordings of all the inhibitors is given Figure 3.2C. Except for TQ 15 mg/kg dose, all other inhibitors, at tested doses, did not show significant % change in the a and b wave amplitude with respect to the baseline suggesting retinal safety. However, TQ at 15 mg/kg showed significant % change in the b-wave amplitude from the baseline recording, at 2h post dosing, suggesting retinal toxicity at this dose. The lower TQ dose was observed to be safe.

3.3.3. Ocular distribution

The substrates and inhibitor doses tested in the present studies were selected from literature reports which demonstrated the impact of P-gp and Mrp1 activity and inhibition in the BBB of WT and/or P-gp or Mrp1 KO rats/mice (76,101-105,119,120).
3.3.3.1. Impact of P-gp activity and its inhibition on the blood-ocular penetration of PTX

a. Plasma concentration of PTX

PTX concentration determined in the plasma of rats from different treatment groups are represented in Figure 3.3. A dose-related increase in the plasma PTX levels was observed in both WT and P-gp KO rats (Figure 3.3A). As the administered dose of PTX increased from 1 mg/kg to 3 mg/kg, a ~3–4-fold increase in the plasma drug concentration was observed in both WT and P-gp KO rats, at both the time points (p<0.05). The plasma PTX concentration in the P-gp KO rats was significantly higher compared to the WT rats, at 60 min (both doses) and 120 min (3 mg/kg dose alone) (p<0.05) (Fig. 3.3A). At 120 min post 1 mg/kg administration, the plasma PTX concentration in the P-gp KO rats were slightly higher over that observed in WT rats but were not statistically different (Fig. 3.3A). These results corroborate with previous reports which demonstrated a ~1.5-fold higher plasma PTX levels in the P-gp KO rats, compared to WT.

Figure 3.3: The plasma concentrations of PTX in the rats from different treatment groups. A) Plasma PTX levels in WT and P-gp KO rats, at 60 and 120 min post administration with 1 mg/kg or 3 mg/kg dose of PTX. B) Plasma PTX levels in the WT rats, at 60 min post co-administration with P-gp inhibitors (EQ or TQ) and PTX (1 mg/kg). α- indicates significant difference at p<0.05, between WT or P-gp KO 1 mg/kg vs WT or P-gp KO 3 mg/kg, at same time point; *-indicates significant difference at p<0.05, between WT vs P-gp KO, at same dose and time point (mean ± SEM, n=6).
This is probably due to reduced renal clearance of PTX in the Mdr1a gene KO rats due to the lack of P-gp expression in the renal cells (88).

At 60 min post PTX (1 mg/kg) administration, the plasma PTX concentration observed in the WT rats pretreated with P-gp inhibitors (either EQ or TQ, at both doses) were slightly higher, but not statistically different, from that observed in the WT rats treated with PTX alone (Fig. 3.3B). This might be due to the partial or incomplete inhibition on P-gp activity, by EQ or TQ (at tested doses), leading to no significant change in the renal clearance of PTX.

b. Blood-to-AH penetration of PTX

To estimate the penetration of PTX into the AH from the systemic circulation, AH PTX and the AH to plasma distribution ratio of PTX ($D_{AH}$), in P-gp KO rats and WT rats in different treatment groups, were estimated and are illustrated in Figure 3.4.

Dose related drug levels were noticed in the AH of WT and P-gp KO rats, at both doses and time points (Fig. 3.4A). At the lower dose (1 mg/kg), PTX concentrations were below LOD in the AH of the WT rats at both time-points (Fig. 3.4A). On the other hand, at the same dose, significant PTX levels were detected in the AH of P-gp KO rats, at the both time points, but, the concentration of PTX noted at120 min were below the LOQ (Fig. 3.4A). The penetration or distribution of PTX from blood into AH was determined as the AH to plasma drug concentration ratio ($D_{AH}$). The $D_{AH}$ of PTX in WT rats treated with the low dose was zero for both the time-points since no drug levels were detected in the AH. The $D_{AH}$ of PTX in the P-gp KO rats injected with low dose (1 mg/kg) was significantly better compared to that of the WT rats, at 60 min (p<0.05) (Fig. 3.4B). At the higher dose (3 mg/kg) treatment, the average concentrations of PTX in the AH obtained in the P-gp KO rats, at 60 min, were ~ 2-fold higher (p<0.05) than that of the WT rat (Fig. 3.4A). The $D_{AH}$ of PTX in P-gp KO rats were significantly higher than that seen in
WT rats, at 60 min. Hence at 60 min point, at both the doses, the influence of P-gp on the transport of PTX across the BAB was clearly evident (p<0.05) (Fig. 3.4A). These findings agree with previous reports on the expression of P-gp at BAB (3,81). At 120 min, no or low $D_{AH}$ in both WT and P-gp KO, compared to that observed at 60 min, suggests the elimination of drug from the blood and the AH, at both the doses of PTX.

Surprisingly, the $D_{AH}$ of PTX in P-gp KO rats administered with 1 mg/kg dose, at 60 min, was significantly higher than that observed in P-gp KO rats treated with 3 mg/kg dose (Fig. 3.4B). This could be possibly due to the damage to the BOB induced by PTX at 3mg/kg dose, as noticed in the ERG studies. Thus, 1 mg/kg PTX dose was selected to investigate the impact of the P-gp
activity inhibition on the blood-AH penetration of PTX in the WT rats pretreated with third generation specific P-gp inhibitors, EQ and TQ.

In the presence of EQ or TQ (at both the doses), the penetration of PTX (1 mg/kg dose) into the AH from blood in the WT rats were significantly higher (p<0.05) compared to that in the WT rats not receiving the inhibitors (Fig. 3.4C). The AH levels and $D_{AH}$ of PTX in WT rats co-administered with P-gp inhibitors and PTX increased with an increase in the EQ or TQ dose (Fig 3.4C). A 2-fold improvement in the AH PTX levels and $D_{AH}$ of PTX were noticed in WT rats, pre-administered with high dose of EQ (15 mg/kg) or TQ (5 mg/kg), compared to that seen in the WT rats administered with low dose of EQ (5 mg/kg) or TQ (2.5 mg/kg) (Fig 3.4C and Fig. 3.4D). These results indicate enhanced plasma to AH penetration of PTX, suggesting effective modulation of P-gp in the BAB by the inhibitors (EQ or TQ). The above findings corroborate with the literature reports which demonstrate the interaction of EQ and TQ with the MDR 1a efflux transporter function. This modulation of P-gp efflux activity is due to blocking of the drug/substrate binding site by EQ or because of blocking of both drug binding and ATP binding sites by TQ, in the P-gp structure (97,122). The AH PTX levels and $D_{AH}$ of PTX in the WT rats administered with EQ, at low dose (5 mg/kg), were not statistically different from those noted in the rats pretreated with TQ, at low dose (2.5 mg/kg) (Fig 3.4C and Fig. 3.4D). Similarly, no statistically significant difference was found between the AH PTX levels and $D_{AH}$ of PTX in the WT rats treated with 15 mg/kg dose of EQ and WT rats injected with 5 mg/kg of TQ (Fig 3.4C and Fig. 3.4D). It has been reported that TQ (IC50 ≤ 0.05 µM) has greater affinity for P-gp compared to EQ (IC50 ≤ 0.1 µM) (97). Hence, even at 2-3-fold lower dose compared to EQ, TQ produced P-gp inhibitory effects similar to that of EQ and no statistical difference in AH PTX levels and $D_{AH}$ was noted (Fig 3.4C and Fig. 3.4D).
The above results confirm that P-gp on the BAB restricts penetration of PTX into the AH from the blood and that effective inhibition of this P-gp activity improves the penetration of PTX across the BAB into the AH.

c. Blood-to-VH penetration of PTX

Dose related VH PTX levels were observed in both WT and P-gp KO rats (Fig. 3.5A). In the VH of WT rats, at 1 mg/kg dose, PTX concentrations were below LOD at both time-points whereas, significant PTX levels were detected in the VH of P-gp KO rats at the 60-min time-point (Fig. 3.5B). Hence, the plasma to VH PTX distribution ratio \(D_{VH}\) in P-gp KO rats receiving the lower dose, at 60 min, was significantly higher compared to that of the WT rats (p<0.05) (Fig. 3.5B).
This demonstrates the superior penetration of PTX from blood to VH through the BRB of subjects lacking P-gp. Moreover, no PTX levels in the VH of WT rats suggests the influence of P-gp, at the blood-VH interface, on the penetration of PTX across the BRB. These results are in agreement with the previous literature reports on the barrier properties of P-gp in the BRB (3,4,80). With the 3 mg/kg dose treatment, the VH PTX concentrations in the WT and P-gp KO rats, were not statistically different from each other, at any time point. Thus, the $D_{VH}$ of drug in WT and P-gp KO rats did not show significant difference at both time intervals post 3 mg/kg treatment (Fig. 3.5B). This could be attributed to the retina structural damage induced by PTX, at 3 mg/kg dose, which was apparent from the ERG studies. Moreover, numerous investigations have reported that PTX, at high doses (3-6 mg/kg), induce retina degeneration in humans and SD rats (117,118). Hence, the 1 mg/kg dose and the 60 min time point were selected to investigate the impact of modulation of P-gp activity, by EQ or TQ, on the penetration of PTX across the BRB.

The WT rats co-administered with high dose of EQ (15 mg/kg) or TQ (5 mg/kg) and PTX (1 mg/kg) showed greater VH PTX levels over that seen in the WT rats administered with PTX alone ($p<0.05$) (Fig. 3.5C). Hence, the VH to plasma PTX distribution ratio ($D_{VH}$) in WT rats receiving high dose of EQ and/or TQ were significantly higher compared to that of the WT rats receiving PTX alone ($p<0.05$) (Fig. 3.5D). As seen in blood-to-AH penetration of PTX, the VH levels and $D_{VH}$ of PTX in rats administered with higher dose of EQ or TQ were significantly higher than that of the rats administered with lower dose of inhibitors, and thus, suggested a dose related inhibition of P-gp activity ($p<0.05$) (Fig. 3.5C & Fig. 3.5D). However, at low dose of P-gp inhibitors, no PTX levels were detected in the VH of rats treated with TQ while the WT rats treated with EQ showed VH PTX levels, which were below the LOQ (Fig. 3.5C). This could be attributed to the partial or no inhibition of P-gp activity in the BRB by the EQ and TQ at low doses. These
results demonstrated effective modulation of P-gp activity in the BRB by EQ and TQ and agreed with the previous literature reports that demonstrate improved penetration of substrates across the BRB on modulating P-gp activity (80, 105).

3.3.3.2. Impact of Mrp1 activity and inhibition on the blood-ocular penetration of MTX

a. Plasma Concentration of MTX

Figure 3.6 delineates the plasma MTX concentrations in the WT rats and Mrp1 KO rats. The plasma concentration of MTX in both WT and Mrp1 KO rats was increased by ~ 2 - 3.5-folds with an increase in the administered MTX dose from 4 mg/kg to 8 mg/kg (Fig. 3.6A). At same dose and time point, the plasma MTX levels in the WT rats were about 1.2-3-fold higher compared to that seen in Mrp1 KO rats (Fig. 3.6A). Mrp1 efflux protein, situated on the basal side of the renal proximal tubular cells, promotes the renal reuptake of substrate drugs (102, 123). Thus, the reduced reuptake of MTX in the Mrp1 KO rats resulted in lower plasma concentrations, compared to WT rats (Fig. 3.6A). The WT rats in the inhibitor treatment group also showed variations in the plasma MTX levels. At 60 min post-treatment with MTX in the WT rats pretreated with MK (at any given dose), the plasma concentration of MTX was not statistically different from that observed in the WT rats injected with MTX alone (4 mg/kg) (Fig. 3.6B). This could be attributed to the low levels of MK in the renal tubules resulting in no or partial inhibition of Mrp1 activity in the kidney and, thus, lead to MTX reuptake. The WT rats pre-administered with PR (at both doses) showed about 1.5 - 2.2-folds higher plasma concentration of MTX compared to that seen in WT
rats in any other group and Mrp1 KO rats injected with 4 mg/kg MTX (Fig. 3.6). This remarkable difference in the plasma MTX levels seen in WT rats pretreated with PR (at both doses) could be attributed to the effectiveness of PR in inhibiting a wide range of Mrp’s, Oat’s, Oatp’s etc (p<0.05) (102). These results support the previous literature reports which demonstrated PR as a potent inhibitor of Mrp2, Oat1 and Oat3 transporters, besides Mrp1, situated on the apical cells of proximal renal tubule and involved in the renal clearance of substrate drugs (102). Hence, the inhibition of Oat1, Oat3 and Mrp2, by PR, in the kidney resulted in decreased renal clearance of MTX and thereby increased plasma MTX levels in the WT rats.

Fig. 3.6: The plasma MTX levels A) WT and Mrp1 KO rats treated with 4 mg/kg or 8 mg/kg, at 60 and 120 min, and B) WT rats co-administered with different doses Mrp1 or PB and MTX. of in different treatment groups. σ - indicates significant difference at p<0.05, between WT or Mrp1 KO 8 mg/kg vs WT or Mrp1 KO 4 mg/kg, at same time point; φ- indicates significant difference at p<0.05, between WT vs Mrp1 KO, at same dose and time point; ∆- indicates significant difference at p<0.05, between MTX (4 mg/kg) and PB (25 mg/kg or15 mg/kg) vs MTX (4 mg/kg) and MK (2.5 mg/kg or 5 mg/kg), WT (4 mg/kg) at 60 min and Mrp1 KO (4 mg/kg), at 60 min (mean ± SEM, n=6).
b. Blood-to-AH penetration of MTX

The AH levels and $D_{AH}$ of MTX in WT rats and Mrp1 KO rats, in different treatment groups, are presented in Figure 3.7. The MTX levels in the AH of WT and Mrp1 KO rats increased, corresponding to the dose administered, at 60 min (both at 4 mg/kg and 8 mg/kg dose) and 120 min (only at 8 mg/kg) (Fig. 3.7A). At the 4 mg/kg dose, the AH MTX levels and the $D_{AH}$ of MTX in Mrp1 KO rats were about 2-fold higher ($p<0.05$) compared to that in the WT rats, at the 60 min time point (Fig. 3.7A and Fig. 3.7B). This suggests Mrp1 mediated efflux activity in the BAB, consistent with the literature reports that demonstrate Mrp1 expression in the apical membrane of
the non-pigmented ciliary epithelial cells (124, 125). Hence, in the absence of Mrp1 efflux protein expression in the KO rats, the AH MTX levels were higher compared to that noted in WT rats, at 60 min post 4 mg/kg dose treatment. At 8 mg/kg dose, the AH concentrations of MTX in WT rats, at both time points, were not significantly different from that observed in the Mrp1 KO rats which could be due to the damage to BOB, as seen in the ERG studies (Fig. 3.2C). Thus, the $D_{AH}$ of MTX estimated in the WT and Mrp1 KO rats, at both time points in 8 mg/kg dose study, were not statistically different (Fig. 3.7B). The AH MTX levels and the $D_{AH}$ of MTX in WT and Mrp1 KO rats, at 120 min, was about 2-4-fold lower compared to that noted at 60 min, at same administered dose, which might be possibly due to the clearance of MTX from plasma and AH. However, at 60 min post 4 mg/kg dose treatment, the higher MTX concentrations in the AH and $D_{AH}$ of MTX in Mrp1 KO rats, over the WT rats, confirmed the active Mrp1 mediate efflux of the MTX across the BAB. Hence, this dose and time point was selected to understand the role of the Mrp1 inhibition on the penetration of MTX across the BAB.

The AH levels and the $D_{AH}$ of MTX estimated in the WT rats co-administered with MTX (4 mg/kg) and MK (5 mg/kg or 2.5 mg/kg) and/or PR (15 mg/kg or 25 mg/kg) were significantly higher compared to that seen in WT rats receiving MTX alone (Fig. 3.7C and Fig. 3.7D). This confirmed effective modulation of Mrp1 activity in the BAB and thereby, improved penetration of MTX into AH from blood. As the administered dose of the MK or PR ~ doubled, the AH MTX levels and $D_{AH}$ in the WT rats increased by about 1.5-2.0-fold and 1.2-1.7-fold, respectively, and suggested an inhibitor dose related modulation of Mrp1 activity at the BAB (Fig. 3.7C). Penetration of MTX into the AH ($D_{AH}$) from the systemic circulation, in WT rats pretreated with low dose of MK (2.5 mg/kg) and PR (15 mg/kg), were similar confirming similar Mrp1 inhibition by these inhibitors at a 5-6-fold dose difference. The AH MTX levels in WT rats administered
with PR high dose (25 mg/kg) were significantly higher compared to that seen in WT rats pretreated with MK high dose (5 mg/kg) (Fig. 3.7C). But, the $D_{AH}$ of MTX in WT rats treated with MK (5 mg/kg) was significantly higher compared to that observed in rats treated with PR (25 mg/kg) (Fig. 3.7D). This could be attributed to the higher MTX levels in plasma of rats pretreated with PR (25 mg/kg) compared to that seen in rats treated with MK (5 mg/kg) (Fig. 3.6B). These results support and corroborate the earlier literature reports which demonstrate effective inhibition of Mrp1 activity in the BBB and other blood-organ barriers by MK (100,101).

c. Blood-to-VH penetration of MTX

In both WT and Mrp1 KO rats, a dose related VH MTX levels were observed at the 60 min time point (Fig. 3.8A). In the 4mg/kg dose study, the MTX concentrations in the VH of Mrp1 KO rats

![Graphs showing VH MTX levels and $D_{AH}$ distribution ratio of MTX in Mrp1 KO rats and/or WT rats in different treatment groups. * - indicates significant difference at p<0.05, between WT or Mrp1 KO 4 mg/kg vs WT or Mrp1 KO 8 mg/kg, at same time point; σ - indicates significant difference at p<0.05, between WT vs Mrp1 KO, at same dose and time point; Φ - significantly different from WT rats treated with MTX (4 mg/kg) alone, at 60 min (p<0.05); φ- significantly different from Mrp1 KO rats treated with MTX (4 mg/kg) alone, at 60 min; ψ - significantly different from all (p<0.05) (mean ± SEM, n=6).]
were significantly higher (p<0.05), at the 60 min time-point, compared to that seen in WT rats
(Fig. 3.8A). Hence, the VH to plasma MTX distribution ratio (DVH) in Mrp1 KO rats, treated with
the lower dose was significantly higher (p<0.05) compared to that of the WT rats, at 60 min (Fig.
3.8B). This suggests that the penetration of MTX across, the BRB, is influenced by Mrp1 efflux
protein activity. These results verify the previous literature reports that demonstrate the expression
of Mrp1 efflux protein on the BRB (78). The MTX concentrations in the VH of the WT and Mrp1
KO rats, at 8 mg/kg dose study, were not statistically different (Fig. 3.8A). Hence, no significant
difference was noticed in the DVH of drug in WT and Mrp1 KO rats at both time intervals post
higher dose treatment (Fig. 3.8B). Reports in the literature demonstrated that MTX induce
reversible optic neuropathies, at higher doses (125). The ERG studies also confirmed that MTX,
at high dose (8 mg/kg) resulted in the damage to the retina architecture and functions. Hence, the
damaged retina could have accommodated MTX in the VH and thus, resulted in similar DVH of
MTX in WT and Mrp1 KO rats.

Considering the remarkable impact of Mrp1 activity at the 60 min time point post 1 mg/kg
MTX dose treatment, this dose and time point was selected to study the effect of Mrp1 inhibition
by MK and PR, on the penetration of MTX across the BRB. Significantly higher (p<0.05) MTX
levels were observed in the VH of rats treated with MTX and Mrp1 inhibitors (MK and PR), over
that seen in the rats administered with MTX alone (4 mg/kg) (Fig. 3.8C). Hence, significantly
higher VH to plasma MTX distribution ratio (DVH) in rats receiving MK or PR (at both doses)
were seen, compared to that observed in the rats dosed with MTX alone (4 mg/kg) (p<0.05) (Fig.
3.8D). The DVH of MTX in WT rats co-administered with MK or PR and MTX were about 2-folds
higher compared to that noted in Mrp1 KO rats (at 4 mg/kg and at 60 min) (Fig 3.8A and Fig.
3.8D). This can be owed to effective inhibition of Oat3, beside Mrp1, in the BRB by MK and PR. The Oat3, an influx transporter positioned on the apical membrane of inner BRB blood vessels, keep the substrate drug in the blood while the Mrp1, an efflux protein located on the basolateral membrane of the RPE (away from VH), prevent the transportation of substrate drugs across the outer BRB into the VH (100-102). Thus, effective inhibition of these transporter proteins by MK and PR allowed greater drug penetration into the VH from blood. These results demonstrate that the effective modulation of Mrp1 activity in the BRB, by MK and PR, improved the blood-VH penetration of MTX.

3.4 Conclusion

The over-all results from the comparative ocular distribution studies in WT, P-gp KO and Mrp1 KO rats delineated the influence of P-gp efflux transporter and Mrp1 efflux protein on the blood-ocular penetration of PTX and MTX, respectively. A dose related effect of PTX and MTX were noticed with respect to the impact of P-gp/Mrp1 activity in the BOBs. The effect of P-gp and Mrp1 on the penetration of these anticancer drugs across the BOB were more evident at the lower dose. The improved distribution of PTX and MTX into the AH and VH from the systemic circulation in the P-gp KO and Mrp1 KO rats, illustrates the existence and magnitude of the efflux proteins (P-gp and Mrp1) barrier characteristics in the BAB and the BRB. An improvement in the blood-ocular penetration of PTX and MTX, in the WT rats, in the presence of specific P-gp inhibitors (EQ and TQ) and Mrp1 inhibitors (MK and PR) point to effective inhibition of P-gp and Mrp1 mediated efflux activity in the BAB and BRB. The ERG studies indicated that both substrates, at higher dose, induced alterations in the retina functions. However, the interactions of PTX/MTX, at the lower dose, and P-gp inhibitors (EQ/TQ) or Mrp1 inhibitors (MK and PR) did not show any toxic
effect on the retinal functions. The findings also suggest that prolonged exposure to the chemotherapeutic agents above a particular dose can lead to damage to the retinal tissue.
CHAPTER 4

COADMINISTRATION OF TOPICAL OCULAR INSERTS OF SPECIFIC INHIBITOR MODULATES P-GP ACTIVITY IN THE BLOOD OCULAR BARRIERS AND IMPROVES BLOOD-TO-EYE PENETRATION OF SUBSTRATE ANTICANCER DRUG

4.1. Introduction

Ocular tumors such as retinoblastoma, uveal melanoma etc. are site- and life-threatening ailments in pediatric and adult patients (126,127). Effective ocular chemotherapy is mainly compromised by P-glycoprotein (P-gp) mediated efflux transportation of chemotherapeutic agents, in the blood ocular barriers (BOB) (128). Upregulation of P-gp in the ocular tumors and its broad substrate specificity restricts the efficacy of the substrate anticancer drugs (128, 76-79). Numerous research investigations demonstrated enhanced substrate drug levels in the target organs by effective inhibition of the P-gp activity at specific blood-organ interface (90,91,129,130). In recent times, the third-generation specific P-gp inhibitors, elacridar (EQ) and tariquidar (TQ), were extensively studied to modulate the P-gp activity and to improve the substrate drug molecules penetration across the blood brain barrier or into the breast cancer cells (76, 97-99). It has been reported that concurrent intravenous or intravitreal administration of substrate drugs and inhibitors, like EQ/TQ demonstrated effective inhibition of P-gp activity in the blood aqueous barrier (BAB) and blood retinal barrier (BRB) (103-105,131). However, the penetration of substrate drug into non-specific organs due to the inhibition of P-gp activity in their plasma membrane is the major drawback associated with concurrent intravenous administration of substrate and inhibitors (132).
Previously, the contribution of topically administered first-generation P-gp inhibitors in improving the blood-ocular penetration of systemically and intravitreally administered substrates has been successfully investigated in New Zealand albino rabbits (106,107).

Ocular drug delivery via topical application, however, is restricted by various physiological, anatomical, static and dynamic barriers (17-19). Most of the conventional ophthalmic dosage forms, upon topical application, are washed out from the precorneal space and thus results in poor ocular bioavailability (5-10%) (17-19). Enhancement in the viscosity and mucoadhesive properties of the formulations prolonged their ocular residence (133). Recently, topical ocular films were explored for sustained ocular drug delivery. They form drug depot in the vicinity of conjunctiva and sclera tissue and thereby favors drug penetration into deeper ocular tissues, probably through conjunctiva-sclera pathway (20,21).

In the current research, EQ and TQ loaded melt cast films were developed using thermoplastic polymers, PolyOx® WSR N-10 (PEO), and Eudragit® FS 100 (FS), for the topical ocular application. Physicochemical properties and transocular membrane permeability of EQ and TQ from the inserts were evaluated. Further, for the first time, the impact of coadministration of EQ/TQ topical ocular inserts on blood ocular penetration of substrate anticancer drug, paclitaxel (PTX), by effective inhibition of P-gp activity in the BOB, was investigated in the Sprague Dawley (SD) rats. Electroretinography (ERG) studies and ultrastructure analyses of retinal pigmented epithelium (RPE) were conducted to determine the safety of PTX-EQ/TQ interactions on the structure and functions of retina.
4.2. Materials and Methods

4.2.1. Chemicals

PTX was procured from Sigma Aldrich (St. Louis, MO). EQ, TQ, were acquired from Alfa Aesar (Haverhill, MA). PEO N 10 [PolyOx® WSR N-10 (PEO N-10), MW: 100,000 Daltons] (PEO) and Eudragit® FS 100 (FS) were generously donated by Dow Chemical Company (Midland, MI) and Evonik (Darmstadt, Germany), respectively. All other chemicals were procured from Fisher Scientific (St. Louis, MO).

4.2.2. Animal tissues

Whole eyes of New Zealand albino rabbits were obtained from Pel-Freez Biologicals® (Rogers, AK). They were shipped overnight in Hanks Balanced Salt Solution (HBSS) over dry ice. On the day of receipt of Whole eye globes, corneas and sclera-retina-choroid (SRC) were excised and utilized in the relevant experiments.

4.2.3. Animals

Six to eight-week-old male wild type SD rats, were purchased from Horizon Discovery (Saint Louis, MO). They were allowed for free access to food and water. The animal experiments were performed according to the approved protocols of the University of Mississippi Institutional Animal Care and Use committee, the University of Tennessee Health Science Center Institutional Animal Care, and Use committee and the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research.

4.2.4. Preparation of EQ and TQ loaded films

EQ and TQ loaded ocular films were prepared by melt cast technology. The physical mixtures of inhibitor/s (EQ or TQ) and polymer/s (PEO alone or PEO:FS (1:1 w/w)) were prepared by mixing
geometric proportions of accurately weighed excipients in the mortar with pestle.

Table 4.1: Composition of elacridar and tariquidar film formulations and evaluation parameters

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Components (% w/w)</th>
<th>Assay (%)</th>
<th>Content Uniformity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PEO</td>
<td>FS</td>
<td>EQ</td>
</tr>
<tr>
<td>EQ PEO-FS</td>
<td>45</td>
<td>45</td>
<td>10</td>
</tr>
<tr>
<td>TQ PEO</td>
<td>90</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TQ PEO-FS</td>
<td>45</td>
<td>45</td>
<td>-</td>
</tr>
<tr>
<td>EQ PEO</td>
<td>90</td>
<td>-</td>
<td>10</td>
</tr>
</tbody>
</table>

- PEO, FS, EQ and TQ indicates poly ethylene oxide N10 (PolyOx® WSR N-10), Eudragit® FS 100, elacridar and tariquidar, respectively.
- EQ-PEO-FS, TQ-PEO-FS, EQ-PEO and TQ-PEO represents elacridar film with polyethylene oxide N10 and Eudragit® FS 100 polymers, tariquidar films with polyethylene oxide N10 and Eudragit® FS 100 polymers, elacridar film with polyethylene oxide N10 polymer and tariquidar films with polyethylene oxide N10 polymer, respectively
- Weight of each film formulation was 200 mg

The physical mixture was then introduced into the center of 13 mm die placed on the brass plate, maintained at 70°C on the hot plate, and compressed with 13 mm punch to form a flat film of 10% w/w EQ or TQ load. The film was further heated for 2-3 min, cooled and cut into inserts of 1 mm x 1 mm x 0.2 mm or 2 mm x 1 mm x 0.2 mm dimensions, weighing approximately 1mg or 2 mg, for further experiments. The composition of the TQ and EQ films are given in Table 4.1.

4.2.5. Analytical procedure to determine EQ and TQ

EQ content in the in vitro samples was estimated by high performance liquid chromatography-ultraviolet spectrometry (HPLC-UV) method. A mobile phase comprising acetonitrile (ACN) and 0.05 M ammonium acetate (45:55 % v/v), pH adjusted to 5.0 with orthophosphoric acid, was passed through a Luna® C18(2) 100 mm column (Phenomenex, 4.6 mm x 250 mm; 5μ particle size), at a flow rate of 1 mL/min, by Waters HPLC system with 600 E controller pump. 25 μL of sample was injected to the flow of mobile phase by Waters 717 plus
Autosampler and the column eluent was monitored at $\lambda_{\text{max}}$ (detection wavelength) of 254 nm using Waters 2487 dual $\lambda$ absorbance detector. The peak response was integrated by an Agilent 3395 integrator (131, 134).

TQ content was estimated in the samples using similar chromatographic conditions except the mobile phase consisted of acetonitrile (ACN) and 0.2 M ammonium (40:60 % v/v) and pH adjusted to 4.5 with glacial acetic acid. The column eluents were tested at $\lambda_{\text{max}}$ of 222 nm (131, 76).

4.2.6. **Assay and content uniformity**

The assay of EQ or TQ ocular films was determined by dissolving the 13 mm film in 10 mL of extracting solvent, equal volumes of dimethyl sulfoxide (DMSO) and methanol. Content uniformity was determined by dissolving four inserts randomly sliced from a single film, in extracting solvent. The samples were diluted and quantified for the EQ and TQ content using HPLC.

4.2.7. **Differential scanning calorimetry (DSC)**

DSC was performed to understand the thermal behavior and chemical compatibility of polymers and inhibitors. DSC thermograms for EQ, TQ, PEO, FS and 10% w/w inserts were obtained on DSC Q20 differential scanning calorimeter (TA instruments, USA). Appropriately weighed samples were sealed in aluminum pans and heated from 0°C to 270°C at 20 °C /min heating rate, under nitrogen purge (20 mL/min).

4.2.8. **In vitro release studies**

*In vitro* release behavior of EQ and TQ from EQ-PEO-FS and TQ-PEO ocular inserts of 2mm x 1mm x 0.2 mm dimensions was tested, as reported earlier (20,21), cross standard US 100 mesh sieve in 20 mL scintillation vials. The insert was taken in 20 mL scintillation vial and a US
100 mesh sieve was placed on the films. 10 mL release medium (2.5 % w/v RMβCD and 1 % w/v Tween 80 in phosphate buffer solution (PBS) (pH 7.4) was added to the vial and a stir bar was placed over the sieve. The whole set up was placed in a thermostatic water bath (Fisherbrand™ Isotemp™ Advanced Stirring Hotplate, HN), maintained at 34°C and under constant stirring, for 2h. 1 mL sample was collected at pre-determined time intervals and replenished with same volume of fresh medium. The samples were analyzed for the EQ/TQ content using HPLC.

4.2.9. Transocular membrane permeability studies

4.2.9.1. In vitro transcorneal permeation study

In vitro transcorneal transport studies were performed using the corneas excised from whole eye globes of New Zealand male albino rabbits. The corneas were mounted on the receptor compartment of the Valia-Chien cells (PermeGear, Inc., Hellertown, PA), such that the epithelial side face towards the donor compartment. The receptor compartment was filled with 5 mL media (2.5 % w/v RMβCD and 1 % w/v Tween 80 in PBS (pH 7.4). 2 mg of EQ-PEO-FS or TQ-PEO insert (equivalent to 0.2 mg of EQ/TQ) was placed on corneas and wetted with 50 µL of media in the donor compartment. The diffusion cells were maintained at 34°C with the help of a circulating water bath for 3h study period. Aliquots of 600 µL were withdrawn from receptor compartment at specific time intervals and replaced with same volume of fresh medium. EQ/TQ content in the samples and cornea tissues were estimated using HPLC.

4.2.9.2. In vitro trans-SCR permeability study

In vitro trans-SCR (sclera-choroidal RPE) permeability of EQ/TQ was determined by using freshly excised scleral tissue with the retinal pigmental epithelium and choroid layers, from whole rabbit eyes. SCR was mounted on receiver compartment of Valia-Chien cells (Perme Gear, Inc®) such that the scleral side was facing to the donor compartment and the RPE-choroidal layers were
located towards the receptor compartment. 5 mL of media (2.5 % w/v RMβCD and 1 % w/v Tween 80 in PBS (pH 7.4) was filled in the receptor chamber. 2 mg of EQ-PEO-FS or TQ-PEO insert (equivalent to 0.2 mg of EQ/TQ) was placed on sclera and wetted with 50 µL of media in the donor compartment. The diffusion cells were maintained at 34°C with the help of a circulating water bath for 3h study period. Aliquots of 600 µL were withdrawn from receiver compartment at specific time intervals and replaced with same volume of fresh medium. EQ/TQ content in the samples and SCR tissue were estimated using HPLC.

4.2.10. Data Analysis

The transocular permeability parameters of EQ/TQ such as the cumulative amount of drug permeated ($M_n$), steady state flux ($J$) and the apparent permeability coefficient ($P_{app}$) were estimated as previously reported (135). Equations 1 was used to integrate the cumulative amount of EQ/TQ permeated across the cornea or SCR.

$$M_n = V_r C_{r(n)} + \sum_{x=1}^{n} V_s(x-1) C_{r(x-1)}$$

Where: $n$ specified sample time interval ($n = 1, 2, 3….8$ corresponding to 15, 30, 45….180 min respectively); $V_r$ and $V_s$ indicates the media volume in the receptor compartments (mL) and sample the volume collected at the $n^{th}$ time interval (mL); and $C_{r(n)}$ represents the EQ/TQ concentration in the receptor compartment medium at $n^{th}$ time interval (µg/mL).

The slope of the cumulative EQ/TQ permeated vs. time ($t$) plot was determined to calculate the steady-state flux (SSF) ($J$) of EQ/TQ was using equation 2:

$$Flux(J) = (dM/dt)/A$$

Where $M$ and $A$ specifies the cumulative amount of drug transported and the surface area of the cornea or SCR utilized in the experiment (0.636 cm$^2$).

The apparent permeability of EQ/TQ across the cornea or SCR was determined from the ratio of
steady state flux \((J)\) and EQ/TQ amount in the donor compartment \((C_d)\) (Eq. (3)) (26, 30).

\[
P_{app} = \frac{Flux (J)}{C_d + 60} \tag{3}
\]

4.2.11. P-gp protein expression by western blotting

The expression of P-gp protein in the BAB and BRB of the SD rats was studied by western blotting. The SD rats were euthanized with excess intraperitoneal (IP) dose of ketamine (35 mg/kg) and xylazine (3.5 mg/kg) combination \((n=6)\), the eyes were enucleated, and immediately the ocular tissue (Iris Ciliary (IC) and Retina choroid (RC)) samples were collected. IC and RC tissues lysates were prepared using 2X lysis buffer (Cell Signaling Technology, USA). Tissue lysates were measured for protein concentrations by Bradford assay (Bio-Rad USA). 20 µg of proteins, from each tissue lysate, were taken into the wells of 10% SDS-PAGE mini-gels (Bio-Rad, USA) and electrophoresed. Then proteins were transferred to nylon membranes (Cell Signaling Technology, USA) according to standard western blot protocol. These membranes were blocked in 5% non-fat dried milk in 1X TBST \([20 \text{ mM Tris–HCl (pH 7.5), 137 mM NaCl and 0.1% (v/v) Tween 20}]\) for 1h at room temperature. Blots were kept overnight with primary antibody MDR1/ABCB1 (Cat # 13978 Cell Signaling Technology) as per the manufacturers protocol. After washing the membranes with 1X TBST, they were probed with anti-rabbit secondary antibody conjugated to HRP (Cat # 7074 Cell Signaling Technology) for 1h. To ensure equal loading, samples were added with β actin antibody (Cell Signaling Technology, USA). The blots were developed with signal flame enhanced chemiluminescence (ECL) TM kit (Cell Signaling Technology, USA). The images were captured, processed and analyzed on ChemiDoC™ MP imaging system (Bio-Rad, USA). Protein band area was computed by using ImageJ and, the ratio of P-gp band area and the corresponding β-actin band area was calculated as relative P-gp protein intensity.
4.2.12. Electroretinography

To determine the PTX-EQ or TQ interactions with the retina functions, electroretinography (ERG) studies were performed in SD rats. 12 SD rats were divided into 2 groups and one group was topically administered with 1 mg EQ-PEO-FS insert (equivalent to 0.1 mg EQ) in each eye while the other group received 1 mg TQ-PEO insert (equivalent to 0.1 mg TQ) in each eye. During the administration of EQ or TQ inserts, the rats were partially anesthetized with ketamine (35 mg/kg) and xylazine (3.5 mg/kg) combination through IP route. Post 30 min EQ/TQ administration in the eyes of the rats in both the groups, they were injected with a specific volume of 3 mg/mL PTX solution (equivalent 1 mg/kg intravenous dose).

ERG was performed as per the standard protocols (115,116). The overnight dark-adapted rats were anesthetized with an I.P. injection of ketamine and xylazine combination. Both the corneas were anesthetized with topical 0.5% proparacaine hydrochloride, the pupil was dilated with 1% tropicamide and gold ring electrodes of 2-3 mm diameter (Roland Consult, Stasche & Finger GmbH), were placed on corneas. Stainless-steel surgical needles were inserted subcutaneously in the forehead and back leg to serve as reference and ground electrodes respectively. Both the eyes were exposed to Ganzfeld light source flashes of different intensities (from −4.0 Log units to 2.88 Log units) and the dark-adapted ERG patterns were measured. The baseline a- wave and b-wave amplitudes were recorded in the rats before the administration of EQ/TQ inserts. The differences in the a- and b- waves amplitudes recorded as baseline and at the of 6h or 12h post PTX (1 mg/kg) treatment were reported as percentage change in the amplitude.

4.2.13. Ultrastructural analyses of retina pigmental epithelium

Ultrastructural analyses of the retina was performed, as per the standard protocols (115, 131), to evaluate the PTX-EQ/TQ interactions with the structure of the retina. Post ERG studies,
the rats from 2 treatment groups were euthanized with excess of ketamine and xylazine combination, the eyes were enucleated and were fixed with a 2% paraformaldehyde and 2% glutaraldehyde mixture in 0.1 M phosphate buffer. These eyes were then embedded in Araldite/Embed812 (Electron Microscopy Sciences) and the anterior-posterior axis of the eye were aligned parallel to the cutting surface block and sections of one-micron thick were sliced through the posterior pole of each eye. The tissue sections were stained in Toluidine Blue O and then seen under a Nikon Eclipse 800 microscope to select the suitable tissues section area for ultrastructural analyses of RPE and retinal blood vessels. These ultrathin sections were observed for any structural changes in the RPE or the retinal vasculature under JEOL JEM1200EX II electron microscope at high magnification.

4.2.14. Ocular distribution

Ocular distribution studies were undertaken in male SD rats to establish the impact of co-administration of specific P-gp inhibitor loaded topical ocular inserts on the blood ocular penetration of PTX through effective modulation of P-gp activity in the BOB. No gender dependent transporter protein expression in the ocular tissues were reported in the literature, thus, the current study was conducted in only male SD rats. 60 SD rats were divided into 2 treatment groups with equal number of rats. Each rat in the first group was administered with 1 mg of EQ-PEO-FS insert (equivalent to 0.1 mg EQ) topically in each eye. The second group rats were administered with topical 1 mg TQ-PEO insert (equivalent to 0.1 mg TQ) in each eye. The rats in each group were partially anesthetized with IP ketamine (35 mg/kg) and xylazine (3.5 mg/kg) combination before the administration of EQ/TQ insert, and they were conscious in 5-10 min post EQ/TQ administration. After 30 min from the EQ/TQ administration, rats in two groups were administered with 1 mg/kg dose of PTX intravenously through tail vein. At 1, 2, 4, 6 and 12h post
PTX treatment, rats (n=6 at each time point) from each group were anesthetized with an I.P. administration of ketamine (35 mg/kg) and xylazine (3.5 mg/kg) mixture, blood was collected via cardiac puncture and were euthanized with excess of ketamine and xylazine combination through I.P. route. The eyes were enucleated immediately. Aqueous humor (AH) and vitreous humor (VH) were collected from both eyes of each rat and was pooled. The blood samples collected were immediately centrifuged at 10,000 rpm for 5 min and the plasma was separated. Plasma, AH and VH samples were stored at -80°C until analysis.

Another group of 12 SD rats, not pretreated with either EQ or TQ insert, were injected with 1 mg/kg dose of PTX through tail vein. At 1h and 2h post PTX dosing, the rats (n=6 at each time point) were processed similarly as mentioned earlier and the plasma, AH and VH samples were collected and stored at -80°C until analysis.

4.2.15. Sample Preparation

10 µL, 2.5 µL, and 5 µL of 1 µg/mL docetaxel (DTX) (internal standard (IS) for PTX) and 1 µg/mL loperamide (LOP) (internal standard (IS) for EQ and TQ) solutions were added to 80 µL plasma, 20 µL AH and 40 µL VH tissue samples, respectively. The proteins in 1 part of sample tissue matrix was precipitated with 2 volumes of ice-cold acetonitrile to extract PTX or EQ and TQ and IS. These samples were vortexed, centrifuged at 13000 rpm for 15 min and the supernatant solution was separated. The PTX, EQ, TQ and their respective IS in the supernatant solution (2 µL) were quantified by the following LC/MS method. The extraction efficiency (EE), limit of detection (LOD) and linearity of PTX, EQ and TQ in plasma (10-100 ng/mL), AH (0.5-40 ng/mL) and VH (0.5-20 ng/mL) was evaluated separately.
4.2.16. Quantification of PTX, EQ and TQ in the biological matrix

The quantity of PTX, EQ and TQ in the plasma, the AH and the VH samples were determined with the ACQUITY UPLC® I-Class System (Waters Corporation, Milford, MA, USA) connected to Waters Xevo TQ-S triple quadrupole tandem mass spectrometer with an electrospray ionization (ESI). Data acquisition and processing were controlled by Waters Xevo TQ-S quantitative analysis TargetLynx software and MassLynx mass spectrometry software, respectively.

Separation of PTX was achieved on an Acquity UPLC™ Cortecs C18 (2.1×100 mm; 2.7 µm) column with operations were accomplished using a C18 column (Acquity UPLC® BEH C18 100 mm×2.1 m, 1.7µm particle size). The mobile phase comprised of water (A) and acetonitrile (B), both containing 0.1 % formic acid, at a flow rate of 0.7 mL/min, which were applied in the following gradient elution: 0 min, 55 % A: 45% B, in next 2.5 min to 50% B, and next 0.2 min to 100 % B, and then to 55% A for next 2.5 min. Each run was followed by a 3 min wash with 100 % acetonitrile, and an equilibration period of 1 min. The column temperature was set at 50 °C. Two microliters of sample was injected into the column and the effluent was directed into the ESI probe (source) operated using following parameters: source temperature 60°C, desolvation temperature 600°C, capillary voltage 2.0 kV, cone voltage 30 V, nebulizer pressure-7 bar, desolvation gas flow 1200 L/h N₂ and cone gas flow 150 L/h N₂. The collision energies ranging from 20 to 64 eV were optimized, for individual analytes. Argon served as the collision gas. All the instrument operations were controlled by MassLynx software (version 4.1, Waters, Milford, MA, USA). Mass spectra of each sample was acquired in positive mode. The precursor to daughter ions transitions (quantifier ion to qualifier ions transitions) of PTX (m/z 854.31 → m/z 286.14, 122.08, 195.06) and DTX m/z 808.37 → m/z 527.23, 327.15, 105.06) was monitored in multiple
reaction monitoring (MRM) mode. The compounds were confirmed in each spectrum by the three daughter ions, each for PTX and DTX.

The peaks of the EQ, TQ and LOP (IS) were quantified with respect to the specific mass to charge \((m/z)\) values \((m/z\) 564, 647 and 477 for EQ, TQ and LOP, respectively). The samples were eluted through BEH C18 (2.1 x 100 mm, 1.7 \(\mu\)m) column and separated using an isocratic mobile phase consisting of with 0.1% v/v formic acid and water with 0.1% v/v formic acid. The method showed a limit of detection (LOD) and limit quantification of (LOQ) of 0.1 ng/mL for EQ and TQ.

4.2.17. Data Interpretation

PTX distribution into the AH and VH from plasma was calculated according to equations 1 & 2 respectively,

\[
D_{AH} = \frac{\text{Concentration of PTX in AH (ng/mL)}}{\text{Concentration of PTX in Plasma (ng/mL)}} \quad (\text{Eq. 1})
\]

\[
D_{VH} = \frac{\text{Concentration of PTX in VH (ng/mL)}}{\text{Concentration of PTX in Plasma (ng/mL)}} \quad (\text{Eq. 2})
\]

Where, \(D_{AH}\) and \(D_{VH}\) designates the distribution ratios delineating the penetration of PTX from plasma into AH and VH, respectively.

4.2.18. Statistical analysis

The data from ERG and ocular distribution studies were processed through one-way analysis of variance (ANOVA) and Tukey’s post hoc HSD (GraphPad Software, San Diego California, USA), to establish the statistical significance. The \(p < 0.05\) were considered statistically significant.

4.3. Results and Discussion

Melt-cast method, a simple and solvent free technique, was adopted to fabricate the ocular films of EQ and TQ (20, 21). In these ocular films, molecular level drug entrapment in carrier
matrix results in higher drug loading. Unlike the conventional ophthalmic dosage forms, the use of excipients such as preservatives are not necessary in the melt cast ocular films (26, 27). PEO and FS, thermoplastic polymers, were used as matrix forming materials due to the thermogelling property and pH 7.4 dependent drug releasing characteristic, respectively (20, 136).

4.3.1. Assay and content uniformity

The assay and content uniformity of the EQ and TQ films are detailed in Table 4.1. EQ-PEO film showed low assay (54.3%) and content uniformity (57%) due to the manufacturing issues like sticking of EQ to motor and picking by 13 mm punch during the film preparation (Table 4.1). No such manufacturing issues were noticed while developing EQ-PEO-FS film and thus, resulted in ~ 100% assay and content uniformity (Table 4.1). On the other hand, loss of TQ was noticed in the development of TQ-PEO-FS film and resulted in low assay (84.68%) and content uniformity (75.84%) (Table 4.1). TQ-PEO film showed acceptable 99.44% assay with uniform distribution of TQ in the PEO matrix suggesting the lack of sticking and picking issues while manufacturing (Table 4.1). EQ-PEO-FS and TQ-PEO films with acceptable assay and uniform distribution of compounds in the polymeric matrix were considered for further studies.
4.3.2. DSC

DSC studies were performed to investigate the thermal behavior and excipient compatibility of TQ-PEO and EQ-PEO-FS ocular inserts. DSC thermograms of EQ, TQ, PEO, FS and PEO-FS (1:1 w/w), TQ-PEO and EQ-PEO-FS are displayed in Figure 4.1. The endothermic peaks seen at 69.49°C and 55.99°C in the PEO and FS thermograms, respectively, corresponds to their melting points (Fig. 4.1). Pure EQ and TQ showed the melting peaks at 227.21°C and 229.03°C, respectively (Fig 4.1). TQ-PEO and EQ-PEO-FS ocular inserts exhibited a melting point peak at 67°C. The absence of native endothermic peak of Eq and TQ in the films indicated the reduction in crystallinity of inhibitors in the polymeric frameworks (Fig. 4.1). No significant
shifts in the characteristic melting points of the polymers and inhibitors in the ocular inserts suggested absence of chemical interactions between the film components.

4.3.3. In vitro release of EQ and TQ

In vitro release profile of EQ and TQ from ocular inserts are presented in Figure 4.2. In vitro release behavior of these P-gp inhibitors were studied in PBS (pH 7.4) with 2.5 % w/v RMβCD and 1 % w/v Tween 80 (pH 7.4) due to relatively high solubility of both EQ (20.14 ± 0.93 µg/mL) and TQ (12.16 ± 0.30 µg/mL). At the end of 2h, the % cumulative release of EQ and TQ, from EQ-PEO-FS and TQ-PEO inserts, were 92.35 ± 4.57 and 87.54 ± 2.56%, respectively (Fig. 4.2). The release of EQ from EQ-PEO-FS insert was comparatively slower than TQ from TQ-PEO insert (Fig. 4.2) which could be attributed to the presence of FS polymer, that helps drug release only at pH 7.0, in EQ-PEO-FS insert (136).
4.3.4. Transocular permeability

*In vitro* transocular membrane permeability parameters of EQ and TQ are given in Table 4.2. Both EQ and TQ showed transcorneal permeability but no penetration was noticed across the SCR (Table 4.2). The steady state flux and $P_{app}$ of TQ-PEO inserts (0.040 ± 0.01 µg/cm²/min and 5.6 ± 2.4 x 10⁻⁶ cm/s), across the rabbit cornea, were significantly higher than EQ-PEO-FS inserts (0.016 ± 0.003 µg/cm²/min and 1.3 ± 0.3 x 10⁻⁶ cm/s). This could be attributed to the higher Log $D_{7.4}$ and topological polar surface area of TQ (5.39 & 111.25 Å²) compared to EQ (3.9 & 89.1 Å²).

It has been demonstrated in the literature that the transmembrane permeability of a compound decreases with the decrease in Log $D_{7.4}$ and the compounds with higher polar surface area can penetrate through the hydrophilic stroma layer in the cornea (137, 138). Additionally, the slow release of EQ from EQ-PEO-FS inserts, compared to TQ from TQ-PEO insert, could have resulted in its lower transcorneal permeation. The SCR tissues treated with corresponding inserts showed EQ and TQ levels suggesting the lateral diffusion of these P-gp inhibitors in the sclera, but they

<table>
<thead>
<tr>
<th>Films</th>
<th>Transcorneal permeability parameters</th>
<th>Trans-SCR permeability parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flux (µg/cm²/min)</td>
<td>$P_{app} \times 10^6$ (cm/s)</td>
</tr>
<tr>
<td>10 % w/w EQ-PEO-FS</td>
<td>0.016 ± 0.003</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>10 % w/w TQ-PEO</td>
<td>0.04 ± 0.01*</td>
<td>5.9 ± 2.4*</td>
</tr>
</tbody>
</table>

- PEO, FS, EQ and TQ indicates poly ethylene oxide N10 (PolyOx® WSR N-10), Eudragit® FS 100, elacridar and tariquidar, respectively.
- EQ-PEO-FS and TQ-PEO represents elacridar film with polyethylene oxide N10 and Eudragit® FS 100 polymers, and tariquidar films with polyethylene oxide N10 polymer, respectively.
- SCR and $P_{app}$ represents sclera-choroidal RPE and apparent permeability coefficient, respectively.
- Weight of each film formulation was 200 mg
- *-indicates level of significance at p<0.05 between transocular membrane permeability of EQ-PEO-FS and TQ-PEO
did not penetrate across the choroidal RPE (Table 4.2). The levels of EQ in the SCR tissue was significantly higher compared to TQ in TQ-PEO treated SCR tissues which could be due to the high the molecular weight of TQ (646 g/mol), compared to EQ (563 g/mol) (Table 4.2). Earlier reports demonstrated poor sclera penetration of high molecular weight compounds (20, 21).

4.3.5. **Electroretinography (ERG)**

ERG was carried out to investigate any toxic effects of PTX-EQ/TQ ocular insert interactions on the functions of photoreceptor cells in the outer segment of the retina and inner retinal cells. The a-wave and b-wave recordings noted in the ERG represents the functioning of photoreceptor cells and bipolar/Müller cells, respectively. Any changes in the a- and b-wave patterns indicate the damage to the outer retina and the inner retina (114-116). The percent change in the amplitude of a- and b-wave examined in the SD rats are delineated in Figure 4.3.

![Figure 4.3: A) Percent changes in the amplitude of ERG wave patterns recorded in the EQ-PEO-FS and TQ-PEO treated rats, at 6h and 12h post PTX (1 mg/kg) intravenous administration (mean ± SEM; n=3).](image)

No significant changes in the a- and b-wave amplitudes were observed, with respect to baseline measurements, in the rats treated with EQ-PEO-FS (equivalent EQ 100µg/eye) and
TQ-PEO (equivalent TQ100µg/eye) ocular inserts, post 6h and 12h PTX (1 mg/kg) intravenous administration, (Fig. 4.3). Thus, ERG studies confirmed that the tested doses of EQ-PEO-FS, TQ-PEO, and PTX did not induce any toxic effects on the functions of the retina.

4.3.6. Ultrastructure analysis of RPE

Figure 4.4 illustrates the ultrastructure of the RPE in rats co-administered with PTX and EQ-PEO-FS or TQ-PEO. RPE was observed under TEM for any morphological changes in choriocapillaris (Ch), Bruch’s membrane (BM), basolateral infoldings of RPE (BNF), pigmented granules (PG), and RPE nucleus. Diffused Ch, reduced BM thickness, absence of BNF, loss of pigmented granules (PG), and appearance of vacuoles in the RPE indicates the damage to retina architecture (139,140). No such observations were noticed in the ultrastructure of RPE in rats topically administered with EQ-PEO-FS and TQ-PEO ocular inserts, at the end of 6h and 12h post PTX (1 mg/kg) IV administration (Fig. 4.4). Moreover, the ultrastructure of RPE in rats pretreated with EQ ocular insert were not different from those treated with TQ-PEO insert (Fig. 4.4). These findings suggested that the PTX-EQ/TQ interactions, at tested doses, did not compromise the structural integrity of retina.

4.3.7. Ocular distribution

Ocular distribution studies were performed to investigate the potential of topical ocular delivery of EQ-PEO-FS and TQ-PEO inserts to improve the blood-ocular penetration of substrate anticancer drug, PTX, by effective modulation of P-gp activity in the BOB. To ensure the inhibition of P-gp activity in the BOB, the rats were pretreated with EQ or TQ inserts 30 min prior to PTX dosing. The ocular pharmacokinetic profile of PTX in the rats co-administered with topical EQ and/or TQ ocular inserts is represented in Figure 4.5. The ocular pharmacokinetic parameters of PTX and P-gp inhibitors (EQ/TQ) are enumerated in Table 4.3 and Table 4.4, respectively.
Figure 4.4: TEM images focusing ultrastructure of retinal pigmented epithelium (RPE) in rats administrated with EQ-PEO-FS and TQ-PEO ocular inserts, taken at 6h (A & C) and 12h (B & D), respectively, post intravenous administration of PTX (1 mg/kg). i and ii – indicates the magnification of 2 µm and 500 nm, respectively. Ch-choriocapillaris; RPE-retinal pigmented epithelium; BM-Bruch’s membrane; BNF-basolateral infoldings of RPE; PG-pigmented granules.
Plasma PTX concentration profile seen in the rats from both EQ-PEO-FS and TQ-PEO groups were not statistically different (Fig. 4.5A). The AH PTX concentration profile seen in the rats treated with TQ inserts were significantly higher (p<0.05), till 4h post PTX administration, over that seen in EQ treated rats (Fig. 4.5B). Similarly, the $D_{AH}$ PTX observed in rats treated with TQ-PEO inserts were comparatively higher (p<0.05), in first 4h post PTX injection, than that noticed in the rats administered with EQ-PEO-FS ocular inserts (Fig. 4.5C). The PTX levels in the

Figure 4.5: Pharmacokinetic profile of PTX in A) plasma, B) AH and E) VH of rats co-administered with EQ-PEO-FS and TQ-PEO topical ocular inserts. D) Distribution of PTX from plasma to AH ($D_{AH}$) and VH ($D_{VH}$) in rats treated with intravenous PTX (1 mg/kg) alone and C) $D_{AH}$, and F) $D_{VH}$ of PTX in rats co-administered with EQ and TQ ocular inserts (mean ± SEM; n=6).

rats treated with TQ inserts were significantly higher (p<0.05), till 4h post PTX administration, over that seen in EQ treated rats (Fig. 4.5B). Similarly, the $D_{AH}$ PTX observed in rats treated with TQ-PEO inserts were comparatively higher (p<0.05), in first 4h post PTX injection, than that noticed in the rats administered with EQ-PEO-FS ocular inserts (Fig. 4.5C). The PTX levels in the
AH of rats treated with PTX (1 mg/kg) alone were below the detectable limits whereas $D_{AH}$ PTX in both EQ and TQ pretreated rats were significantly higher (p<0.05) compared to that found in these rats and confirmed the effective inhibition of P-gp activity in the BAB (Fig. 4.5C & Fig. 4.5D). These results corroborate with the earlier literature reports which demonstrated the enhanced penetration of substrate drug into the AH due to the effective modulation of the P-gp expression in the BAB, post concurrent intravenous or intravitreal administration of P-gp substrate and inhibitor (103,104, 131). The PTX profiles in the VH of rats administered topically with either

<table>
<thead>
<tr>
<th>Pharmacokinetic parameters</th>
<th>PTX (1 mg/kg) + EQ (100 µg/eye)</th>
<th>PTX (1 mg/kg) + TQ (100 µg/eye)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC$_{0-12}$ (ng h/mL)</td>
<td>AH 31.5 ± 2.5 VH 35.3 ± 4.6</td>
<td>AH 39.5 ± 2.3 VH 34.7 ± 1.6</td>
</tr>
<tr>
<td>$t \frac{1}{2}$ (h)</td>
<td>AH 2.4 ± 0.4 VH 2.3 ± 0.5</td>
<td>AH 1.9 ± 0.2 VH 2.0 ± 0.3</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>AH 3.1 ± 0.4 VH 3.1 ± 0.3</td>
<td>AH 3.0 ± 0.2 VH 3.0 ± 0.4</td>
</tr>
</tbody>
</table>

- PTX, EQ, TQ, AH and VH indicates paclitaxel, elacridar, tariquidar, aqueous humor and vitreous humor, respectively
- AUC, $t \frac{1}{2}$ and MRT represents area under curve, elimination half life and mean residence time, respectively

<table>
<thead>
<tr>
<th>Pharmacokinetic parameters</th>
<th>EQ (100 µg/eye)</th>
<th>TQ (100 µg/eye)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC$_{0-12}$ (ng h/mL)</td>
<td>AH 77.0 ± 2.8*</td>
<td>AH 36.5 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>VH 82.9 ± 4.6*</td>
<td>VH 12.5 ± 1.1</td>
</tr>
<tr>
<td>$t \frac{1}{2}$ (h)</td>
<td>AH 2.8 ± 0.5</td>
<td>AH 2.6 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>VH 4.2 ± 0.8*</td>
<td>VH 1.8 ± 0.6</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>AH 4.5 ± 0.8</td>
<td>AH 5.8 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>VH 5.2 ± 0.7*</td>
<td>VH 3.5 ± 0.3</td>
</tr>
</tbody>
</table>

- EQ, TQ, AH and VH indicates elacridar, tariquidar, aqueous humor and vitreous humor, respectively
- AUC, $t \frac{1}{2}$ and MRT represents area under curve, elimination half life and mean residence time, respectively
- * indicates the level of significance at p<0.05 between pharmacokinetic parameters seen EQ (100 µg/eye) and TQ (100 µg/eye) treated rats
EQ-PEO-FS and TQ-PEO ocular inserts were similar (Fig. 4.5E). Thus, the $D_{VH}$ PTX in both EQ and TQ treated rats were not statistically different (Fig. 4.5F). On the other hand, no drug penetration was noticed from plasma to VH of the rats administered with PTX alone (Fig. 4.5D). Hence, the $D_{VH}$ PTX in rats from both EQ-PEO-FS and TQ-PEO treatment groups were significantly higher ($p<0.05$) compared to the rats treated with PTX alone suggesting an improved blood-to-VH penetration of substrate anticancer drug by inhibiting the P-gp activity in the BRB, as reported in the earlier literature (105-107,131).

AUC of PTX in the AH of rats administered with TQ (39.5 ± 2.3 ng. h/mL) was slightly higher, but statistically not different, compared to that found in the rats treated with EQ (31.5 ± 2.5 ng. h/mL) (Table 4.3). The AUC of EQ and TQ in the AH of rats treated with P-gp inhibitor loaded ocular inserts were 77.5 ± 2.8 ng h/mL and 36.5 ± 2.8 ng h/mL, respectively (Table 4.4). Even at ~2 fold lower AUC in AH of rats, TQ produced P-gp inhibitory effect similar to that of EQ and hence, no statistical difference was noticed in the AUC of PTX in the AH of rats from both EQ and TQ treatment groups. Similarly, at 2-6 fold lower AUC of TQ in VH, rats treated with TQ showed similar AUC of PTX in VH compared to that found in EQ treated rats (Table 4.3 and Table 4.4). These results can be related to high affinity of TQ (IC50 ≤ 0.05 µM) for P-gp compared to EQ (IC50 ≤ 0.1 µM) (95). It has been reported that the TQ modulates the P-gp efflux activity by blocking both drug and ATP binding sites, whereas, EQ blocks only the drug/substrate binding site in the P-gp structure (97,121). Hence, TQ showed effective inhibition of P-gp activity in the BOB and thus, enhanced the blood ocular penetration of PTX.

The $D_{AH}$ and the $D_{VH}$ of PTX in the rats treated with TQ-PEO insert was reduced, after 4h timepoint, while they sustained for 6h in the rats administered with EQ-PEO-FS inserts (Fig. 4.5C and Fig. 4.5F). This can be related to the elimination half-life ($t_\frac{1}{2}$) of TQ and EQ, in
the AH and the VH of rats. The t½ of TQ in AH and VH was short whereas the EQ was longer in these ocular tissues (Table 4.4). The longer t½ of EQ in the AH and the VH could be attributed to the slow release of EQ due to the presence of FS polymer which helps drug release at pH 7.0 (136, 21). TQ-PEO and EQ-PEO-FS ocular inserts form a localized depot in the vicinity of conjunctival-scleral tissue and thereby distribute into the deeper ocular compartments (20, 21). Hence, the MRT of EQ and TQ in the AH and VH was 4.5-5.8h ensuring the blood ocular penetration of PTX for 6h by effective modulation of P-gp activity in the BOB (Table 4.4).

4.4. Conclusion

EQ and TQ loaded ocular inserts were successfully prepared by hot melt technology using PEO and FS thermoplastic polymers as matrix forming materials. TQ-PEO and EQ-PEO-FS ocular inserts showed acceptable assay and content uniformity. These ocular inserts showed ~ 90 % cumulative amount of EQ and TQ release in the in vitro release studies. Both EQ and TQ demonstrated the transcorneal permeability characteristics but poor trans-SCR permeability behavior. ERG studies and ultrastructure analyses of RPE revealed that the PTX, a substrate anticancer drug, and EQ/TQ interactions did not induce any toxic effects on the structure and functions of retina. Improved penetration of PTX into the AH and the VH from systemic circulation indicated the effective inhibition of P-gp activity in the BOB and exemplified the concurrent administration of topical ocular inserts of EQ/TQ, and intravenous anticancer substrate drug, as a potential strategy to enhance the ocular chemotherapy.
5.1. Introduction

Inflammation of the anterior and posterior segment ocular tissues are often associated with most of the eye ailments (140). Numerous steroidal and non-steroidal anti-inflammatory agents are being used for the management of ocular inflammations (141). Triamcinolone acetonide (TA) is a potent synthetic corticosteroid utilized to ameliorate the inflammation of ocular compartments (141). Currently, TA is available in the market as an intravitreal injection (Kenalog®, Kenacort®, Tricinolon®, Flutex®) for the treatment of deeper ocular tissue inflammations (140,142). Despite efficient therapy, intravitreal drug delivery encounters poor patient compliance due to post injection associated complications such as retinal detachment, postoperative infections etc. (19, 144-146). Thus, topical ocular drug delivery is a more preferred route of administration. However, the ocular bioavailability of topically instilled drugs is hampered by various anatomical and physiological, constraints (19,133).

In recent times, drug delivery through the eyelid has been investigated as an alternative, noninvasive, drug delivery platform that can maintain and sustain therapeutic drug levels in the eye (147-150). The thickness of the skin and stratum corneum on the eyelid is very thin compared to the skin in any other region (148,149,151). The drug applied to the upper eyelid penetrates into the conjunctiva and from there it will move into the inner ocular tissues, either through the corneal or through the scleral route (147-150).
It is also likely that this route of administration will maintain a steady drug concentration in the tear film (148). To the best of our knowledge, there have been few literature reports that studied trans-eyelid drug delivery (147-150). The data demonstrated prolonged drug levels on the ocular surface following application of a semisolid dosage form applied on the eyelid compared to the conventional formulation instilled in the eye (147,150).

Protransfersome gels were extensively explored for superior transdermal drug delivery (152). These formulations rapidly convert into transfersomes, ultradeformable and ultraflexible vesicles, by absorbing water from the skin, and efficiently penetrates into the deeper skin layers (43,152-155). Unlike the proliposome and proniosome systems, these gels have an edge activator alongside the vesicle forming components, which provides the flexibility to the transfersomes and, thus, enhances the transcellular permeation across the intact skin (43,156-159). Various mechanisms have been proposed to explain the ability of vesicles to modify drug diffusion through the skin (156-160). Principally, fluidization of the regular skin structure, because of the inclusion of the vesicle into the intercellular lipid layers of the skin, is important in overcoming the barrier property of the stratum corneum (157-161). The higher concentration gradient, between the skin and drug loaded vesicles, is another driving force that improves drug permeation through the skin (152,157-161).

The effectiveness of a protransfersome gel formulation for eyelid drug delivery, loaded with TA, has not been investigated till date. Thus, the goal of the current research was to develop and investigate the TA loaded protransfersome gel (PTF) as effective eyelid drug delivery system for improved ocular pharmacotherapy.
5.2. Materials and Methods

Phospholipon 90H (PL) was procured from Lipoid, LLC (Newark, NJ, USA), Kolliphor TPGS (D-alpha tocopheryl polyethylene glycol 1000 succinate) was obtained from BASF Corporation (NJ, USA), (Sorbitan monostearate (Span 60 (S60)) was received from Sigma Aldrich (St. Louis, MO, USA. Cholesterol (CH) was procured from Alfa Aesar (Ward Hill, MA, USA). TA, Amicon® Ultracentrifugal filter devices with regenerated cellulose membrane (molecular weight cut off 100 kDa), high performance liquid chromatography (HPLC) - grade solvents, and other chemicals (analytical grade) were supplied by Fisher Scientific (Hampton, NH, USA).

5.2.1. Preparation of TA loaded Protransfersome gels

PTF were manufactured as per previously reported technique (152,158). Briefly, the quantities of TA, the vesicle forming components (PL, CH and S60) and, the edge activator

<table>
<thead>
<tr>
<th>Component/Evaluation parameter</th>
<th>PTF 1</th>
<th>PTF 2</th>
<th>PTF 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA (mg)</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>PL (mg)</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>CH (mg)</td>
<td>34</td>
<td>34</td>
<td>34</td>
</tr>
<tr>
<td>S60 (mg)</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>TPGS (mg)</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Ethanol (mg)</td>
<td>100</td>
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<td>400</td>
</tr>
<tr>
<td>Water (mg)</td>
<td>500</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>Assay (%)</td>
<td>92.1 ± 3.2</td>
<td>94.5 ± 2.6</td>
<td>95.7 ± 2.2</td>
</tr>
<tr>
<td>Content Uniformity (%)</td>
<td>80.2 ± 11.5</td>
<td>89.5 ± 3.1</td>
<td>92.4 ± 2.7</td>
</tr>
<tr>
<td>Particle size (nm)</td>
<td>366.4 ± 6.6</td>
<td>184.5 ± 6.8</td>
<td>235.5 ± 4.6</td>
</tr>
<tr>
<td>PDI</td>
<td>0.52 ± 0.13</td>
<td>0.30 ± 0.08</td>
<td>0.30 ± 0.05</td>
</tr>
<tr>
<td>ZP</td>
<td>-21.7 ± 1.7</td>
<td>-21.4 ± 2.3</td>
<td>-25.3 ± 3.6</td>
</tr>
<tr>
<td>%EE</td>
<td>64.3 ± 4.4</td>
<td>89.4 ± 2.2</td>
<td>76.4 ± 2.1</td>
</tr>
</tbody>
</table>

- TA, PL, CH, S60 and TPGS indicates triamcinolone acetonide, phospholipon 90H, cholesterol, span 60, and TPGS, respectively.
- ZP and %EE represents zeta potential and % entrapment efficiency, respectively.

(TPGS) were accurately weighed in a clean glass vial and then, appropriate volume of
alcohol was incorporated. Later, the glass vial was closed with the screw cap and placed in a water bath maintained at 60-70°C.

The contents in the glass vial was melted to form a homogenous viscous fluid, to which, appropriate quantity of water (maintained at same temperature) was added in a dropwise manner with constant mixing till the translucent gel was formed. Further, the PTF was allowed to attain room temperature and utilized for further studies. The composition of PTF formulations is given in Table 5.1.

5.2.2. Quantification of TA in in vitro samples

TA content in all the in vitro samples were analyzed using high performance liquid chromatography (HPLC) method reported earlier (162). The HPLC system comprising of Waters 717 plus auto sampler, Waters 2487 dual absorbance detector, 600 Waters controller pump, and an Agilent 3395 Integrator. A mobile phase of a 1:1 isocratic solution of water and acetonitrile was passed through a Luna® C18 (4.6 mm × 250 mm) column at a flow rate of 1 mL/min; 25µL of sample was injected into the mobile phase and, the column eluents were detected for TA at wavelength (λmax) of 254 nm.

5.2.3. Assay, Content Uniformity and Entrapment Efficiency

To determine the assay and content uniformity in the PTF, known amount of formulation was dissolved in methanol and centrifuged (AccuSpin 17R centrifuge, Fisher Scientific, USA) at 13,000 rpm for 15 min. The supernatant was separated, and TA content was quantified using HPLC.

Entrapment efficiency (EE (%)) was determined by dispersing known amount of PTF (equivalent 1 mg TA) in 1 mL water and then vortexed to form a homogenous dispersion. Further, a 500 uL aliquot of this dispersion was added to the sampler holder in 100-kDa centrifugal filter
unit (Amicon Ultra) and centrifuged at 13,000 rpm for 15 min. The drug in the filtrate was estimated by HPLC method and the %EE was calculated by using the equation 1:

\[
\frac{0}{0}\text{EE} = \left(\frac{w_i - w_f}{w_i}\right) \times 100
\]

Where, \(w_i\) is the total drug content and \(w_f\) is the free drug present in the aqueous phase.

5.2.4. Morphometric characterization

The hydrodynamic radius, zeta potential (ZP) and polydispersity index (PDI) of the transfersomes formed from the PTF formulations were determined by photon correlation spectroscopy using a Zetasizer Nano ZS Zen3600 (Malvern Instruments, MA, USA) at 25°C in clear, disposable folded capillary cells. Known amount of PTF formulation was dispersed in 1 mL of water and vortexed to get a homogenous dispersion. This transfersome dispersion was diluted 10,000 times with bi-distilled water, and the particle size and PDI measurements were obtained using a helium-neon laser of 633 nm. Zeta potential (ZP) measurements were also carried out at 25°C using the laser Doppler velocimetry function of the same instrument.

5.2.5. Polarized Microscopy

The crystalline nature of pure TA and the protransfersome gels was observed under optical polarized microscopy, as reported earlier (163,164). Briefly, the test samples were placed on the rectangular cover slips and then loaded onto the optical microscope with uncrossed polarizer (Nikon Eclipse E600 Pol, Nikon Instech Co., Japan). The images with optical birefringence of drug was recorded.

5.2.6. Optical microscopy to observe the formation of transfersomes from PTF

The formation of transfersomes from PTF was observed under optical microscopy, as reported earlier, (Carl Zeiss AxioLab A1, CA) and the images were captured with live capture
module software (Zen, CA) (158). The protransfersome gel was hydrated with 5 mL water and gently agitated. Then the gel was placed in the well of a concave glass slide, cover slip was placed on the slide and placed under microscope. Microphotographs of transfersomes formation were captured at 45X magnification.

5.2.7. *In vitro* trans-eyelid permeability studies

*In vitro* trans-eyelid flux was measured using isolated rabbit eyelids as per the methods established for assessing flux across the skin (147,152). In brief, the rabbit eyelids will be washed with IPBS (pH 7.4) after removing the hair on the eyelid with epilator and mounted, as an inverted cup, on the Valia-Chien diffusion cells, such that the conjunctival side of the eyelid face towards receiver chamber. The donor chamber was then tightly secured so that the eyelid surface will be exposed to the donor compartment. Accurately weighed optimized PTF or marketed formulation (Triamcinolone Acetonide cream USP, 0.5 % w/w), equivalent to 1 mg of TA, was applied on the eyelid surface in the donor chamber. At predetermined time points, 600 µL aliquots was collected from the receiver cell and replaced with equal volume of fresh medium. The samples were quantified for the drug content using HPLC-UV method.

Cumulative amount of drug permeated \( (M_n) \), steady state flux \( (J) \) and trans-eyelid permeability \( (P_{app}) \), across the rabbit cornea, were estimated in order to study the transport of TA across rabbit eyelid (147,148). The cumulative amount of TA was calculated as per the equation:

\[
M_n = V_r C_{r(n)} + \sum_{x=1}^{n} V_s(x-1) C_{r(x-1)} \quad (2)
\]

Where, \( n \) is sampling time point; \( V_r \) and \( V_s \) are the volume in the receiver chamber (mL) and the volume of the sample collected at the nth time point (mL), respectively and \( C_{r(n)} \) is the concentration of the drug in the receiver chamber medium at nth time point (µg/mL).
The rate of TA transported across rabbit eyelid was calculated using the slope of the cumulative amount of TA transported versus time plot. The steady state flux of TA was determined using the following equation:

\[ \text{Flux}(J) = \frac{(dM/dt)}{A} \]  

(3)

Where, \( M \) is the cumulative amount of drug transported and \( A \) is the surface area of the eyelid (0.636 cm\(^2\)).

The transcorneal permeability of TA was calculated by the following equation:

\[ \text{Permeability (Papp)} = \frac{\text{Steady state flux}}{\text{Donor concentration}} \]  

(4)

5.2.8. Ocular Distribution Studies

Ocular penetration and distribution of TA from optimized PTF and marketed cream formulations was evaluated on recently sacrificed New Zealand male albino rabbits (within 30 min after euthanization with excess of pentobarbital). The hair on the right upper eye lid in rabbits were removed with the aid of epilator and accurately weighed amount of optimized PTF formulation and marketed TA 0.5 % w/v cream formulation, equivalent to 1 mg of TA, was applied. At the end of 1h and 2h post application, the eyes were enucleated, cleaned with ice cold IPBS (pH 7.4) and the ocular tissues were collected. These ocular tissue samples were stored at -80°C until further analysis.

5.2.9. Sample preparation

Protein precipitation technique was adopted to extract TA from the ocular tissues. Briefly, the isolated ocular tissues: conjunctiva, cornea, iris ciliary (IC), retina choroid (RC) and the sclera were transferred to separate eppendorf tubes. The tissues were dissected into small pieces and 100 \( \mu \)L of 1 \( \mu \)g/mL prednisolone (PR) was added as the internal standard (IS) to each of the tissue samples. After a while, 1mL ice cold methanol with 0.1% formic acid was added to precipitate the
proteins. Two hundred microliters of aqueous humor (AH) and 500 µL vitreous humor (VH) tissue proteins were precipitated using 200 µL and 500 µL respectively, of ice-cold methanol. All samples were vortexed for about 30 sec and sonicated for 5 min, to extract the drug into the solvent. Then the samples were centrifuged for 15-30 min at 13,000 rpm, the supernatant was collected and analyzed for TA content using the UPLC-triple quadrupole (TQ)-MS system.

5.2.10. **Bioanalytical method**

TA concentration in all the ocular tissues was quantified using Ultra Performance Liquid Chromatography system coupled with a triple quadrupole mass spectrometer (UPLC-TQ-MS) (Waters, USA). The peaks of both the drug and the IS (PR) were quantified with respect to the specific mass to charge (m/z) values (m/z 435 and 361 for TA and PR, respectively). Two microliters of the sample were eluted through BEH C18 (100mm×2.1 m, 1.7 µm) Acquity UPLC® column and separated using an isocratic mobile phase consisting of acetonitrile with 0.1% formic acid and water with 0.1% formic acid in the ratio of 98:2 respectively. The method showed a limit of detection (LOD) and quantification (LOQ) of 0.1 ng/mL for TA and IS respectively. All the instrument functions were operated and managed by Mass Lynx software (version 4.1, Waters, Milford, MA, USA).

5.2.11. **Statistical analysis**

A one-way analysis of variance (ANOVA) along with Tukey’s post hoc test (version 5.00; GraphPad Prism Software, San Diego, CA, USA) was used to analyze the data obtained from the ocular distribution studies, and the statistically significant difference between the set of formulations was observed at a ‘p’ value less than 0.05 (p<0.05).
5.3. Results and Discussion

5.3.1 Physicochemical properties of PTF

PTF formulations were successfully developed changing the ethanol composition, keeping all other components at same proportions. The physicochemical evaluation parameters are enumerated in Table 5.1. The drug loading in all the PTF formulations (~ 1% w/w) were ~ 2 folds higher, compared to the topical triamcinolone USP marketed cream dosage form (0.5 % w/w), with ~92- 96 % assay (Table 5.1). The content uniformity of the PTF 1 formulation was 80.2 ± 11.5, which was lower than that seen in PTF 2 and PTF 3 formulations. This suggests poor distribution of TA in the lipid matrix at this proportion of ethanol. The particle size of the PTF formulations (184.5 – 336.4 nm) decreased to some extent as the ethanol composition increased but, it increased again upon further increase in the ethanol proportion (Table 5.1). In contrast, the % EE in the PTF formulations increased upon increase in the amount of ethanol to an extent but it decreased again upon further increase in the ethanol amount in the formulation (Table 5.1). It has been reported that in the vesicular systems, ethanol helps in i) lowering the transition temperature of the lipids, and thereby, allows the lipophilic drug partitioning into the phospholipid bilayers; ii) attaining the gel phase due to regular arrangement of bilayer components, unlike liquid crystalline phase where irregular arrangement of PL and CH is seen; and, iii) permeability of the bilayer membrane (158, 165-167). Thus, at lower ethanol proportion the TA partitioning into lipid components was less which resulted in larger transfersomes with less drug entrapment. While at higher concentration of ethanol, the %EE dropped due to increased permeability of the bilayer membrane. Hence, PTF 2 formulation, which formed 184.5 nm size transfersomes with ~90% EE, was optimized and taken for further studies (Table 5.1).
5.3.2 Polarized Microscopy

Polarized microscopic images of pure TA and PTF 2 formulations are presented in Figure 5.1. The optical birefringence of pure TA showed interference colors (Fig. 5.1A). The polarized optical microscopic images of PTF 2 formulation revealed the absence of crystalline drug in the gel formulation, confirming the solubility of drug in lipid phase (Fig. 5.1B).

5.3.3 Formation of Transfersomes from PTF

Figure 5.2: Photomicrographs showing the formation of transfersomes under optical microscope: (A) gel surface immediately after hydration, B) Tubular structure formation, C) Transformation of tubular projection to vesicular form and D) Multilamellar transfersomes.
The formation of transfersomes from PTG formulation are illustrated in Figure 5.2. The optical microscopic studies demonstrated projection of globular structures on the surface of gel matrix (Fig. 5.2A), initially upon hydration of PTF 2, and they transformed into tubular structure (Fig. 5.2B and Fig. 5.2C) and finally, formed as multilamellar transfersomes (Fig. 5.2D). These findings were consistent with the earlier reports which demonstrated the formation of liposomes from proliposomes gel formulation (158).

### 5.3.4 *In vitro* trans-eyelid permeability study

Trans-eyelid permeability parameters of TA from marketed and PTF 2 formulations are given in Table 5.2. The results from *in vitro* trans-eyelid permeation studies suggested a ~2-fold improvement in TA flux and permeability across the eyelid with the PTF 2 (p<0.05) formulation compared to marketed dosage form (Table 5.2). This demonstrates efficient partitioning of the lipid vesicles into the intercellular lipid layers in the eyelid - thus, increasing the permeation (150, 152,1754). Reports in the literature demonstrated immediate formation of vesicles from the pro-vesicular gel formulation and increased vesicle-skin interaction (152, 154). The phospholipid in the vesicle structure allows immediate partitioning of this flexible vesicle, transfersomes, into the lipid layers of the eyelid through the intercellular passages (17-19). Moreover, the destabilization

| Table 5.2: *In vitro* trans-eyelid permeability parameters of TA from optimized PTF and marketed formulations (mean± SD; n=3) |
|-------------------------|-------------------------|-------------------------|
| Formulation              | Flux (µg/h/cm²)         | P<sub>app</sub> x 10<sup>5</sup> (cm²/h) |
| Triamcinolone acetonide  |                         |                         |
| USP, 0.5% w/w            | 0.31 ± 0.16             | 6.3 ± 3.2               |
| PTF 2                    | 0.69 ± 0.28*            | 13.8 ± 5.6*             |
| - PTF 2 and P<sub>app</sub> indicates the optimized protransfersome gel formulation and apparent permeability, respectively. |
| - *- represents the level of significance at p<0.05 between PTF 2 and triamcinolone acetonide marketed formulation. |
of skin layers by Span 60 and TPGS results in improved permeation of TA across the eyelid (155, 157-159,161).

5.3.5 Ocular Distribution Studies

TA levels in different ocular tissues of the rabbits (carcass) treated with PTF 2 and marketed formulation was delineated in Table 5.3. ~2-13 fold higher TA levels were seen in the conjunctiva, cornea and sclera of the rabbits receiving the PTF 2, in contrast to the marketed formulation (p<0.05) (Table 5.3). This suggests continuous absorption of TA from PTF, through the eyelid, into conjunctiva and other exterior ocular tissues, which will thus allow a sustained ocular delivery platform.

Furthermore, at any given time point, the TA levels in the rabbits receiving the protransfersome gel formulation was significantly better than those treated with marketed formulations (p<0.05) (Table 5.3). The results demonstrated greater permeability characteristics, of protransfersome gel formulation (p<0.05) (Table 5.3). The TA levels seen in these ocular tissues of the rabbits treated, with PTF 2, were upto 2-fold higher at the 2h time point, compared to 1h (Table 5.3).

This suggests continuous absorption of TA from PTF, through the eyelid, into conjunctiva and other exterior ocular tissues, which will thus allow a sustained ocular delivery platform.

<table>
<thead>
<tr>
<th>Ocular tissues</th>
<th>Formulations</th>
<th>PTF 2</th>
<th>Marketed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time (h)</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Conjunctiva (µg/g)</td>
<td>4.78 ± 1.04 *</td>
<td>8.5 ± 2.79 *</td>
<td>2.3 ± 1.19</td>
</tr>
<tr>
<td>Cornea (µg/g)</td>
<td>4.24 ± 0.96 *</td>
<td>13.04 ± 0.56 *</td>
<td>0.32 ± 0.32</td>
</tr>
<tr>
<td>IC (µg/g)</td>
<td>0.68 ± 0.079*</td>
<td>0.88 ± 0.061*</td>
<td>0.20 ± 0.056</td>
</tr>
<tr>
<td>AH (ng/mL)</td>
<td>54.03 ± 7.43 *</td>
<td>125.32 ± 27.91 *</td>
<td>21.19 ± 12.37</td>
</tr>
<tr>
<td>Sclera (µg/g)</td>
<td>11.07 ± 5 *</td>
<td>11.45 ± 1.8 *</td>
<td>0.45 ± 0.15</td>
</tr>
<tr>
<td>RC (µg/g)</td>
<td>1.07 ± 0.003*</td>
<td>1.79 ± 0.4</td>
<td>0.21 ± 0.14</td>
</tr>
<tr>
<td>VH (ng/mL)</td>
<td>6.25 ± 1.37 *</td>
<td>43.66 ± 17.84 *</td>
<td>1.20 ± 0.40</td>
</tr>
</tbody>
</table>

IC-Iris-ciliary body; AH-aqueous humor; VH-vitreous humor; RC-retina-choroid

*-indicates significantly different with respect to the marketed formulation, at respective time point (p<0.01)
system, through the eyelid, thus presented drug to the deeper ocular tissues (147,150,152). The only in vivo trans-eyelid delivery investigation reported dexamethasone levels in the anterior segment ocular tissues following the application of a poloxamer gel formulation on the eyelid of conscious rabbits (150). The permeability of TA and dexamethasone through ocular tissues has been reported to be largely similar due to the similar physicochemical behavior (168,169). In view of this, the levels of TA seen in the conjunctiva, cornea and AH with the protransfersome gel formulation were comparable with those obtained with dexamethasone from the poloxamer based gel formulation at the 2h time point. TA levels were also noticed in back-of-the eye tissues, the vitreous humor and retina-choroid. Furthermore, the levels of TA in ocular tissues, especially in AH and VH were significantly higher than the minimum therapeutic concentrations of TA - 1.7 and 6.5 ng/mL, respectively (170-172).

5.4. Conclusion

TA protransfersome gel formulations were successfully prepared with at least a two-fold higher drug load, compared to the marketed topical dosage form, which allows higher, localized, concentration gradients. Microscopic studies confirmed complete drug dissolution in the lipid phase and rapid formation of transfersomes on hydration. Significantly higher trans-eyelid TA permeation with the protransfersome gel formulation – demonstrated their potential in enhancing drug delivery to the ocular surface and deeper tissues via the eyelid. Experiments in rabbit carcasses confirmed that TA efficiently penetrated into the eyelid and formed a depot leading to improved concentration-time profiles in all ocular tissues tested. The overall results suggest that trans-eyelid protransfersome gel formulations can provide a platform for the sustained delivery of therapeutic agents to both the surface of the eye as well as the anterior and posterior segment ocular tissues.


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