DELIVERY OF THE ANTIFUNGAL AGENTS NYSTATIN AND VORICONAZOLE BY IONTOPHORESIS IN AN EX VIVO RABBIT CORNEA MODEL

By

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To Mom and Dad
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Rabbit cornea immediately after being treated with nystatin and iontophoresis. Note the circular, yellow haze of nystatin still left on the cornea.
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<td>N.B.</td>
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<td>P or P group</td>
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<td>G or G group</td>
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<td>HPLC</td>
<td>High-performance liquid chromatography</td>
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Chair: Caryn Plummer
Major: Veterinary Medical Sciences

The objective of this research was to determine the potential for electromigration of antifungal chemotherapeutic agents and evaluate iontophoretic delivery of nystatin and voriconazole to cornea.

Phase I – Nystatin was chosen for further analysis for its opacity and successful electromigration in agarose. Phase II – delivery of nystatin (100,000 IU/mL) by iontophoresis to ex vivo rabbit corneas was tested using a current of 4mA for 2.5min, 5min, 7.5min, or 10min. Application of the iontophoretic device and nystatin for 5min without current was used as negative control. For each group, n= 5, except for the 10min timepoint (n=6). Corneas were homogenized and drug concentration and mass were determined by HPLC. The same protocol was carried out for phase III except n=6 for each group, 1% voriconazole was evaluated, and negative control groups included no current for 5min, 7.5min, and 10min.

Phase II – detectable levels of 100IU/mL nystatin in ex vivo rabbit cornea. Phase III – one-way ANOVA – p-value of 0.0237. Two sample student t-Tests assuming unequal variances – statistically significant results were as follows (p-values): no current ↔ 7.5min – 0.0149, no current ↔ 10min – 0.0285, 2.5min ↔ 10min – 0.0208, 2.5min ↔ 10min – 0.0472. Two more control groups were run – 7.5min and 10min. No statistical significance from their iontophoresis
7.5min and 10min counterparts. One-way ANOVA for homogenization methods – p-value of 0.004.

Phase II – 100IU/mL nystatin able to be electromigrated into corneal tissue and reach detectable levels. Phase III – 1% voriconazole able to be electromigrated into corneal tissue, although results were not statistically significant when compared to topical control groups. Glass on glass pulverizers seem to be superior in extracting 1% voriconazole from tissue as compared to plastic on glass pulverizers.
Delivering drugs to the cornea and the interior of the eye is far more difficult a task than might be expected. There exist a number of barriers that limit the interaction of the ocular tissues with both the external environment and the rest of the body including the blood-aqueous barrier, the blood-retinal barrier, the blood-brain barrier and the multi-layered biphasic structure of the cornea itself. These barriers exist to protect the eye from inflammation and infections that may lead to sight-threatening damage. However, they also prevent the delivery of beneficial therapies to the eye, limiting our ability to medically intervene effectively in diseased states.

When one attempts to deliver therapeutic agents to the eye, several factors must be considered including the target tissue, the formulation of the drug (size, charge, lipophilidity, and hydrophilidity), tissue penetration, route and duration of delivery, potential adverse effects and owner and/or patient compliance.

The Cornea and Challenges to Ocular Drug Delivery

In most cases of corneal disease, topical medications are usually preferred because the drug can be applied directly to the ocular surface in high concentration with relative ease and most patients are reasonably amendable to this form of therapy. However, the effective delivery of drug into the corneal tissues is limited, sometimes drastically, by the inherent physiologic, chemical and metabolic barriers that exist in the cornea. The cornea is the transparent structure of the eye that makes up the anterior portion of the outer fibrous layer of the globe. In the dog, the cornea measures approximately 0.5 – 0.6mm thick in the periphery and approximately 0.6 – 1.0mm thick in the central cornea depending on the dog’s size. In the horse, multiple different modalities have been utilized to measure peripheral and central corneal thickness. These include but are not limited to ultrasonic pachymetry, high-frequency/high-
resolution ultrasound, confocal microscopy, and spectral-domain optical coherence tomography (SD-OCT). Studies using these different modalities have revealed the peripheral cornea to measure slightly above 0.8mm (range from 0.793mm – 0.917mm). The central cornea was measured to be approximately 0.8mm (range from 0.725mm – 0.920mm).(5-9) The cornea can arguably be divided into five layers: the precorneal tear film, epithelium, stroma, Descemet’s membrane, and the corneal endothelium. Furthermore, an epithelial basement membrane, referred to as Bowman’s layer, has been described in large, herbivorous mammals such as giraffes and in various species of whales and birds.(10)

The precorneal tear film is continuously and uniformly spread across the cornea by the eyelids and nictitating membrane during blinking. The tear film serves several functions, which include maintaining an optically uniform corneal surface, removing material from the cornea and conjunctival sac, allowing passage of nutrients, such as oxygen, to the cornea, and providing antimicrobial substances to protect the cornea and surrounding tissues from infection. The tear film is actually made up of multiple layers, three to be exact, consisting of a lipid layer, aqueous layer, and mucin layer.(10)

The different tear film layers were originally described as stratified, but in reality, the tear film is an admixture of the three. The outermost tear film layer is a thin, oily layer produced by the sebaceous glands of Zeis and the meibomians glands. The purpose of this layer is to reduce evaporation and to form a barrier along the lid margins to prevent tear overflow.(10)

The middle layer is made up of aqueous fluid produced by multiple glands. The lacrimal gland produces 61% of the aqueous layer, the gland of the nictitans produces 35%, and the accessory glands of Kraus and Wolfing are species dependent on their production.
(approximately 3%).(10, 11) Uptake of oxygen for corneal metabolism occurs through this tear film layer.(10)

The innermost layer consists of mucin, which is produced mainly by the conjunctival goblet cells. Mucin is also contributed to the tear film by lacrimal and accessory-lacrimal glands that have mucous secreting cells. Mucin accumulates on the corneal epithelial surface and is distributed evenly by the eyelids during blinking. Due to the mucin’s hydrophilic surface, the aqueous layer becomes intermixed with this inner layer to where it contributes the most to the tear film thickness.(10, 12)

The corneal epithelium is approximately 25 – 40μm thick in the domestic carnivore and two to four times that in ungulates.(10) The most prevalent type of collagen is type IV, VI, and VII, and the most prevalent type of glycans are laminin, fibronectin, and hyaluronans. This layer is stratified squamous epithelium, is lipophilic and made up of eight to twelve layers of non-keratinized squamous cells (superficial), wing cells (intermediate), and basal cells (deep).(13) These cells are arranged in this manner to provide order to surface cell replacement via desquamation. The basal cells firmly attach to a basement membrane beneath the epithelium by hemidesmosomes, anchoring collagen fibrils, and laminin.(10) Through these attachments, the basement membrane attaches the epithelium to the stroma, forming the epithelium/basement membrane/stromal adhesion complex. The epithelial cells have good regenerative power, with basal cell turnover being approximately 7 days. If the basement membrane basal lamina is removed, though, weeks to months are needed for it to reestablish. In this time period, the epithelium is not attached tightly to the deeper stroma and can easily be removed.(10, 13)

The corneal stroma comprises 90% of the corneal thickness and is composed of approximately 75 to 80% water, making it hydrophilic.(13) It is in a relatively dehydrated state,
termed deturgence, compared to most tissues of the body. Deturgence is accomplished by Na\textsuperscript{+}/K\textsuperscript{+} adenosine triphosphatase (ATPase) pumps in the corneal epithelium and endothelium, but being most active in the endothelium.(10)

The prevailing types of collagen in the stroma include I, III, V, VI, and XII, and the most prominent glycans include chondroitin 4- and 6-sulfate and dermatan sulfate. This layer is composed of transparent, structureless lamellae of fibrous tissue, which lie in sheets and easily split into individual planes. These lamellae travel the full diameter of the cornea. Keratocytes are located in between the lamellae and contribute to formation and maintenance of the lamellar sheets. When deep corneal injury occurs, keratocytes may form into myofibroblasts and cause corneal scars leading to corneal opacification. Otherwise, the parallel arrangement and integration of collagen and specific proteoglycans, such as lumican, keratocan, osteoglycin, and decorin, of the lamellae contribute to the clarity of the cornea.(10, 14) Because of this parallel arrangement and homogeneity of the collagen fibers’ size and diameter, it is believed that the stroma permits 99% of light moving through the cornea to pass without scatter.(15)

Descemet’s membrane is the basement membrane of the corneal endothelial layer. Because it is produced by the corneal endothelium throughout life, it becomes thicker as an animal ages.(10, 13) It exhibit modest elasticity and contains collagen I, III, IV, V, VI, and VIII and glycans laminin, fibronectin tenascin, P component, heparan sulfate.(10) Type VIII collagen is unique as it is found in the iridocorneal angle and in Descemet’s membrane, but nowhere else in the cornea. Descemet’s membrane ends at the apex of the trabecular meshwork in the limbal region and is relatively similar in composition to the trabeculae of the iridocorneal angle. This homogenous, acellular membrane is under a constant state of tension and if ruptured, curls like a scroll.
The endothelium is the innermost layer of the cornea. It consists of a single layer of flattened, interdigitating, hexagonal cells creating a barrier between the aqueous humor in the anterior segment of the globe and the rest of the corneal layers. Along the lateral cell margins are cell junctions, zonulae occludentes, maculae adherentes, and nexi that help keep fluid and solutes out of the cornea to maintain clarity. In addition, the corneal endothelium utilizes \( \text{Na}^+/\text{K}^+ \)-ATPase pumps to actively keep the cornea in a relatively dehydrated state.\(^{(10, 13)}\) The presence of cofactors in the aqueous humor such as calcium, glutathione, bicarbonate, and endothelial cell size, shape, and density all determine the efficiency of the barrier and pump system.\(^{(13)}\) There is a gradual loss in the hexagonal shape of the endothelial cells due to a decrease in density with age. A study performed in 2001 revealed that the mean endothelial cell density in normal horses is 3,155 cells/mm\(^2\), with this number declining as age increased but no variation was noted between gender.\(^{(16)}\) As an animal ages, endothelial cell density may eventually lower to 50% or less. When this occurs, the cells spread out and produce more pumps in order to compensate for an increase in fluid and solute leakage.\(^{(10)}\) An interesting finding by Andrew et al was that although endothelial cell density decreased with age, there was little to no change in corneal thickness; so despite decrease in number, there remains sufficient function.\(^{(16)}\) If loss of cell density does continue, though, the cells become unable to compensate, leading to leakage due to insufficient pumps and an increase in corneal thickness and opacity. This is known as corneal decompensation and occurs when the cell density falls below the critical value of 400 to 700 cells/mm\(^2\) \(^{(10, 16, 17)}\).

When considering the anatomy and physiology of all of the corneal layers, it is evident why there is such difficulty for a topical medication to penetrate through such a relatively short distance. The constant turnover of tears from the ocular surface limits drug contact time. The
lipophilic, negatively charged corneal epithelium repels or fails to admit certain drugs into the deeper tissues (stroma, Descemet’s membrane, endothelium, and anterior chamber) beneath. The hydrophilic corneal stroma has a vastly different affinity for charged molecules than either the epithelium or the lipophilic endothelium and their respective basement membranes. This complex arrangement makes formulation of topical medication very difficult. (2, 3, 18) To further complicate penetration, the drug chosen must not only be able to reach the intended target, it also must have the desired action, such as negating an inflammatory cascade or acting to slow or stop the proliferation of a pathogen. (19, 20) It has been reported in humans that ocular drug bioavailability is extremely poor due to these different barriers and is on the order of 5% or less. (4)

**Iontophoresis as a Treatment Modality**

Iontophoretic delivery of drug is a non-invasive procedure, and while it has been investigated in human medicine for many years, it has never been adopted as a standard of care in ophthalmology. (18) With its ease of application, minimal systemic side effects, and improved drug penetration, it has been used in transdermal application of medication for quite some time. (18). Ocular iontophoretic experimentation began around 1908 when the German investigator Dr. R. Wirtz passed an electric current through electrolyte-soaked cotton sponges that were placed over the globe attempting to treat corneal ulcers, keratitis, and episcleritis. (18, 21) At this time, the procedure was not referred to as iontophoresis, but catapheresis. An example of this “catapheresis” set can be seen in Figure 1-1. By the end of the century, iontophoretic experiments were being extensively conducted for the delivering of ophthalmic medications such as antibacterials, dyes, antiviral medications, antifungal medications, steroids, antimetabolites, and genes. This treatment modality seemed to be an answer to the low bioavailability of topically administered drugs and to the possible complications after intraocular
injections. Due to the lack of controlled trials and toxicity data, it has never been completely accepted as an alternative for traditional topical ocular drug delivery. (18) It has been considered but never used as a drug delivery device in the treatment of ocular disease in veterinary medicine, despite the promise that laboratory testing in rabbits has demonstrated. (18, 22-24) The rabbit corneal model has been studied for iontophoretic delivery of riboflavin for corneal collagen cross-linking to stop the progression of keratoconus. (22-24) Previous ex vivo and in vivo studies that involved the iontophoretic delivery of riboflavin and oligonucleotides suggest that this modality can be safe and effective at delivering drugs into the deep cornea that would not otherwise advance there with topical application alone. (22, 25)

The basic principle behind iontophoresis is that oppositely charged ions attract and similarly charged ions repel. More eloquently stated, iontophoresis is the electromechanical delivery of an agent via electromigration: a drug is applied to the corneal surface followed by an electric current that forces ions to move through the different layers of the cornea. (2, 18) Electrorepulsion occurs at the anode for positively charged drugs and at the cathode for negatively charged drugs. The *Nernst-Planck effect* is what drives the ionic-electric field interaction. (18) Two studies demonstrating enhanced delivery of neutral species by applying an electric field brought about modifications to the classical Nernst-Planck flux equation to include motion of the solvent. (18, 26, 27) This is termed *electroosmotic flow* and is defined specifically as the bulk fluid flow, which occurs when a voltage difference is forced across a charged membrane. (18, 28) A third mechanism, which aids in drug penetration is the *damage effect* of the electrical current. This increases tissue permeability. (18, 29) Along this same principle, a recent study with delivery of RNA interference-based oligonucleotides via iontophoresis suggests that delivery of solute increases tissue osmolarity. (30) Current density, duration of application, drug
concentration, and drug ionization must all be considered when planning iontophoretic delivery. (2)

When considering variables such as current density, duration of application, drug concentration, and drug ionization, it has been shown that increasing current density and duration of application will improve drug penetration. (31, 32) In one study using negatively charged hemisuccinate methyl prednisolone (HMP) for transscleral cathodal iontophoresis, it was found that aqueous humor concentrations reached 16.1 +/- 7.6µg/mL, 32.9 +/- 12.8µg/mL, and 46.0 +/- 27.6µg/mL when electric currents of 0.8, 2.0, and 4.0mA/cm², respectively, were applied to the rabbit globe for 4.0 minutes. (32) When applied topically without iontophoresis, aqueous humor concentrations only reached 0.6 +/- 0.5µg/mL.

If current density and duration of application is increased, drug penetration will be enhanced. The difficulty in dealing with these variables, though, is trying to establish reasonable parameters wherein no side effects are caused by large current densities or lengthy applications. In addition, particularly in veterinary medicine, prolonged procedures cannot occur without heavy sedation or even general anesthesia for the patient. The optimal conditions for iontophoretic use in veterinary ophthalmology would be an adequate current density that could penetrate to the target ocular tissue in a short amount of time so that only topical anesthetic or topical anesthetic plus a mild amount of sedation would be needed. Obviously, this treatment would need to have minimal to no side effects.

Multiple studies have been performed which show promise for successful tissue penetration in a reasonable time frame with minimal to no side effects. One study revealed that transscleral application of current densities up to 4mA/cm² for 10 minutes caused no adverse effects to the function or structures of rabbit globes. Furthermore, the authors treated one group
of rabbits at 4mA/cm² for 10 minutes and analyzed the globe after 24 hours. Two more groups were treated for 4 and 10 minutes at 4mA/cm² for three consecutive days, and the globes were analyzed 24 hours after the last treatment. No lesions were found at the application site in any group, and no changes were seen histologically. (32) In another study carried out with healthy human volunteers, a commercial transcleral iontophoretic device (OcuPhor – Salt Lake City, UT, USA) was used in applying currents from 0.1 to 4mA/cm² for time periods ranging from 20 to 40 minutes. (33) After application, the patients were to fill out a questionnaire reporting items such as blurred vision, pain, and sensitivity. A thorough ophthalmic examination performed by a boarded ophthalmologist was also carried out. Results from the questionnaire and ophthalmic examination showed that currents of up to 3.0mA for 20 minutes or 1.5mA for 40 minutes were well tolerated and caused no significant adverse effects in the ophthalmic assessment. (33) Another example was a study performed on both mouse cornea and skin and rabbit cornea using iontophoresis to deliver RNA interference-based oligonucleotides. Current densities of up to 5mA for 5 minutes were tested on both ex vivo rabbit and mouse corneas and 4mA for 5 minutes on in vivo rabbit corneas. The results showed no chemical or thermal damage to the corneas. The only side effect noted was immediate corneal edema, suggesting that the majority of the effect was due to the osmotic pressure of the delivered oligonucleotide. (30)

Not only have the effects of electric current density over time been studied, but the ionization of macromolecules and the mechanism at which they may contribute to corneal iontophoretic delivery has also been examined. (2) A study was performed with dextran and glyceraldehyde-3-phosphate dehydrogenase (GADPH) siRNA in C57BL/6 mice and both cathodal and anodal iontophoresis was carried out. (25) When anodal iontophoresis was performed, dextran, which is a non-ionized molecule, was more effectively delivered. This
revealed the possibility of electroosmosis in uncharged particles. On the other hand, GAPDH siRNA, a negatively charged molecule, was more effectively delivered using cathodal iontophoresis. This study demonstrated the ability of macromolecules to penetrate the cornea via electromigration.(25)

The basic design for an iontophoretic device is a direct current power source and two electrodes (See Figure 1-2). The ionized drug is placed in an electrode compartment that should bear the same charge, and the ground electrode is placed on the body. There are two approaches for drug retention in an iontophoretic device: an eye cup and a drug saturated gel as the delivery probe.(18)

An eye cup seems to be the most common example of drug retention for an iontophoretic device. An example of this system is the “Eyegate” made by Optis, France. In this system, the eye cup is filled with the drug to be delivered and placed on the surface of the cornea or sclera. Each eye cup has variable internal diameters depending on size of globe and if the drug is to be delivered transsclerally or transcorneally. A metal electrode is attached to the eye cup and submerged in the drug solution. There are two ports to the eye cup in which one delivers the drug solution while the other holds the metal electrode and aspirates air bubbles. Slight negative pressure is maintained in order for the eye cup to remain in contact with the treated surface. The ground electrode is usually attached to the ear of the animal, as close as possible to the eye cup electrode in order to avoid resistance.(18, 34-38) The Eyegate II system has been developed (EyeGate Pharma, Waltham, MA, USA) in order to deliver medications to both the anterior and posterior segments of the eye.(39, 40) See Figure 1-3 and 1-4. A phase II trial utilizing this system investigated the ocular iontophoretic delivery of dexamethasone phosphate in 102
patients with dry eye disease. The results showed that this drug delivery method significantly reduced the patients’ symptoms. (39)

Although not as common, the drug saturated delivery probe seems to be becoming more popular. One of the first described uses was in a study by Jones and Maurice who delivered fluorescein in the anterior chamber via a transcorneal approach with a fluorescein-saturated agar gel. (41) The gel was placed in a plastic tube and a small amount extruded to make direct contact with the cornea. Other similar studies were performed by Grossman et al and Frucht-Pery et al with gentamicin-saturated agar to perform transcorneal and transscleral iontophoresis in a rabbit model. (42, 43) The agar delivery probes were found not to be a good device as agar is too fragile and would remain on the cornea or sclera after the procedure was finished.

More recent studies have used drug-loaded hydrogels with more successful outcomes. An example is OcuPhor™ by Iomed Inc., Salt Lake City. This system is for transscleral iontophoresis. The drug applicator is made of a small silicone shell containing a patented silver-silver chloride ink conductive material, a hydrogel pad to absorb the drug, and a small, flexible wire to connect the conductive material to the dose controller. (33, 44-46) Before administration, the dry hydrogel matrix is hydrated with the drug solution to be used and placed against the sclera in the conjunctival fornix of the patient. See Figure 1-5. The ground electrode can be positioned anywhere on the body.

Another example is the Visulex™ by Aciont Inc., USA for use in drug delivery to the posterior segment via transscleral iontophoresis. See Figures 1-6 – 1-8 to view loading and application of the system. A study by Hastings et al reported on its efficacy. Its applicator has been engineered with a selective membrane in order to increase drug transport by excluding non-drug ions. The system has two compartments, one in which contains the drug ion to be delivered
and the other that contains a counter ion that will precipitate the drug after the interaction. This makes the drug ions to be delivered the primary carrier of electrical current through the sclera and aids in getting the drug to the target tissue rather than being lost to superficial tissues. This also reduces the need for frequent applications.\(^{(2, 47)}\) Multiple studies have used a polyacrylic-porous hydrogel saturated with gentamicin or dexamethasone for transcorneal or transscleral iontophoresis.\(^{(31, 43, 48, 49)}\) When comparing the two different iontophoretic application modalities, the hydrogel applicators may be more convenient, have less current interruptions, and be less harmful to the ocular tissues compared to the eye cup.\(^{(18)}\)

**Keratomycosis**

Keratomycosis is a disease that is prevalent in both human and veterinary medicine. It is a cause of morbidity and frustration, as treatment for the disease can be difficult and protracted. In veterinary ophthalmology, keratomycosis seems to be most prevalent in equines and can result in loss of vision, eye removal, and in the case of some patients, euthanasia due to vision loss in one or both eyes. Horses seem to be more susceptible to keratomycosis because of multiple factors: commensal but opportunistic fungi of the corneal and conjunctival surfaces, the large amount of fungi in the environment, prominent globes with a large corneal surface, use of topical corticosteroids and antimicrobials, local or systemic immunodeficiencies, and hereditary factors.\(^{(50-55)}\) A lack of stability and integrity of the precorneal tear film and corneal injury can predispose and encourage fungal adhesion, invasion, and colonization as well.\(^{(51, 56)}\) Keratomycosis can manifest as microerosions, ulcerative keratitis, fungal plaques, keratomalacia, and stromal abscess.\(^{(50)}\)

There are a variety of ophthalmic diseases that could benefit from iontophoretic drug delivery including, but not limited to, fungal keratitis, uveitis, retinitis, retinoblastoma, proliferative vitreal retinopathy, and various retinal degenerations.\(^{(1)}\) Ocular fungal disease is a
particularly frustrating condition that is difficult to manage medically in part because of the limited ability of antifungal drugs to penetrate into the deeper layers of the cornea. In addition, the equine cornea has limited ways of reacting to injury. The epithelial layer can increase up to fifteen layers after an injury. For superficial, noninfected ulcers, a rapid and linear response is usually seen over the first five to seven days. Epithelial cell migration of midstromal, noninfected ulcers is approximately 0.6mm/day in horses.(57) The epithelial basement membrane does not completely adhere to the stroma for approximately six weeks post-injury.(58)

Keratomycosis can affect the more superficial layers of the cornea, but in many cases, the fungi seem to have an affinity for the deeper layers, particularly the posterior stroma adjacent to Descemet’s membrane and Descemet’s membrane itself.(19, 20) As the organisms move through the stromal collagen lamellae, destructive proteases such as matrix metalloproteinases, including MMP-2 and MMP-9, alkaline protease, and elastase are secreted. The MMPs and serine protease neutrophil elastase are thought to be the major components to collagenolysis or corneal “melting” (ie keratomalacia) based on a study in which there were elevated levels of these enzymes in affected equine tear film.(50, 59) Moreover, inflammatory cytokines are released and with their release, polymorphonuclear leukocytes and T lymphocytes are recruited. More stromal damage occurs from degranulation of the recruited cells. This vicious cycle continues until medications and/or the immune response can stop the enzymatic activity.(19, 20) Tissue degradation can continue even if sterility of the corneal surface is achieved. Lastly, it is not uncommon for keratomalacia to be rapid and progressive of a superficial ulcer can develop into a descemetocele or a full-thickness perforation in a matter of 24-48 hours.(50)
Keratomycosis has a worldwide distribution, with variations in pathogenic fungal species, clinical manifestations, treatment strategies, and visual outcomes. (19, 60-62) The disease appears to be highly prevalent in warmer, temperate, and/or humid environments (61, 63) and is particularly problematic in human beings and horses. (64-67) Other species can be affected as well with reported cases in the literature in canines, (68-70) felines, lagomorphs, (71) camelids, (72) reptiles, avian species and a bovine. (73) Keratomycosis is a significant cause of corneal blindness and can threaten the integrity of the entire globe as well. Horses seem especially prone to reflex uveitis from corneal disease. When injured, nerve endings in the cornea arising from the ophthalmic branch of the trigeminal nerve trigger an inflammatory response in the iris and ciliary body (ie iridocyclitis). The inflammation causes iris and ciliary body muscle spasm and miosis. Additionally, ciliary body spasm as a result of the inflammatory process causes severe pain. (50, 56) Pain is manifested as blepharospasm, excessive lacrimation, photophobia, and self-trauma. As the severity of uveitis increases, signs such as aqueous flare, hypopyon, and/or hyphema, may be evident. Although keratomycosis-induced uveitis can be variable, medical management of the intraocular inflammation is necessary when treating keratomycosis either medically or in the post-operative period as permanent ocular sequelae such as synechia and cataract can cause visual compromise or blindness. (50, 56)

The three most prevalent fungal pathogens associated with keratomycosis in equine patients are Aspergillus spp., Fusarium spp., and Candida spp. (60-63, 65, 74) Other species that have been reported with regularity include Papulaspora (63, 67) and Mortierella spp., (60) while Alternaria, Eurotium, Rhizopus, Cladosporium, and Penicillium spp. were frequently found in the conjunctival flora in European horses. (75) Although not as common, many different fungal species have been reported to cause keratomycosis in dogs, but not one particular organism
seems to predominate. The fungi identified in canine cases include *Aspergillus*, *Scedosporium*, *Candida*, *Cladosporium*, *Chrysosporium*, *Penicillium*, *Curvularia*, and *Phialemonium* spp. (68-70) *Aspergillus* and *Fusarium* spp. (72) were most frequently cultured in alpacas, (72) *Aspergillus fumigatus* has been reported in a pet rabbit, (71) and *Eurotium amstelodami* was reported in a Brown Swiss cow. (73)

Diagnostic testing can be important when trying to identify etiology of keratitis and differentiate between a sterile keratitis, bacterial keratitis, fungal keratitis, or combination of bacterial and fungal infections. These tests may include fluorescein staining, rose Bengal staining, tear film breakup time, superficial and deep corneal cytology, superficial and deep corneal cultures for identification of pathogens and determination of their susceptibility patterns, and corneal histopathology if surgery is performed. (51, 56) Rose Bengal retention is indicative of poor quality of tear film or tear film instability and can be associated with a poor prognosis for healing. (56, 76) Cytology should be performed with a sterile handle of a scalpel blade, Kimura cytology spatula or cytology brush. (77) Although fungal organisms may not always be present on a cytologic example, this does not necessarily exclude the possibility of fungal infection. When fungal organisms are found, this is a significant finding usually indicative of causative or complicating pathology. (56) Laser scanning confocal microscopy can aid in the diagnosis of equine fungal disease and confirm the presence of organisms in the deeper layers of the cornea without the need for histopathology. (78-82) Even in cases where culture or histopathology may not identify fungal involvement, fungal DNA may be present and identified by polymerase chain reaction. (50, 78)

As with any microbial infection, effective treatment of keratomycosis requires selection of an antifungal chemotherapeutic agent that has an appropriate spectrum of action. Additionally,
the selected agent must be able to penetrate the cornea in order to reach therapeutic levels at the affected location (i.e. superficial, middle, or deep cornea or the anterior chamber). Currently, voriconazole is the most commonly employed topical antifungal drug due to its broad spectrum of activity (covers both filamentous fungi and yeasts) and its ability to penetrate the cornea relative to other drugs. However, voriconazole is not appropriate in every case of keratomycosis (susceptibility and resistance patterns, etc.) and does not have the ability to penetrate completely unhindered. For instance, a study of 68 horses with keratomycosis in the Northeastern United States showed that natamycin, nystatin, or clotrimazole were recommended as initial topical treatment. Studies have also shown that agents that may seem unconventional or are not traditionally classified as antifungal agents, such as povidone-iodine and silver sulfadiazine, can have success in treating fungal keratitis. There is a great need to develop drugs or new strategies to medically manage keratomycosis. If antifungal drugs or unconventional antimicrobial agents that would have efficacy against the offending fungi could be delivered to the site of disease in effective concentrations, the medical management of keratomycosis would improve greatly and result in better outcomes with fewer lost globes and less vision limiting scar formation. The iontophoretic delivery of antifungal agents into the cornea appears to be a promising approach.

**Objective**

The purpose of this project is to utilize iontophoresis to improve delivery of antifungal agents to the deeper layers of the cornea, with the ultimate goal of improving the success of medical management of keratomycosis.

**Specific Aims and Hypotheses**

Specific Aim 1: To determine if it is possible to electro-migrate a variety of anti-fungal chemotherapeutic agents within an agarose gel via electrophoresis.
**Hypothesis 1:** Movement of the anti-fungal chemotherapeutic agents will be possible and visualized within their respective agarose gel lane.

**Specific Aim 2:** To evaluate Nystatin and Voriconazole concentrations in ex vivo rabbit corneas via an iontophoretic delivery method. In addition, to determine the optimal parameters for this iontophoretic drug delivery.

**Hypothesis 2:** Adequate tissue concentrations of antifungal chemotherapeutics in the cornea will be achieved via iontophoresis.
Figure 1-1. Example of Dr. R. Wirtz Catapheresis Set c.1908. (Photo courtesy of http://eyeantiques.com/shop/museum/other-ophthalmic-objects-museum/ocular-iontophoresis-set/)
Figure 1-2. Basic design of iontophoretic device. (Photo courtesy of author)

Figure 1-3. EyeGate II Applicator. (Photo courtesy of http://www.biotuesdays.com/features/2016/1/19/eyegate-developing-non-invasive-ophthalmic-treatments)
Figure 1-4. Eyegate II Iontophoresis Delivery System. (Photo courtesy of http://www.eyegatepharma.com/technology/iontophoresis-delivery-system/)

Figure 1-5. OcuPhor applicator placed in the lower conjunctival fornix of a human patient. (Photo courtesy of https://www.researchgate.net/figure/OcuPhor-iontophoresis-system-inserted-in-the-human-eye-Applicator-is-placed-on-the_fig9_221979693)
Figure 1-6. Loading Visulex System with drug. (Photo courtesy of http://www.aciont.com/technologies/visulex)

Figure 1-7. Placing Visulex System on human patient’s cornea. (Photo courtesy of http://www.aciont.com/technologies/visulex)
Figure 1-8. Visulex System adhered to patient’s cornea. (Photo courtesy of http://www.aciont.com/technologies/visulex)
CHAPTER 2
IN VITRO PREPARATORY EXPERIMENTATION WITH ELECTROMIGRATION AND EXTRACTION METHODS

In establishing an experimental protocol, it is important to run preliminary testing so that experiments can be run efficiently and effectively. With the objective in mind of driving an antifungal agent into the deeper layers of the cornea via iontophoresis, preliminary testing was performed with agarose gel electrophoresis. The theory behind utilizing agarose gel electrophoresis initially was to establish if the antifungal agents showed movement in a medium when a voltage was applied, thus could the chemotherapeutic agents be electromigrated at all? Furthermore, electrophoresis and iontophoresis are related as both utilize electromigration to move ions through a specific medium. Electrophoresis electromigrates ions through a polymer where iontophoresis electromigrates ions through biologic tissue.(18, 84)

Amphotericin B (AmB) was used in preliminary experiments because of its size, charge, and coloration. It is a very large molecule (924.091g/mol) with a formal charge of 0.(85) It was believed that if this molecule was able to be electromigrated through an agarose gel medium, then smaller antifungal agents could be moved through the medium with minimal difficulty. In addition, its amber color allowed for visualization of the medication through the gel.

Gel Electrophoresis and Amphotericin B

Part I

An agarose gel was created using \textbf{100mL} of tris acetate EDTA (TAE) (1:50 strength) and \textbf{1g} agarose powder. The two components were mixed thoroughly in a beaker and then placed into a microwave for \textbf{2 minutes}. The gel was then plated in the EasyCast electrophoretic device (Owl Separation Systems, LLC, Newington, NH, USA), and a \textbf{20-well} comb was inserted into the gel as it was allowed to solidify. Once in solid form, the comb was removed, leaving the wells. The amphotericin B (AmB) was reconstituted to an unknown concentration. The TAE buffer was
then poured into the electrophoretic apparatus until the gel mold was immersed in the liquid. The AmB was mixed with 30% sucrose. Approximately 10μL of AmB and 10μL of 30% sucrose were pipetted (Gilson Pipetman Pipette, Gilson, Inc., Middleton, WI, USA) into every other well (10 total wells with 20μL/well). The apparatus was set to 80mV and allowed to run. When no movement was seen at 20 minutes, the voltage was increased to 100mV. When no movement was seen at 30 minutes, the voltage was increased to 130mV. The apparatus was allowed to run for 3 hours.

Part II

An agarose gel was created using 50mL of TAE (1:50 strength) and 0.5g agarose powder. The two components were mixed thoroughly in a beaker and then placed into a microwave for 2 minutes. The gel was then plated in the EasyCast electrophoretic device (Owl Separation Systems, LLC, Newington, NH, USA), and a 10-well comb was inserted into the gel as it was allowed to solidify. Once in solid form, the comb was removed, leaving the wells. The TAE buffer was then poured into the electrophoretic apparatus until the gel mold was immersed in the liquid. The AmB was mixed with 30% sucrose. Approximately 2.5μL of AmB and 2.5μL of 30% sucrose were pipetted (Gilson Pipetman Pipette, Gilson, Inc., Middleton, WI, USA) into the first 5 wells (5μL/well), and then approximately 5μL of AmB and 5μL of 30% sucrose were pipetted (Gilson Pipetman Pipette, Gilson, Inc., Middleton, WI, USA) into the next 5 wells (10μL/well). The apparatus was set to 130mV and allowed to run. The apparatus was allowed to run for 1.5 hours.

Gel Electrophoresis and Gentamicin, Fluorescein Dye, and Nile Blue

Just as AmB was used initially in the preliminary electrophoresis experiments, gentamicin, fluorescein dye, and Nile blue were also utilized to improve the experimental
procedure. Similarly to AmB, fluorescein dye and Nile blue were utilized due to their vibrant coloration and thus ability to visualize their electromigration. Gentamicin was used due to frequent application with iontophoresis in lab animal models, such as rabbits and rats, and in vivo studies dealing with delivery of the drug via transcleral and transcorneal routes.\textsuperscript{(38, 42, 43, 49, 86, 87)}

An agarose gel was created using 100\\text{mL} of TAE (1:50 strength) and 1g agarose powder. The two components were mixed thoroughly in a beaker and then placed into a microwave for 2 minutes. The gel was then plated in the EasyCast electrophoretic device (Owl Separation Systems, LLC, Newington, NH, USA), and a 16-well comb was inserted into the gel as it was allowed to solidify. Once in solid form, the comb was removed, leaving the wells. 0.3g of gentamicin powder was reconstituted with 1\\text{mL} of double distilled water (ddH2O). A fluorescein strip (BioGlo 1mg Fluorescein Sodium Ophthalmic Strip, HUB Pharmaceuticals, LLC, Rancho Cucamonga, CA) was added to 25\\text{mL} of ddH2O until the liquid was a fluorescent yellow. 0.25g of Nile Blue chloride powder and approximately 5\\text{mL} ddH2O were placed in a beaker. A magnet was placed in the beaker, and the beaker was placed on a Nuova II stir plate (Analytical Instruments, LLC, Minneapolis, MN, USA) for approximately 20-30 minutes while the rest of the equipment was made ready. The TAE buffer was then poured into the EasyCast electrophoretic apparatus (Owl Separation Systems, LLC, Newington, NH, USA) until the gel mold was immersed in the liquid. 10\mu L of each substance (gentamicin, fluorescein, and Nile Blue) was pipetted (Gilson Pipetman Pipette, Gilson, Inc., Middleton, WI, USA) into 6 wells. Approximately 10\mu L of 30\% sucrose was added via pipette into the previously mentioned 6 wells.

a. Gentamicin – Well 1, 2
b. Fluorescein – Well 3, 6
c. Nile Blue – Well 7, 8
Note: Wells 4 and 5 were skipped due to blue appearance to the gel in these lanes.

The apparatus was set to 139mV and allowed to run for approximately 80 minutes. The gel was removed from the apparatus and the cathode lanes, anode lanes, and wells were separated via a reinforced 1.5in single edge razor blade (Stanley Black & Decker, Inc, New Britain, CT, USA) for each substance (18 samples in total). The lanes were placed in 15mL plastic vials (Southern Labware, Cumming, GA, USA). The wells were placed in 2mL Eppendorf tubes (Thermo Fischer Scientific, Wilmington, DE, USA). Approximately 1mL of gentamicin, 1mL of fluorescein, and 1mL of Nile Blue were also placed in 2mL Eppendorf tubes (controls). The samples were all placed in a -20°C freezer.

Continued Experimentation with Gentamicin, Nile Blue, and Fluorescein

The vials with the gel lanes and the pure liquid gentamicin, fluorescein, and Nile blue (N.B.) were removed from the freezer and allowed to thaw. The vials that contained the wells were left in the freezer until needed. Each agarose lane was separated into 4 equal portions (approx. 1.5cm in length but ranged from 1.5 – 1.6 – 1.75cm) with a 1.5in single edge razor blade. Each portion was placed in a 2mL Eppendorf tube and labeled as such: Drug Name, # (lower # closer to well, higher # farther away from well), Cat or Ano (Cathode/Anode Side), and circled # (signified the lane). The Freeze n’ Squeeze (FnS) method was implemented. One sample was placed in freezer for 12 minutes, then spun for 12 minutes; the second sample was kept in the freezer for 24 minutes, then spun for 24 minutes. The wells were kept in the freezer the whole time while processing and performing FnS on lanes. The wells were then removed from the freezer and placed in the centrifuge for 12 minutes. All pure samples were run through the NanoDrop™ 2000 (Thermo Fisher Scientific, Wilmington, DE, USA) approximately 3 times; lane samples were run through the NanoDrop™ 2000 (Thermo Fisher Scientific, Wilmington,
DE, USA) approximately 2 times (possibly 3 – 4 if problems occurred with the machine). Data was then recorded and saved via Excel.

Another two-part experiment/practice was carried out in which processing was performed about one month after preparation. Previously used gentamicin (300mg/mL) was inoculated in well 3, 6, and 9; previously used fluorescein (unknown concentration) was inoculated in well 12 and 15. 10μL of gentamicin was initially inoculated into their respective wells, then 10μL of 30% sucrose was then added. The same process was performed with the fluorescein. After the electrophoretic device was allowed to run for 45 minutes at 128mA, the lanes were separated according to cathode and anode side - separated into 4 equal portions (approx. 1.5cm in length but ranged from 1.5 – 1.6 – 1.75 cm) with a 6500 Beaver blade (Beaver-Visitec International, Inc., Waltham, MA, USA). Each portion was placed in a 2mL Eppendorf tube and labeled as such: Drug Name, # (lower # closer to well, higher # farther away from well), Cat or Ano (Cathode/Anode Side), and circled # (signified the lane. The wells were placed in 2mL Eppendorf tubes. The vials were stored in -80°C freezer. This is where the preparation ended.

NOTE: Before cutting of the lanes began, residual liquid was removed from each well and stored in a 1.5mL plastic vial. These vials were placed in the refrigerator rather than freezer. Only gentamicin lanes were removed. The fluorescein was used as a control to ensure drug was moving through gel as it is a smaller molecule and tends to migrate through agarose gel easily. See Figure 2-1. Total samples included 3 liquid gentamicin, 3 wells, 12 anode sections, 12 cathode sections. The FnS method was implemented. The samples were removed from the freezer, then spun in the centrifuge for 12 minutes at 13,000 X g. Supernatant was removed from centrifuged tubes and measured via NanoDrop™ 2000 (Thermo Fisher Scientific, Wilmington, DE, USA). Data was recorded and saved via Excel.
Creating a Serial Dilution Standard Curve Using Gentamicin and Finding the Most Appropriate Extraction Method

Not only was it important to try and establish an efficient and effective protocol for moving a chemotherapeutic agent through an agarose gel medium, it was just as important to establish the appropriate protocol to extract the agent from the medium. This is vital in establishing that an appropriate amount of drug, such as a minimum inhibitory concentration, is reaching the target tissue. The serial dilution standard curve was created in order to know exactly how much drug, in this case gentamicin, was being admixed into the agarose gel medium. In addition, the serial dilution standard curve served to be an evaluation as to which extraction method was generally the most accurate and which extraction method could record the smallest amount of drug.

An agarose gel was created using 25mL of TAE (1:50 strength), 0.375g agarose powder, and 0.25g gentamicin powder. The three components were mixed thoroughly in a small plastic weigh dish and placed into a microwave for 30 seconds. After removing from the microwave, the gel was then allowed to solidify. A serial dilution was made with TAE and gentamicin. The concentrations were as follows:

a. 500mg/mL  
b. 250mg/mL  
c. 125mg/mL  
d. 62.5mg/mL  
e. 31.25mg/mL  
f. 15.625mg/mL

An 8.0mm punch biopsy (Integra®/Miltex®, York, PA, USA) was used to remove samples from the gel. A cotton-tipped applicator was used to push the samples from the punch biopsy into 2mL Eppendorf tubes and Freeze N Squeeze Gel Extraction Spin Columns/centrifuge tubes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). A 1.5in single edge
razor blade was used to cut the circular samples in half (the samples were too wide to fit in the centrifuge tubes), and the two halves were placed in the centrifuge tubes. 9 samples were collected in total. 6 samples were placed in 2mL Eppendorf tubes and 3 samples were placed in centrifuge tubes. 1mL of acetone was mixed with 3 samples in the 2mL Eppendorf tubes. 1mL of acetonitrile was mixed with 3 samples in the 2mL Eppendorf tubes. Nothing was added to the centrifuge tubes – 3 samples in total. The filter tubes were placed in a -20°C freezer for 5 minutes. These samples were then placed in an Eppendorf centrifuge (Eppendorf North America, Hauppauge, NY, USA) and allowed to spin for 5 minutes at 13,000 x g. The time was increased from 3 minutes to 5 minutes due to the still “wet” appearance of the gel during the last experiment. The samples with the acetone and acetonitrile were spun in a centrifuge for 5 minutes at 13,000 x g. This method known as the “freeze-squeeze” recovery method was described by Thuring et al. (88) The supernatant was pulled from all tubes (both 2mL and centrifuge tubes) and placed in 1.5mL vials. All samples (serial dilution, supernatants, precipitates, and agarose gel) were stored in a refrigerator. Samples were removed from the refrigerator the following day.

Spectrophotometric measurements were taken via NanoDrop™ 2000 (Thermo Fisher Scientific, Wilmington, DE, USA) and its associated data collection program on all samples. Approximately 3 measurements were taken from each sample. More measurements were taken on certain samples in which the machine revealed an abnormal measurement (“flat line” or “sawtooth” appearance to curve). The goals of the spectrophotometric measures were to find the absorption spectrum of the serial dilution of gentamicin, find the absorption “peak” of gentamicin, and compare the different extraction methods’ absorptions at this particular peak. Sample measurements for the serial dilution were averaged via Excel and linear and curvilinear
regression lines were created through the data points. The slope, y-intercept, and correlation coefficient were calculated for both. The gentamicin absorbance at the absorbance peak was then compared to the serial dilution standard curve.

**Continued Development of the Appropriate Extraction Method**

The serial dilution made with TAE and gentamicin was carried out to two more concentrations (1/64th and 1/128th). The concentrations were as follows:

a. 500mg/mL  
b. 250mg/mL  
c. 125mg/mL  
d. 62.5mg/mL  
e. 31.25mg/mL  
f. 15.625mg/mL  
g. 7.8125mg/mL  
h. 3.90625mg/mL

This time no filter was used for the freeze n’ squeeze (FnS) method; the centrifuge tubes were placed in a -20°C freezer for **5 minutes**. These samples were then placed in a centrifuge and allowed to spin for **5 minutes** at 13,000 x g. No supernatant was visualized, so the samples were again placed in the -20°C freezer for **6.5 minutes** and then re-run in the centrifuge for **6 minutes** at 13,000 x g. Spectrophotometric measurements were taken via NanoDrop™ 2000 (Thermo Fisher Scientific, Wilmington, DE, USA) and its associated data collection program on all samples. Approximately **3 measurements** were taken from each sample. More measurements were taken on certain samples in which the machine revealed an abnormal measurement (“flat line” or “sawtooth” appearance to curve). This time before measuring the acetone and acetonitrile samples, the machine was properly calibrated. The gentamicin absorbance at the absorbance peak (**307nm**) was then compared to the serial dilution standard curve, which was created with the two new concentrations added to the data. The concentration of each sample was also calculated via the new linear regression equation (standard curve).
Continued Development of the Freeze n’ Squeeze Extraction Method

An agarose gel was created using **25mL** of TAE (1:50 strength) and **0.375g** agarose powder. The two components were mixed thoroughly in a small plastic weigh dish and then placed into a microwave for **30 seconds**. The gel then was allowed to solidify. **6 samples** were taken via an **8.0mm** punch biopsy and placed in different **1.5mL** plastic vials. 2 different Freeze n’ Squeeze (FnS) methods were implemented:

a. 6.5 minute freeze, 6 minute centrifuge; 6 minute freeze, 6 minute centrifuge (FnS X 2 method)

b. 12.5 minute freeze, 12 minute centrifuge (FnS Long method)

These samples were spun at 13,000 x g in the centrifuge.

Spectrophotometric measurements were taken via NanoDrop™ 2000 (Thermo Fisher Scientific, Wilmington, DE, USA) and its associated data collection program on all samples. **3 measurements** were taken from each sample. The gentamicin absorbance at the absorbance peak (**307nm**) was then compared to the serial dilution standard curve. The concentration of each sample was also calculated via the previously created linear regression equation (standard curve).

Testing Accuracy and Precision with Different Concentrations of Gentamicin Spiked Agarose Gel

Agarose gels were created using **10mL** of TAE (1:50 strength) and **0.15g** agarose powder. The two components were mixed thoroughly in small plastic weigh dishes and then placed into a microwave for **12 seconds**. The gel then was allowed to solidify. **6 total gels** were created with the following concentrations of gentamicin: **0mg/mL, 2.5mg/mL, 5mg/mL, 10mg/mL, 15mg/mL, and 20mg/mL**. Three **1.5mL** vials were labeled for each concentration (18 total). The gels were placed in a refrigerator. The following day, an **8.0mm** punch biopsy was used to take samples from each of the **6 concentrations** of agarose gel (18 total samples). A
cotton-tipped applicator was used to push the samples from the punch biopsy into the pre-labeled 1.5mL plastic vials. The 1.5mL tubes were placed in a -20°C freezer for 12 minutes. These samples were then placed in a centrifuge and allowed to spin for 12 minutes at 13,000 x g. The supernatant was hard to visualize so the process was repeated (12min freezer, 12min centrifuge).

Spectrophotometric measurements were taken via NanoDrop™ 2000 (Thermo Fisher Scientific, Wilmington, DE, USA) and its associated data collection program on all samples. 3 measurements were taken from each sample. The gentamicin absorbance at the absorbance peak (307nm) was then compared to the previously created serial dilution standard curve. The concentration of each sample was also calculated via the previously created curvilinear regression equation (standard curve).

**Electrophoresis of Antifungal Drugs**

5 anti-fungal medications were reconstituted to the concentrations listed below:

a. 5% natamycin
b. 100,000IU/mL nystatin
c. 1% miconazole
d. 2% miconazole
e. 10mg/mL posaconazole
f. 1% ketoconazole
g. 4% ketoconazole

Spectrophotometry was used to analyze the natamycin, nystatin, posaconazole, and both ketoconazole concentrations. Error messages were received when analyzing the natamycin, nystatin, and 4% ketoconazole likely due to the opacity of the solutions. Measurements could be taken for the posaconazole (consistent results) and 1% ketoconazole (inconsistent results). The miconazole and ketoconazole reconstituted poorly (miconazole worse than ketoconazole). When trying to mix the powdered drug with sterile water, heavy particulate matter resulted and would not mix into solution or form a suspension. Because of this heavy particulate seen in both
miconazole concentrations, the drug was left overnight on a rotator/mixer to see if constant agitation would help create a better suspension. It was decided that due to the opacity of most of the antifungals that had been reconstituted, the researchers would proceed by trying to visualize movement rather than measure concentrations of drug within the gel or within the respective well.

An agarose gel was created with **10 wells**. 1% ketoconazole was inoculated into lane 1, 4% ketoconazole in lane 3, natamycin in lane 5, nystatin in lane 7, and 2% miconazole in lane 9. The electrophoretic device was set to **135-137 mA** and was allowed to run for **30 minutes**.

**Electrophoresis of 1% and 4% Ketoconazole, 5% Natamycin, and 100,000 IU/mL Nystatin**

An agarose gel was created with **10 wells**. 1% ketoconazole was inoculated into lane 1, 4% ketoconazole in lane 3, natamycin in lane 5, and nystatin in lane 7. All of the antifungal agents sit well distributed throughout their respective wells. The electrophoretic device was set to **139 mA** and was allowed to run for **30 minutes**. The gel was made with less agarose during this experiment to decrease the gel density. Making a **0.5%** agarose gel was attempted, but it was too soft and fell apart. A **0.75%** agarose was created that did not fall apart and was used in the experiment.

**Continued Electrophoresis of 1% and 4% Ketoconazole, 5% Natamycin, and 100,000 IU/mL Nystatin**

A **0.75%** agarose gel was created with ten wells. 1% ketoconazole was inoculated into lane 1, 4% ketoconazole in lane 3, natamycin in lane 5, and nystatin in lane 7. **5 μL** of antifungal and **5 μL** of **15%** sucrose were inoculated into each well. All of the antifungal agents sit well distributed throughout their respective wells.

The electrophoretic device power setting started at **118 mA**, then reached **146 mA** twenty-five minutes into the experiment, then was decreased to **135 mA** at this time. Total running time
of electrophoresis was **45 minutes**.

**Continued Electrophoresis of 5% Natamycin, and 100,000 IU/mL Nystatin**

The natamycin and nystatin were centrifuged at 13,000 × g for **5 minutes** and supernatant was pulled from each sample. A **0.75%** agarose gel was created with ten wells. Natamycin was inoculated in lane 1, a supernatant of natamycin was inoculated in lane 3, nystatin in lane 5, and a supernatant of nystatin in lane 7. **5μL** of antifungal and **5μL** of **15%** sucrose were inoculated into each well. Immediately after power source was turned on it was noted that the nystatin was already attracted to the anode side. The power setting started at **103mA** and reached a peak of **133mA**. Total running time of electrophoresis was about **50 minutes**.

This type of experiment was carried out approximately five more times in order to confirm that the nystatin had the most movement in the agarose gel well.
Figure 2-1. Fluorescein movement visualized in gel electrophoresis lanes highlighted by red arrows (Photo courtesy of author)
CHAPTER 3
RESULTS

Phase One Experimentation: In Vitro Gel Electrophoresis

Initial Antifungal Testing

Five anti-fungal medications were reconstituted to respective concentrations utilized clinically both in the human and veterinary literature - 5\% Natamycin, 100,000 U/mL Nystatin, 1\% and 2\% Miconazole, 10mg/mL Posaconazole, and 1\% and 4\% Ketoconazole. The miconazole and ketoconazole reconstituted poorly (miconazole worse than ketoconazole). Heavy particulate could be seen in both miconazole concentrations, so the drug was left overnight on a rotator/mixer to see if constant movement would help create a better solution. It was decided that due to the opacity of most of the antifungals that had been reconstituted, the researchers would proceed by trying to visualize movement rather than measure concentrations within the agarose gel medium. An agarose gel was created with ten wells. 1\% ketoconazole was inoculated into lane 1, 4\% ketoconazole in lane 3, natamycin in lane 5, nystatin in lane 7, and 2\% miconazole in lane 9. Figure 3-1 represents the gel prior to initiation of current. The electrophoretic device was set to 135-137mA and was allowed to run for 30 minutes. Figure 3-2 shows the gel after running the device for 30 minutes. No movement from the wells was apparent with any medication.

Use of More Opaque Antifungal Medications

An agarose gel was created with ten wells. 1\% ketoconazole was inoculated into lane 1, 4\% ketoconazole in lane 3, natamycin in lane 5, and nystatin in lane 7 (seen as bottom to top in initial picture). Figure 3-3 demonstrates the gel appearance prior to initiation of current. The electrophoretic device was set to 139mA and was allowed to run for 30 minutes. The gel was made with less agarose during this experiment to decrease the gel density. Initially, a 0.5\%
agarose gel was made, but was too soft and fell apart. A 0.75% agarose gel was then created that did not fall apart and thus was used in the experiment. Figure 3-4 represents the gel after running the device for **30 minutes**. The reasoning for making a less dense gel was the hope that it was the molecular weight causing the medication to remain in the well. If the gel was less dense, it would allow the larger molecules to move. Once again, no movement was seen with any of the antifungal medications from their respective wells.

**Initial Movement**

A 0.75% agarose gel was created with ten wells. 1% ketoconazole was inoculated into lane 1, 4% ketoconazole in lane 3, natamycin in lane 5, and nystatin in lane 7. 5μL of antifungal and 5μL of 30% sucrose were inoculated into each well. Figure 3-5 demonstrates the gel appearance prior to initiation of current. The power setting started at 118mA, then reached 146mA twenty-five minutes into the experiment, then was decreased to 135mA at this time. Total running time of electrophoresis was **45 minutes**. Figure 3-6 shows the gel after running the device for **45 minutes**. The 1%/4% ketoconazole did not appear to have any movement. They were still dispersed at the bottom of wells #1 and #3. The natamycin and nystatin appeared to move within their respective wells. While they did not move out of their wells and into a cathode or anode “lane,” they were pressed against the anode side of their respective wells. The nystatin seemed to be completely pressed against the anode side of the well, while the natamycin was partially pressed against the anode side of the well (some drug remained at the bottom of the well).

**Gel Electrophoresis with Natamycin and Nystatin Only**

The natamycin and nystatin were centrifuged at 13,000 X g for **5 minutes** and supernatant was pulled from each sample. A 0.75% agarose gel was created with ten wells.
Natamycin was inoculated in lane 1, a supernatant of natamycin was inoculated in lane 3, nystatin in lane 5, and a supernatant of nystatin in lane 7. 5µL of antifungal and 5µL of 30% sucrose were inoculated into each well. Figure 3-7 represents the appearance of the gel prior to initiation of current. The power setting started at 103mA and reached a peak of 133mA. Total running time of electrophoresis was approximately 60 minutes. Figure 3-8 represents the gel after running the device. Once again, it appeared that the nystatin and natamycin moved within their respective lanes. The supernatants could not be visualized, as there was no cloudiness or opacity to these liquids. This experiment was carried out five more times with the same results: the natamycin and nystatin moved within their respective wells. Supernatant was no longer examined, as it could not be visualized under white light or UV light conditions. The nystatin seemed to have the most movement as all of the solution would electromigrate to the side of the well. The natamycin would indeed show movement, but some of the solution would also remain at bottom of its respective well. One last thing to note in this experimental series is that when reproducing these results for the fifth consecutive time, the nystatin seemed to have some movement outside of its well. This can be seen in Figure 3-9 and 3-10. After establishing that nystatin had the most movement among the opaque antifungal medications, the second phase of experimentation was begun: iontophoresis of nystatin on the ex vivo rabbit cornea.
Figure 3-1. Experiment 1 – Initial Antifungal Testing. Location of antifungals before current was applied - 1% ketoconazole was inoculated into lane 1, 4% ketoconazole in lane 3, natamycin in lane 5, nystatin in lane 7, and 2% miconazole in lane 9. (Photo courtesy of author)
Figure 3-2. Experiment 1 – Initial Antifungal Testing. No movement from the wells was apparent with any medication. (Photo courtesy of author)
Figure 3-3. Experiment 2 – Use of More Opaque Antifungal Medications. Location of antifungals before current was applied. 1% ketoconazole was inoculated into lane 1, 4% ketoconazole in lane 3, natamycin in lane 5, and nystatin in lane 7 (seen as bottom to top in initial picture). (Photo courtesy of author)
Figure 3-4. Experiment 2 – Use of More Opaque Antifungal Medications. No movement from the wells was apparent with any medication. (Photo courtesy of author)
Figure 3-5. Experiment 3 – Initial Movement. Location of antifungals before current was applied. 1% ketoconazole was inoculated into lane 1, 4% ketoconazole in lane 3, natamycin in lane 5, and nystatin in lane 7. (Photo courtesy of author)
Figure 3-6. Experiment 3 – Initial Movement. Movement within the wells were seen for Natamycin and Nystatin, wells 5 and 7 respectively. Note the slight migration of natamycin up against the left side of its respective well (red arrow) and the moderate to significant migration of nystatin against the left side of its respective well (red arrow). (Photo courtesy of author)
Figure 3-7. Experiment 4 – Gel Electrophoresis with Natamycin and Nystatin Only. Location of antifungals before current was applied. Natamycin was inoculated in lane 1, a supernatant of natamycin was inoculated in lane 3, nystatin in lane 5, and a supernatant of nystatin in lane 7. (Photo courtesy of author)
Figure 3-8. Experiment 4 – Gel Electrophoresis with Natamycin and Nystatin Only. Movement seen within wells 1 and 5 (natamycin and nystatin, respectively). The natamycin and nystatin supernatants in well 3 and 7, respectively, could not be visualized. (Photo courtesy of author)
Figure 3-9. Experiment 4 – Gel Electrophoresis with Natamycin and Nystatin Only. Movement seen within well 1 (natamycin) and outside of well 6 (nystatin). The red arrows are pointing to a faint opaque line of nystatin. (Photo courtesy of author)
Figure 3-10. Experiment 4 – Gel Electrophoresis with Natamycin and Nystatin Only. Movement seen within well 1 (natamycin) and outside of well 6 (nystatin). The red arrow and red circle are highlighting the movement of nystatin. (Photo courtesy of author)
CHAPTER 4
MATERIALS AND METHODS – EX VIVO RABBIT CORNEA MODEL

No live animals were used for the purposes of this research. All animal tissues were handled in accordance and protocols for this experiment were approved by the University of Florida Institutional Animal Care and Use Committee.

Once it was established that nystatin could be electromigrated in an agarose gel medium, the next phase of testing was conducted with biologic tissue, specifically rabbit corneas. The iontophoretic device was used to electromigrate nystatin into this ex vivo rabbit cornea model. In addition, the iontophoretic device was used to electromigrate 1% voriconazole into the ex vivo rabbit cornea model in the third phase of this research study. The corneas were then homogenized and the resulting homogenate was evaluated via high-performance liquid chromatography (HPLC). The purpose was to separate, identify, and quantify the nystatin and the voriconazole in the corneal tissue.

**Basic Iontophoresis Protocol**

A previously frozen then thawed rabbit globe (Pel-Freez, LLC., Rogers, AR, USA) with the optic nerve facing down was placed in a Styrofoam tray with wells (test tube shipping package). See Figure 4-1. Gauze was placed into the well before the globe was added so that the globe would not sink down into the well. Three gauze 2X2 sponges (Medtronic, Minneapolis, MN, USA) were placed in a plastic weigh boat and soaked with 1X basic HEPES solution (pH 7.85). Circular holes were cut in the center of the sponges and Parafilm squares (Bemis Company, Inc., Oshkosh, WI, USA). Approximately 5-10 mL of acidic HEPES solution was loaded in a syringe and set aside. A pipette (Gilson Pipetman Pipette, Gilson, Inc., Middleton, WI, USA) was set to 250 µL, and 100,000IU/mL nystatin plus 15% sucrose was drawn up and set aside. For phase three of the study, a pipette (Gilson Pipetman Pipette, Gilson, Inc.,
Middleton, WI, USA) was set to 250µL, and 1% voriconazole plus 15% sucrose was drawn up and set aside. Two gauze 2X2’s that had been soaked with the basic solution were placed over the iontophoretic device cup and then placed onto the rabbit cornea. The cup was sitting centered on the cornea and the sponges were wrapped snuggly around the device. The thin platinum outer ring attached to the anode (red) was placed back around the cup and sat on top of the 2X2’s. Another soaked 2X2 was placed on top of the platinum electrode. Lastly, a piece of Parafilm (Bemis Company, Inc., Oshkosh, WI, USA) was placed on top of the upper 2X2. See Figure 4-2. The power on the Phoresor II Auto PM850 (Iomed, Inc., Salt Lake City, UT, USA) was set. For example, a power setting of 20mA X min meant 4mA for 5 minutes. With a gloved hand, pressure was applied to the cup meaning the cup was slightly pinched while simultaneously pressing down on Parafilm. See Figure 4-3. This created a seal between the cup and the cornea so that no liquid could leak while the iontophoresis was occurring. Approximately 1mL of acidic buffer was added to the cup from the syringe that had been previously set aside. The drug/sucrose solution that had been set aside in the pipette was then added to the base of buffer inside cup, closest to the corneal surface to minimize mixing. See Figure 4-4. The delivery electrode attached to the cathode (black) was placed inside the cup and then the iontophoresis process was begun. When completed, the cup was removed, and pictures were taken before the cornea was rinsed with ddH2O, after the cornea was rinsed with ddH2O, and after the cornea had been removed at the limbus from the rest of the globe. When the cornea were removed, pictures were taken with ambient lighting and with UV lighting. See Figures 4-5 – 4-7. In addition, corneal “buttons” were created with an 8.0mm punch biopsy (Integra®Miltex®, York, PA, USA) and placed in a -80°C; high-performance liquid chromatography (HPLC) was performed within 24 hours. Corneal buttons were protected from ambient lighting.
Preparation of Standard Solution

A 100,000IU/mL nystatin suspension was created from nystatin powder (Sigma Aldrich, St. Louis, MO, USA) and ddH2O. This concentration was obtained from The Code of Federal Regulations, Title 21 – Food and Drugs, Subchapter E – Animal Drugs, Feeds, and Related Products, Part 524 – Ophthalmic and Topical Dosage Form New Animal Drugs, Section 524.1600b. There is no other current literature regarding nystatin concentrations for ophthalmic use. Each container of nystatin powder (Sigma Aldrich, St. Louis, MO, USA) contained 5 million IU of drug and weighed 852mg. Simple mathematics was performed to establish the concentration as 17.04 mg/mL and taken one step further, 85mg/5mL to create a 100,000IU/mL suspension. Once the ddH2O was added to the nystatin powder, the container holding the suspension was placed on a Fisher Vortex Genie 2 (Scientific Industries, Inc., Bohemia, New York, USA) for 5 minutes to break up larger particles. Once this was accomplished, the stock solution was stored in the freezer, protected from ambient light, in aliquots to be used for individual experiments.

A 1% voriconazole solution was created from voriconazole powder (Vfend® I.V., Pfizer Pharmaceuticals, New York, NY, USA) and ddH2O. This concentration was obtained from multiple studies found in the current veterinary literature. In these studies, this concentration of voriconazole was administered topically, subconjunctivally, and intrastromally. All three forms of drug administration proved to be safe for the corneal surface and anterior chamber (75, 89). Each container of voriconazole powder (Vfend® I.V., Pfizer Pharmaceuticals, New York, NY, USA) contained 200mg of drug. It was reconstituted with 9.5ml of ddH2O in order to obtain 10ml of a 20mg/ml solution. (90) Once this was accomplished, the stock solution was stored in the freezer, protected from ambient light, in aliquots to be used for individual experiments. A 2% preparation was created so that once the 30% sucrose was added to the stock voriconazole
before performing iontophoresis, the sucrose concentration would be 15% and the voriconazole concentration would be 1%.

**Homogenization of Corneal Tissue**

For the nystatin treated corneas, corneal buttons were allowed to thaw which took approximately 5 – 10 minutes. Each corneal button was placed in a Safe-Grind plastic coated tissue grinder (Wheaton Industries Inc., Millville, NJ, USA) with 250μL Phosphate Buffered Saline with Tween 20 (PBS-T). The tissue was ground for 3 minutes by manual force. The homogenate was then stored either in the freezer, protected from ambient light, or used for HPLC experimentation that day. Each corneal button was placed in a Safe-Grind plastic coated tissue grinder (Wheaton Industries Inc., Millville, NJ, USA) with 250μL Phosphate Buffered Saline with Tween 20 (PBS-T). The tissue was ground for 3 minutes by manual force. The homogenate was then stored either in the freezer, protected from ambient light, or used for HPLC experimentation that day.

For the voriconazole treated corneas, the same process was performed on three corneal buttons. The other three corneal buttons were placed in a PYREX® Ten Broeck Homogenizer (Corning Inc., Corning, NY, USA). The tissue was ground for 3 minutes by manual force. The homogenate was then stored either in the freezer, protected from ambient light, or used for HPLC experimentation that day.

**Chromatographic System**

A Waters Alliance 2695 Separations Module was used with a Waters Alliance 2996 Photodiode Array Detector (PAD) (Waters Corporation, Milford, MA, USA). Chromeleon 7.2 Chromatography Data System Software (ThermoFisher Scientific, Waltham, MA, USA) was used to analyze all the HPLC data. The parameters were as follows:
• PAD detection at 306nm was used.
• Gradient HPLC was performed at 40°C at a flow-rate of 1.0mL/min.
• Mobile phase was constituted by acetonitrile:water.
• Retention time was about 14 – 15min.
• Injection volume was 100uL.
• Total running time was about 29min.

Source (91). See Figures 4-8 – 4-11.

**Experimental Groups**

There were a total of 5 experimental groups. The first group was a control group. This group consisted of globes that had drug in contact with the corneal surface for 5 minutes, but no current was delivered by the iontophoresis device. During the voriconazole testing, two more control groups were added: no current for 7.5 minutes and no current for 10 minutes. The next experimental group consisted of globes that had drug in contact with the corneal surface for 2.5 minutes. The following group contained globes that had drug in contact with the corneal surface for 5 minutes. The next group consisted of globes that had drug in contact with the globe for 7.5 minutes. The last group contained globes that had drug in contact with the globe for 10 minutes. These four groups had 4 mA of current delivered to the cornea for each respective amount of time.
Figure 4-1. Rabbit globe placed in Styrofoam tray at beginning of iontophoresis run (Photo courtesy of author)

Figure 4-2. Platinum loop attached to anode has been added over the iontophoresis cup, and gauze 2X2 has been placed over it. (Photo courtesy of author)
Figure 4-3. Pressure being applied to the iontophoresis cup. (Photo courtesy of author)
Figure 4-4. Nystatin being added to the iontophoresis cup. The tip of the pipette is close to the corneal surface. (Photo courtesy of author)

Figure 4-5. Ex vivo cornea prior to rinsing. (Photo courtesy of author)
Figure 4-6. Ex vivo cornea following rinse. (Photo courtesy of author)

Figure 4-7. Ex vivo cornea imaged with UV illumination. (Photo courtesy of author)
Figure 4-8. Waters Alliance HPLC System. (Photo courtesy of author)

Figure 4-9. Waters Alliance HPLC System with attached computer and Chromeleon software. (Photo courtesy of author)
Figure 4-10. Waters Alliance 2695 Separations Module. (Photo courtesy of author)

Figure 4-11. Waters Alliance 2996 Photodiode Array Detector. (Photo courtesy of author)
CHAPTER 5
RESULTS

Phase Two Experimentation: High-Performance Liquid Chromatography (HPLC) with an Ex Vivo Rabbit Cornea Model - Nystatin

A serial dilution of nystatin, specifically a ten-fold serial dilution, was performed in order to create a standard curve for HPLC measurements. By using Chromeleon 7.2 Chromatography Data System Software (ThermoFisher Scientific, Waltham, MA, USA), the following equation was created:

\[ y = 1656.7464x + 11.9921 \]

where 1656.7464 is the slope and 11.9921 is the y-intercept. An R-squared value of 0.9994 was also created with the software. See Figure 5-1.

Iontophoresis was performed on a total of eighteen corneas. The procedure was carried out as described in Basic Iontophoresis Protocol in the Materials and Methods section of this publication. There were a total of five corneal groups: one control group in which drug contacted the corneal surface for five minutes but no current was delivered and four experimental groups. The experimental groups consisted of corneas in which a 4mA current was applied for 2.5, 5, 7.5, and 10 minutes. The appearance of a cornea before and after nystatin was rinsed from the surface can be seen in Figures 5-2 and 5-3. Five corneas were used per group, except for the 10-minute experimental group in which six corneas were used due to abnormal results from the initial three corneas tested. Data was collected via software for the height and area under the curve found for the nystatin peak on the chromatogram. All data are listed in Table 5-1.

For the control group with no iontophoretic current, a mean height of 18.431mAU and mean area of 10.350mAU*min was found. The mean height and mean area of the 2.5-minute iontophoretic group was 25.427mAU and 15.184mAU*min, respectively. For the 5-minute iontophoretic group, a mean height of 21.354mAU and mean area of 12.651mAU*min was
found. For the **7.5-minute** iontophoretic group, a mean height of **65.856mAU** and mean area of **37.486mAU*min** were found. The mean height and mean area for the **10-minute** iontophoretic group was **19.806mAU** and **11.150mAU*min**, respectively.

From the equation derived from the standard curve, concentrations for samples were calculated. These concentrations were calculated by utilizing both height and area values. The mean concentration when using height values for the control and the **2.5-minute**, **5-minute**, **7.5-minute**, and **10-minute** experimental groups were **30,546.9328IU/mL**, **42,138.6352IU/mL**, **35,389.6025IU/mL**, **109,118.1308IU/mL**, and **32,826.0636IU/mL**, respectively. The mean concentration when using area values for the control and the **2.5-minute**, **5-minute**, **7.5-minute**, and **10-minute** experimental groups were **17,159.870IU/mL**, **25,168.582IU/mL**, **20,971.491IU/mL**, **62,117.340IU/mL**, and **18,485.267IU/mL**, respectively.

Lastly, the mass was calculated for all samples using the following equation:

$$(\text{Extracted Mass/Extracted Volume}) / 2 = \text{HPLC Concentration}$$

Mass = HPLC concentration * 2 * Extracted Volume or

Mass = HPLC Concentration * 0.2mL

where **0.2mL** represented 2 multiplied by an extracted volume of **0.1mL**. The HPLC device would extract a volume of **0.1mL** per sample, so when creating a sample, **0.1mL** of homogenate was mixed with **0.1mL** PBS to ensure an adequate volume was delivered to the HPLC system. Therefore, the extracted mass/extracted volume had to be divided by a value of 2. When utilizing concentrations derived from height values, the mean mass was **6,109.387IU** for the control, **8,427.727IU** for **2.5 minutes** of iontophoresis, **7,077.920IU** for **5 minutes** of iontophoresis, **2,182.626IU** for **7.5 minutes** of iontophoresis, and **6,565.213IU** for **10 minutes** of iontophoresis. The mass for the control was **3,431.974IU** and masses for the experimental groups
of 2.5 minutes, 5 minutes, 7.5 minutes, and 10 minutes were 5,033.716IU, 4,194.298IU, 12,423.468IU, and 3,697.053IU, respectively. This was using concentrations derived from the area under the curve. If all drug was delivered into the cornea, a theoretical value of 25,000IU should have been obtained. This was derived from the equation:

\[ \text{Mass} = 0.250\text{mL} * 100,000\text{IU/mL} \]

where 0.250mL was the volume of nystatin injected into the iontophoretic device and 100,000IU/mL was the concentration of the stock solution to be delivered. By examining this value and all values obtained through calculation, no drug had 100% yield in the cornea. Figures 5-4 and 5-5 show what appears to be drug deposition in the cornea illuminated by white and UV light, respectively.

**Phase Three Experimentation: High-Performance Liquid Chromatography (HPLC) with an Ex Vivo Rabbit Cornea Model – Voriconazole**

Iontophoresis was performed on a total of thirty corneas. The procedure was carried out as described in *Basic Iontophoresis Protocol* in the Materials and Methods section of this publication. There were a total of five corneal groups: one control group in which drug contacted the corneal surface for five minutes but no current was delivered and four experimental groups. The experimental groups consisted of corneas in which a 4mA current was applied for 2.5, 5, 7.5, and 10 minutes.

Six corneas were used per group. Data was collected via software for the area under the curve found for the voriconazole peak on the chromatogram. All data are listed in Table 5-2. Data was also evaluated and compared for corneas that were homogenized with the plastic on glass (P) homogenizer and glass on glass (G) homogenizer. These data are listed in Table 5-3.

For the control group with no iontophoretic current, a mean area of 22.301mAU*min was found. The mean area of the 2.5- minute iontophoretic group was 22.402mAU. For the 5-
minute iontophoretic group, a mean area of **31.529mAU*min** was found. For the 7.5-minute ionotophoretic group, a mean area of **40.552mAU*min** was found. The mean area for the 10-minute iontophoretic group was **36.390mAU*min**. When comparing the homogenization methods, the P group area had a value of **25.766mAU*min** and the G group had a mean area of **35.504mAU*min**.

After obtaining these numbers from the HPLC software, a more thorough statistical analysis was performed. First a one-way ANOVA was performed for the control and experimental groups. A p-value of **0.0237** was obtained, signifying a statistically significant difference among these groups (see Table 5-4). Due to this result, two sample student t-Tests assuming unequal variances were run comparing all the groups head-to-head. The statistically significant results were as follows (p-values): no current ↔ 7.5min – **0.0149**, no current ↔ 10min – **0.0285**, 2.5min ↔ 10min – **0.0208**, 2.5min ↔ 10min – **0.0472**. The p-value obtained between the homogenization method groups was **0.00404** signifying a statistically significant difference (see Table 5-5).

Lastly, **12** more corneas were run from experimentation to homogenization and through HPLC. Six corneas acted as a control with no current for 7.5min, and six corneas acted as a control with no current for 10min. The p-value obtained between the 7.5min no current and 7.5min with current was **0.319** (see Table 5-6), and the p-value obtained between the 10min no current and 10min with current was **0.410** (see Table 5-7). Therefore, there was no statistically significant difference among these groups.
Figure 5-1. Graph of standard curve with values for slope, y-intercept, and R-Square. (Photo courtesy of author)
Figure 5-2. Rabbit cornea immediately after being treated with nystatin and iontophoresis. Note the circular, yellow haze of nystatin still left on the cornea. (Photo courtesy of author)

Figure 5-3. Rabbit cornea immediately after being treated with nystatin and iontophoresis. Note the circular, yellow haze of nystatin still left on the cornea. (Photo courtesy of author)
Figure 5-4. Rabbit cornea after removing from the globe and illuminated with white light. Note the corneal haze or opacification. (Photo courtesy of author)

Figure 5-5. Rabbit cornea after removing from the globe and illuminating with UV light with the goal of highlighting opacities not visualized with white light. Note the multifocal to coalescing corneal opacities. (Photo courtesy of author)
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<td>4009.737</td>
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</tr>
<tr>
<td>Avg</td>
<td>11.086</td>
<td>6.214</td>
<td>18378.35134</td>
<td>10307.346</td>
<td>3675.670</td>
<td>2061.469</td>
</tr>
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</tr>
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<tr>
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<td>1.564</td>
<td>6025.981532</td>
<td>2603.143</td>
<td>1205.196</td>
<td>520.629</td>
</tr>
<tr>
<td>Avg</td>
<td>11.086</td>
<td>6.214</td>
<td>18378.35134</td>
<td>10307.346</td>
<td>3675.670</td>
<td>2061.469</td>
</tr>
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<td>1.564</td>
<td>6025.981532</td>
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<td>1205.196</td>
<td>520.629</td>
</tr>
<tr>
<td>Sealed 5min (11/2018)</td>
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<td>0</td>
<td>11.9921</td>
<td>11.9921</td>
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<td>2.398</td>
</tr>
<tr>
<td>Avg</td>
<td>14.015</td>
<td>8.118</td>
<td>23231.2929</td>
<td>13461.791</td>
<td>4646.259</td>
<td>2692.358</td>
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<tr>
<td>Avg</td>
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<td>13461.791</td>
<td>4646.259</td>
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</tr>
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<td>11.9921</td>
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<td>2.398</td>
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Table 5-2. Area Under the Curve (mAU*min) – Control vs Experimental Groups for Voriconazole Testing

<table>
<thead>
<tr>
<th>Sealed No Current</th>
<th>Sealed 2.5min</th>
<th>Sealed 5min</th>
<th>Sealed 7.5min</th>
<th>Sealed 10min</th>
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</thead>
<tbody>
<tr>
<td>9.237</td>
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<td>30.192</td>
<td>24.955</td>
<td>34.461</td>
</tr>
<tr>
<td>25.076</td>
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<td>14.532</td>
<td>25.228</td>
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<td>33.139</td>
<td>26.326</td>
<td>46.081</td>
<td>44.536</td>
<td>28.633</td>
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<tr>
<td>22.355</td>
<td>33.146</td>
<td>44.899</td>
<td>49.466</td>
<td>51.067</td>
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<td>22.637</td>
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<td>52.107</td>
<td>34.236</td>
</tr>
<tr>
<td>21.363</td>
<td>27.458</td>
<td>31.780</td>
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<td>47.073</td>
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Table 5-3. Area Under the Curve (mAU*min) – Plastic on Glass Homogenizer vs Glass on Glass Homogenizer for Voriconazole Testing

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<thead>
<tr>
<th>Plastic on Glass</th>
<th>Glass on Glass</th>
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<td>18.28</td>
<td>33.146</td>
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<tr>
<td>2.948</td>
<td>26.256</td>
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<tr>
<td>26.326</td>
<td>27.458</td>
</tr>
<tr>
<td>30.192</td>
<td>44.899</td>
</tr>
<tr>
<td>14.532</td>
<td>21.689</td>
</tr>
<tr>
<td>46.081</td>
<td>31.780</td>
</tr>
<tr>
<td>24.955</td>
<td>49.466</td>
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<td>44.536</td>
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Table 5-4. ANOVA: Voriconazole Control and Experimental Groups – Area Under the Curve (mAU*min)

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<th>MS</th>
<th>F</th>
<th>P value</th>
<th>F crit</th>
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</thead>
<tbody>
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<td>303.0029</td>
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<tr>
<td>Within Groups</td>
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<td></td>
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</table>

Table 5-5. ANOVA: Voriconazole Homogenization Techniques – Area Under the Curve (mAU*min)

<table>
<thead>
<tr>
<th>Sources</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P value</th>
<th>F crit</th>
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<tr>
<td>Within Groups</td>
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<td>136.008745</td>
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<td></td>
</tr>
<tr>
<td>Total</td>
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### Table 5-6. t-Test: Two-Sample Assuming Unequal Variances Between 7.5min Control Group with No Current vs 7.5min Iontophoresis Group (Voriconazole)

<table>
<thead>
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<th></th>
<th>Control 7.5min</th>
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</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean</strong></td>
<td>33.03266667</td>
<td>40.55233333</td>
</tr>
<tr>
<td><strong>Variance</strong></td>
<td>159.0778199</td>
<td>149.7603923</td>
</tr>
<tr>
<td><strong>Observations</strong></td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td><strong>Hypothesized Mean Difference</strong></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>df</strong></td>
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<td>10</td>
</tr>
<tr>
<td><strong>t Stat</strong></td>
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<tr>
<td><strong>P(T&lt;=t) one-tail</strong></td>
<td>0.159628599</td>
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<tr>
<td><strong>t Critical one-tail</strong></td>
<td>1.812461123</td>
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<tr>
<td><strong>P(T&lt;=t) two-tail</strong></td>
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<tr>
<td><strong>t Critical two-tail</strong></td>
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### Table 5-7. t-Test: Two-Sample Assuming Unequal Variances Between 10min Control Group with No Current vs 10min Iontophoresis Group (Voriconazole)

<table>
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</thead>
<tbody>
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<tr>
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<td><strong>Observations</strong></td>
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</tr>
<tr>
<td><strong>Hypothesized Mean Difference</strong></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>df</strong></td>
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<td>10</td>
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<td><strong>t Stat</strong></td>
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<td><strong>P(T&lt;=t) one-tail</strong></td>
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<td>0.409716106</td>
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<tr>
<td><strong>t Critical one-tail</strong></td>
<td>1.812461123</td>
<td>2.228138852</td>
</tr>
<tr>
<td><strong>P(T&lt;=t) two-tail</strong></td>
<td>0.409716106</td>
<td>0.409716106</td>
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<tr>
<td><strong>t Critical two-tail</strong></td>
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CHAPTER 6
DISCUSSION

Drug delivery and pharmacokinetics have a foundation laid by biology, chemistry, physiology, and physics. Whether a disease has generalized impact throughout the body or is localized to a specific organ, the proper medication must be chosen and delivered in a manner that ensures it reaches its target. Therefore, drug delivery and pharmacokinetics must be both efficacious and efficient to most effectively treat a disease or condition.

Research and development of anti-infective drugs is very expensive. Initially, a drug must be created or discovered that will cause an interaction or an effect on a specific pathogen. Will the drug eradicate an organism (“cidal” drug)? Will the drug stop an organism from multiplying (“static” drug)? These questions are critical to the research and development process and inform questions of dose and delivery. Then, once a drug has proven its efficacy, the next questions posed are, “How can this drug be delivered to the target site, or biophase, and are the drug concentrations high enough to treat the pathogen once it gets there?”(92)

No matter the part of the body a drug is being delivered to, it will encounter physical, mechanical, and chemical barriers. Barriers to ocular drug delivery include, but are not limited to, corneal membrane barriers, conjunctiva and scleral membrane barriers, blood-ocular barriers, efflux transporters, tear washout, blinking and nasolacrimal drainage.(92) These barriers may affect a drug by a small amount, large amount, or not at all. An advantage to ocular pharmacotherapy, whether in the human or veterinary field, is that in most cases, medications are applied close to the biophase with minimal systemic side effects. By the time a drop is absorbed by the pericorneal or ocular tissue, diluted by the precorneal tear film, or washed away by mechanical forces (ie. blinking), there is minimal drug to be administered systemically. Systemic routes may include drainage into the nasolacrimal system and then ingestion, absorption through
mucous membranes, absorption by ocular vasculature, or intraocular penetration and diffusion through the blood-aqueous barrier if there is compromise to this biologic wall. While the likelihood of systemic absorption is low, it can occur and has been documented in both human and veterinary medicine. At-risk patients include those with lower body weights, infants, elderly people, and pregnant or nursing patients. (93) Some systemic diseases associated with ocular administration seen both in the human and veterinary sector have been renal tubular acidosis with carbonic anhydrase inhibitor use, bradycardia with beta blocker use, and hypertension with phenylephrine use. (93-96) A major problem also arises to pharmacotherapy in human and veterinary medicine in compliance. In veterinary medicine, this can be seen through owner compliance as the patients do not administer their own medications, while in human medicine it can be seen through patient compliance to the physician’s prescribed instructions. (92)

The objective of this study was to utilize iontophoresis to improve delivery of antifungal agents to the deep layers of the cornea, with the ultimate goal of improving the success of medical management of keratomycosis. The first goal was to determine if electromigration of at least one antifungal agent in a test medium, specifically agarose gel, is possible. The researchers then took the information they gained from the gel electrophoresis testing and applied it to iontophoresis testing of nystatin in an ex vivo rabbit cornea model. While the researchers were not able to quantify the depth to which the drug reached after electromigration, they were able to demonstrate that a large, uncharged molecule, specifically nystatin, can be successfully delivered into the corneal tissue via iontophoresis. Furthermore, the researchers were able to use the information gained from the assays in the first and second phase of this study to successfully electromigrate a more clinically relevant antifungal medication such as voriconazole. To the authors’ knowledge, no studies have been initiated to specifically address the utility of
Iontophoresis for ophthalmic clinical cases in veterinary medicine. However, studies have been performed in the laboratory animal sector in order to validate this type of drug delivery method for human use in ophthalmology and dermatology.\(^{(18)}\)

Iontophoresis for ophthalmic purposes has been studied for over one hundred years but has never been adopted as a standard of care due to the lack of controlled trials and toxicity data. It was originally investigated for the treatment of corneal ulcers, keratitis, and episcleritis. Electric current was passed through electrolyte-saturated cotton sponges that were placed over the globe. Wirtz had particularly beneficial effects on serpiginous corneal ulcers treated with 0.5% zinc sulfate at 2mA for 1 minute, interstitial keratitis treated with 1% sodium iodide solution, and episcleritis treated with chlorine ions from 0.9% sodium salt solution.\(^{(18, 97)}\) As more research was carried out, iontophoresis was found to be useful in treating both anterior and posterior segment disease.\(^{(18)}\)

In veterinary ophthalmology, advancements in drug delivery systems are necessary since traditional methods of administering topical medications can be challenging. Applying topical medications such as drops or ointments can be cumbersome for a client in addition to being irritating for the animal. Compliance is improved if frequency is decreased or sustained delivery can be achieved. In horses, the subpalpebral lavage system was developed so as to ensure drug delivery to the ocular surface when patient resistance may be an impediment.\(^{(98)}\) Furthermore, there have been previous studies to develop continuous infusion systems for topical medications. A subconjunctivally implanted micro-osmotic pump has been evaluated. This device was developed to reduce labor of treatment and improve pharmacologic effects of the administered drug. These devices seemed to be well tolerated by horses in a study by Blair et al. and allowed effective delivery of atropine.\(^{(99)}\) Another study evaluating continuous medication delivery
utilized a pump connected to a subpalpebral lavage system. In this study, tear film kinetic of fluorescein was assessed. Fluorescein was applied at a constant rate to evaluate if a stable drug concentration could be maintained at the ocular surface. (100) Recently, thermogels for depot delivery in the subconjunctival space have been investigated. Cuming et al. investigated a voriconazole releasing thermogel in horses. The voriconazole diffused through the cornea and sclera in all groups, but permeation seemed greater through the sclera than the cornea. (101) Allbaugh et al. evaluated voriconazole concentrations in equine tears following subconjunctival injection of antifungal medication combined with thermosensitive poloxamer gel. These researchers found that the subconjunctival injection of the combined voriconazole and gel only had a drug presence of about three hours, which was not the prolonged effect that they were aiming for. (102) While iontophoresis is not a continuous infusion of medication, it is a relatively non-invasive drug delivery method, which if successful, could prove to decrease the frequency of medications and prevent the need for surgical intervention in cases of keratomycosis.

The first aim of this study was to try and electromigrate a variety of antifungal agents within an agarose gel via electrophoresis. This initial step was vital in the study to determine if drug would move when a current was applied to it. If this phase in the research process was left out, then many inappropriate antifungal agents would have been tested in the second phase of the study. These drugs would have likely had minimal to no corneal penetration, as the applied current could not drive them.

Initially, seven antifungal agents were evaluated. These agents included: 5% natamycin, 100,000IU/mL nystatin, 1% and 2% miconazole, 10mg/mL posaconazole, and 1% and 4% ketoconazole. These concentrations were chosen because they are routinely used or are available for ophthalmic use. After performing the first experiment with these medications, the field was
reduced to the four most opaque antifungal chemotherapeutic agents. Posaconazole was a clear solution and could not be visualized after being applied or loaded into its respective agarose gel well. Miconazole was eliminated from the study because it did not reconstitute into a homogenous solution well. Instead, large particulate matter could be seen floating in the solvent even after continual mixing on a platform rotator. The major advantages of using the most opaque antifungal agents was that their movement caused by electromotive forces could be directly visualized. Thus, further microscopic or chemical analysis was not necessary in evaluating whether or not an agent actually traveled in the testing medium. This made preliminary testing more efficient and financial resources were able to be allocated toward the ex vivo testing. A major disadvantage of using the most opaque antifungal agents was that these drugs were not the most clinically relevant for treating equine keratomycosis.

The four antifungal agents that were then evaluated were 5% natamycin, 100,000IU/mL nystatin, and 1% and 4% ketoconazole. These antifungal agents were the most opaque and could be visualized the best when delivered into their respective gel wells. Because drug movement could be visualized, it was not necessary to quantify drug concentrations in the respective gel lanes. Photographs were taken before and after current was applied to the gel to record the extent of movement in each respective gel lane. Once again the field was narrowed down after this step. 5% natamycin and 100,000IU/mL nystatin were further tested as they appeared to show movement. While they did not initially appear to migrate out of their respective well, they did show movement within their wells.

Before moving on to the HPLC phase of the research study, multiple electrophoresis runs were carried out with 5% natamycin and 100,000IU/mL nystatin. Consistent migration out of the wells was not observed. However, nystatin did appear to be electromigrated into the anode side
of the gel during one experimental run. See Figure 6-1 as this shows a cloudy, fluorescent material that has migrated out of what appears to be the nystatin well. The common finding that was seen during the gel electrophoresis phase of the study was that as soon as current was applied to the gel, nystatin would dramatically shift to the anode side of its well. Natamycin would also shift to the anode side of the well, but its movement was more gradual and not as profound. See Figure 6-2 and note how nystatin seems to be pushed against the left side of its respective well, while natamycin has slowly migrated toward that side of its respective well.

With the timeliness and degree of movement seen by the nystatin, it was decided that this antifungal chemotherapeutic agent would be utilized in phase two of this experimental study. This antifungal agent is classified as a polyene macrolide antibiotic and was discovered around 1950. Nystatin has gone by the trade name Mycostatin. The polyene antifungal agents were the first major group of antifungal drugs to be discovered and are a group of structurally similar products of *Streptomyces* spp. Other well-known antifungal agents in this group are amphotericin B and natamycin. (64) This group is poorly absorbed and has limited penetration into the eye, cerebrospinal fluid, and joint capsule. Nystatin, specifically, is too toxic for parenteral administration and it is not absorbed well after oral administration.

Polyene agents are fungicidal in nature, and they employ a barrier disruption mechanism of action. Polynes irreversibly bind to ergosterol, the main sterol in the fungal cell membrane. This binding leads to increased membrane permeability, inhibition of cytochrome P-450, and inhibition of the electron transport chain. (64) Because the fungal cell membrane is more permeable, this allows for leakage of potassium and essential metabolites and damage to the concentrating mechanisms of the organism. This leads to loss of ammonium, inorganic phosphate, low-molecular-weight carboxylic acids, phosphate esters, nucleotides, and proteins.
Aerobic and anaerobic glycolysis and respiration is caused by cation leakage.(64) Hydrogen ions replace the lost cations and decrease the internal pH until there is lysosome disruption, autolysis, and cell death. Susceptibility of fungal organisms is determined by amounts of sterol in the cell membrane.

Polyenes have a broad spectrum of activity against antifungal agents. Species include *Aspergillus*, yeast (especially *Candida* spp. – except *Candida lusitaniae*), *Mucor*, *Rhisopus*, *Blastomyces*, *Coccidioides*, *Histoplasma*, *Paracoccidioides brasiliensis*, *Sporotrichum*, *Torulopsis*, and *Sporothrix schenckii*. Activity varies against *Prototheca*, *Curvularia*, *Alternaria*, *Wangiella*, and *Cladosporium*, but nystatin is active against *Cryptococcus*, *Prototheca*, some filamentous fungi, some dimorphic fungi, and *Trichophyton*. All polyenes are cytotoxic to some degree but cytotoxicity may be decreased by their higher affinity for fungal ergosterol compared to mammalian cholesterol.(64)

Although a topical formation is described by Ford 2004, little is known about nystatin and appropriate dosing.(64) To the authors’ knowledge, minimal to no research has been performed with nystatin for veterinary use. The formulation for nystatin topical ophthalmic suspension is as follows: pure nystain, 100,000 IU, in 5 mL of sterile isotonic, isohydric phosphate buffer solution [20mL of NaH$_2$PO$_4$ (8.0g in 1L of H$_2$O) and 80mL NaH$_2$PO$_4$ (9.47g in 1L of H$_2$O)] are mixed; NaCl (0.44g) is added, and the solution is sterilized.(64) Another topical formulation is described in the United States Code of Federal Regulations. It is described as an ophthalmic ointment that includes nystatin, neomycin sulfate, thiostrepton, and triamcinolone. The formulation is as follows: Each cubic centimeter of ointment contains – 100,000 units of nystatin, neomycin sulfate equivalent to 2.5 milligrams of neomycin base, 2,500 units of thiostrepton, and 1.0 milligram of triamcinolone acetonide.(103) The conditions for use included
anti-inflammatory, antipruritic, antifungal (*Candida albicans*), and antibacterial for keratitis and conjunctivitis in dogs and cats and for infectious keratoconjunctivitis (pink eye) in cattle.

Directions stated that it could be used as frequently as two to three times daily and as infrequently as once daily to once weekly. (103)

Based on this information, it was decided that an appropriate nystatin dose for this study would be 100,000IU/mL. This was the dose described by the Code of Federal Regulations for the ophthalmic ointment and a similar dose as described by Ford 2004. (64, 103) The researchers recognize that nystatin was not the optimal antifungal drug to utilize for antifungal research due to minimal use in both human and veterinary medicine, but it was appropriate for this proof of concept study. Nystatin has a molecular weight of 926.107g/mol and a formal charge of 0. (104) So this drug not only could be visualized well, but also showed that a large, uncharged molecule could be electromigrated via electrophoresis in an agarose gel medium. This showed promise for future studies in which using a smaller, charged molecule should lead to increased efficiency in drug penetration.

The second aim of this study was to evaluate nystatin and voriconazole concentrations in ex vivo rabbit corneas via an iontophoresis delivery method. By completing the initial aim of the study, the researchers were able to determine that nystatin was an opaque antifungal agent that could be electromigrated. They could then focus on a more clinically relevant model with ex vivo rabbit corneas. In this phase of the study, the researchers wanted to evaluate the concentration and mass of the drug that was actually found in the cornea after iontophoresis via high-performance liquid chromatography.

Current to electromigrate both the nystatin and voriconazole into the rabbit corneas was held constant during the second and third phases of the study, while amount of time for drug
delivery was changed per experimental group. The current was chosen from previous success with iontophoretic delivery of RNA interference-based oligonucleotides in ex vivo mouse and rabbit corneas and in vivo rabbit corneas. The amount of current (4mA) in these previous experiments proved safe for the corneal surface and also did not interfere with corneal wound healing after drug delivery.(30) Choosing the correct current was extremely important as the researchers did not want to cause more harm to the cornea and did not want to impede healing due to iontophoretic delivery of a medication. Fungal keratitis, whether ulcerative or non-ulcerative, has a protracted course of healing, so setbacks caused by the iontophoretic delivery device would not be appropriate for clinical use.

The experimental time groups chosen were 2.5, 5, 7.5, and 10 minutes. Just like the chosen applied current, these time periods were chosen due to success with iontophoretic delivery of RNA interference-based oligonucleotides. Furthermore, the researchers wanted to test clinically relevant treatment periods, as the ultimate aim of iontophoretic drug delivery in veterinary medicine is a short and safe outpatient procedure. Ideally, this would be 10 minutes or less, especially with equine patients, in order to perform the procedure under sedation rather than general anesthesia.

The control groups consisted of corneas in which drug was loaded into the iontophoretic delivery system but no current was applied. For these groups, the drug was allowed to make contact with the cornea for 5, 7.5, and 10 minutes before being removed. Note the 7.5 minute and 10 minute groups were only seen in the voriconazole testing. The control groups were supposed to simulate a simple topical delivery and to evaluate drug concentration and mass found in the cornea without electromechanical aid.
In hopes of improving intracorneal drug concentration and mass, the iontophoretic device was loaded in two phases. Initially, a buffering solution was added to the iontophoretic cup, then a suspension of 15% sucrose and nystatin was administered near the corneal surface. Because the suspension of 15% sucrose and nystatin was more dense than the buffering solution, it would sit at the bottom of the iontophoretic cup atop the cornea. Due to its location in the cup, the nystatin had less distance to travel to be administered to the corneal layers. The researchers developed this dual phase delivery system in previous iontophoresis testing. (30)

Nystatin was chosen to be the antifungal administered to the ex vivo rabbit cornea model due to its opaque nature (better visualization) and electromigration capabilities. One other advantage to this drug for topical delivery is that when reconstituted in the 100,000IU/mL concentration, it is a suspension not a solution. With ophthalmic suspensions, drug delivery follows first-order kinetics (see Figure 6-3) in that the applied drug that is available for the target tissue declines dramatically as it is lost through the nasolacrimal system and washed out via tear production. (105) Suspensions are advantageous as compared to solutions as the retention of particles and their dissolution prolong contact time with the corneal surface. This allows the drug to stay in the tear film longer than a solution. In addition, suspensions can show enhanced ocular bioavailability if the dissolution rate of particles is greater than the rate of their clearance. (105)

The mean concentration for the 7.5 minute experimental group was the highest among all groups at 62,117.340IU/mL. The next highest concentration was the 2.5 minute experimental group at 25,168.582IU/mL, followed by the 5 minute group (20,971.491IU/mL), 10 minute group (18,485.267IU/mL), and lastly, the control group (17,159.870IU/mL). Because the mass was directly correlated to the intracorneal concentration, the same order from highest to lowest was noted for these values. The 7.5 minute time experimental group had a mass of 12,423.468IU,
followed by the 2.5 minute group with a mass of 5,033.716IU, then the 5 minute group with a mass of 4,194.298IU, then the 10 minute group with a mass of 3,697.053IU, and lastly 3,431.974IU for the control group.

The researchers encountered both expected and unexpected results when evaluating the intracorneal concentrations and masses of nystatin. In regards to the control group, it was expected that a lower amount of drug would penetrate the cornea. Although nystatin was not electromigrated in this particular group, the drug’s bioavailability would be relatively higher when compared to topical use in vivo. With the ex vivo rabbit corneas, we did not have to worry about drug loss due to nasolacrimal drainage or tear washout. Therefore, with no biomechanical losses, nystatin was expected to stay in contact with the cornea for the full experimental time period and penetrate the corneal layers.

It was also expected that iontophoresis would produce higher intracorneal concentrations and masses, as our goal was to successfully electromigrate antifungal to deeper corneal layers. If higher concentrations and masses were not seen with iontophoresis, then electromechanical delivery would be no better than topical administration. When comparing the descriptive statistics among the control and experimental groups, the mean concentrations and masses of the iontophoretic experimental groups were higher than the “topical” control group. We would have expected more of a direct relationship in regards to the experimental groups. For instance, as time increased with the same current, it would be expected that the nystatin concentration and mass would increase as well. Instead decreases in concentration and mass were seen between 2.5 minute and 5 minute and 7.5 minute and 10 minute (see Figures 6-4 and 6-5).

The most surprising result was the dramatic decrease of concentration and mass between 7.5 minute and 10 minute experimental groups. There was a slight decrease in the 2.5 minute to 5
minute experimental groups, but the decrease between the 7.5 minute and 10 minute groups was much more dramatic. In fact, the mean concentration and mass for the 10 minute group was quite comparable to the control group. Therefore, three more corneas were tested under the 10 minute iontophoretic conditions, processed, and separated, identified, and quantified with HPLC. The same results occurred with a dramatic decrease in the concentration and mass.

It is difficult to say what caused the dramatic decrease from 7.5 minute to 10 minute in the iontophoresis experimental groups. Several theories have been posed among the researchers. The first theory is that the majority of the nystatin actually migrated through all the corneal layers. It could be that 10 minutes is actually too long for the iontophoretic device to be run. If it is allowed to run for this amount of time, the drug being electromigrated may bypass the target tissue. This is actually a positive result as this could mean that the device should be run for shorter periods of time, thus accomplishing our goal of delivering drug in an efficient manner.

Another theory is that the drug was electromechanically pushed deep into the corneal stroma and thus was more difficult to extract during tissue homogenization. This would also be a positive result because as previously stated, fungal organisms seem to have an affinity for the deeper stromal lamellae and are drawn to Descemet’s membrane (19, 20). If the researchers were successful in electromigrating the antifungal deep into the stromal layers, this would be ideal and an appropriate target tissue. A different homogenization routine may need to be utilized in future experiments.

The last theory for the dramatic decrease in nystatin concentration and mass from the 7.5 minute to 10 minute experimental groups was that the drug may be binding to the corneal epithelium and/or endothelium, and then there is a sloughing of these respective layers during processing. If one or both of these layers were lost during processing, there would be a dramatic
decrease in drug concentration and mass. This would be difficult to prove or correct during the experimental process. Slit-lamp biomicroscopy was performed on the second set of corneas and no epithelial damage or sloughing was noted immediately after iontophoresis.

While only descriptive statistics were used, the researchers showed a proof of concept that nystatin, an antifungal chemotherapeutic agent, could be electromigrated into an ex vivo rabbit corneal model. Even though nystatin is not an antifungal that is typically used in either human or veterinary medicine, it proved suitable for the researchers’ assays. The opaque nature of the drug helped us visualize movement in our early assays (phase one). It is a large, uncharged molecule, so by showing it could be electromigrated in both an agarose gel media and a biologic tissue media, the foundation was laid to use more clinically relevant drugs with more compact molecular structure and lower molecular weight. Due to this success, the researchers moved on to testing with the antifungal chemotherapeutic agent voriconazole.

In phase three of this research, voriconazole was electromigrated into the corneas of the ex vivo rabbit model. Voriconazole is part of the azole antifungal family, which is the class of antifungal agents most widely used in veterinary ophthalmology. Azoles can be broken down further into two subclasses: the imidazoles and triazoles. Along with itraconazole, fluconazole, and terconazole, voriconazole is part of the triazoles. Azoles are fungistatic chemotherapeutic agents that inhibit ergosterol biosynthesis and disturb lipid organization in cell membranes. They inhibit the cytochrome P450 3A (CYP3A) enzyme lanosterol 14-α-demethylase. This action prevents lanosterol from converting to ergosterol and upsets the integrity of membrane-bound enzymes and fungal cell membranes. This results in increased membrane permeability and leakage of small ions, amino acids, and protein from the fungal organisms. Azoles are a group of broad spectrum antifungal agents
that cover both yeasts and filamentous fungi. These organisms include *Coccidioides, Candida, Cryptococcus, Histoplasma* spp., *Paracoccidia, Paecilomyces lilacinus, Scopulariopsis brevicaulis, Aspergillus, Mucor, Fusarium* spp., *Sporothrix, Alternaria, Blastomyces, Sporotrichum* spp., and *Prototheca.* (64)

The molecular weight of nystatin is almost three times that of voriconazole at 926.107g/mol versus 349.311g/mol, respectively. In addition, voriconazole is a much more compact molecule. Please see Figures 6-6 and 6-7. Taking this into account, along with the recommendations of this drug as a first line therapeutic for keratomycosis, voriconazole was the drug of choice for phase three testing of this research. (74, 89, 101) The same experimental design was employed during phase three in which a control group consisted of corneas in which drug was loaded into the iontophoretic delivery system with no application of current. The drug was allowed to make contact with the cornea for 5 minutes before being removed. Once again, the experimental time groups chosen were 2.5, 5, 7.5, and 10 minutes and a 4mA current was applied to all groups.

By performing more than just descriptive statistics, the researchers were able to find a statistically significant difference among the groups for the phase three data with voriconazole. An alpha of 0.05 was used as the cutoff for statistical significance. A one-way ANOVA was performed, and the p-value for this dataset testing was 0.0237. Since there was a statistically significant difference among groups, the next step was to identify which specific groups were statistically significantly different from one another.

Two sample student t-Tests were run between groups assuming unequal variances. An alpha of 0.05 was used as the cutoff for statistical significance. Statistically significant differences were found between the control or “topical” delivery group and the groups that
delivered current for longer periods of time (7.5 minutes and 10 minutes). In addition, statistically significant differences were found between the group that delivered current for the shortest amount of time (2.5 minutes) and the groups that delivered current for the longer periods of time (7.5 minutes and 10 minutes). This was a substantial finding as it initially appeared to demonstrate delivery of this antifungal agent into the corneal model, and the data that was obtained was not simply the result of chance. The researchers felt one step closer in showing that iontophoretic delivery of multiple antifungal chemotherapeutic agents could be performed in an ex vivo rabbit corneal model.

While there was a statistically significant difference in the 7.5 minute and 10 minute iontophoresis groups versus the 5 minute control group, the researchers felt it necessary to evaluate side-by-side comparison of passive diffusion (ie topical delivery) and iontophoretic delivery. The researchers’ final goal was to evaluate if there was a statistically significant difference between a 7.5 minute control group compared to the 7.5 minute iontophoresis experimental group and between a 10 minute control group compared to the 10 minute iontophoresis experimental group. In other words, was there a statistically significant difference in placing the drug on the cornea for an extended period of time versus iontophoretic delivery of the drug for that same time period? Two sample student t-Tests were run between groups assuming unequal variances. An alpha of 0.05 was used as the cutoff for statistical significance. The p-value for the 7.5 minute comparison was 0.319, and the p-value for the 10 minute comparison was 0.410. Therefore, there was no statistically significant difference among these groups. This revealed that more research into this modality should be performed before it can be considered as a viable topical delivery system.
One theory for lack of a statistically significant difference between iontophoretic delivery and topical delivery of 1% voriconazole is the drug’s ability to penetrate the cornea. This coincides with the study by Clode et al 2006 in which concentrations of voriconazole were detected in aqueous humor of horses after being topically administered. It has been proven in this study that voriconazole can penetrate the cornea well and concentrations can be found in the aqueous humor. In the researchers’ present study, voriconazole was allowed to sit on the corneal surface undisturbed, therefore it would only make sense that transcorneal penetration would improve with a longer contact time. It would be interesting to evaluate whether the same results would occur in vivo if there was an active blink response, patent nasolacrimal system, and excessive lacrimation.

In considering ways to improve delivery of medications as compared to increased contact time, a drug could be reconstituted into a vehicle that created an increase in formal charge. Then, a better protocol could be established in regards to placing the anode or cathode side to the studied tissue. This would provide assistance in knowing whether or not appropriate repulsion of ions is occurring so as to appropriately electromigrate medication into the tissue.

Another thought on the use of iontophoresis in transcorneal delivery of medications is that this protocol may only be appropriate for medications that have been proven to poorly penetrate the cornea. Since voriconazole is a small molecule proven to have clinically effective corneal penetration, future studies may need to be conducted in which chemotherapeutic agents known to have poor corneal penetration are used. This would show that these types of molecules would benefit from electromigration.

One other finding that the researchers deemed significant was regarding the homogenization methods. During phase three testing, all seven groups consisted of six samples.
Of the six sample corneas per group, three corneas were homogenized with a glass on glass tissue grinder, and the other three corneas were homogenized with a plastic on glass tissue grinder. Please see Figures 6-8 and 6-9 for examples of these devices. A one-way ANOVA was run between the two types of tissue grinders, and a statistically significant difference was found between the groups (p-value = 0.004). The numbers appeared more variable in the plastic on glass tissue grinder group. Please see Figure 6-10 for a box-and-whisker plot of the data. The researchers felt that the glass on glass tissue grinder provided better breakdown of the tissue being homogenized, and therefore, more drug was being extracted. In addition, a study conducted by Smith et al in 2014 revealed that voriconazole precipitated in silicone subpalpebral lavage tubing.(107) A similar event could have happened during the homogenization process in which the voriconazole precipitated and adhered to the plastic pulverizer. Another explanation could have been that voriconazole precipitated and was left in the bottom of the glass pestle when transferring the homogenate to a centrifuge tube or the precipitate was left in the centrifuge tube when transferring to HPLC vial. It was concluded that glass on glass tissue grinders should be used in future studies when tissue homogenization is necessary.

The use of iontophoresis in veterinary ophthalmology is a novel technique and only has been described or performed in the lab animal sector, never in a clinical setting. The researchers achieved their initial aims and learned vital information during the research process regarding this delivery system, the most important arguably being the delivery of large molecules that have minimal to no charge into the target tissue is possible. Furthermore, the researchers were able to electromigrate a more clinically relevant drug, voriconazole, and lay the foundation for a novel approach to treating equine corneal disease. The researchers will continue their work with optimization of this non-invasive medication delivery system. Future studies will be designed in
order to establish the most efficient yet effective treatment time period, to continue evaluating additional chemotherapeutic agents, to develop a prototype for an iontophoretic cup to be used in equine veterinary medicine, and to test this prototype and delivery method on an in vivo equine model.
Figure 6-1. Photo of natamycin and nystatin after gel electrophoresis with UV assistance. Natamycin is in the first well and the red arrow is pointing to the nystatin well. The red circle is highlighting the fluorescent opacity in the gel that is presumed to be nystatin that has migrated out of its respective well. (Photo courtesy of author)
Figure 6-2. Natamycin can be seen in well #3 and nystatin can be seen in well #7. Note that natamycin seems to have slightly migrated to the left side of its well, while nystatin has “plastered” itself to the left side of its well. In addition, the nystatin well appears to be bowed toward the anode. (Photo courtesy of author)
Figure 6-3. First-Order Kinetics for topical ophthalmic medications in which a drug’s concentration is high on initial instillation, then decreases exponentially when lost to nasolacrimal drainage and tear washout. (Photo courtesy of Veterinary Ophthalmology, 5th Ed.)

Figure 6-4. Plot of nystatin concentrations among the experimental groups; 1 – control; 2 – 2.5min; 3 – 5min; 4 – 7.5min; 5 – 10min. (Chart courtesy of author)
Figure 6-5. Plot of nystatin masses among the experimental groups; 1 – control; 2 – 2.5min; 3 – 5min; 4 – 7.5min; 5 – 10min. (Chart courtesy of author)

Figure 6-6. Molecular structure of nystatin with molecular weight equal to 926.13g/mol. (Photo courtesy of https://dailymed.nlm.nih.gov/dailymed/fda/fdaDrugXsl.cfm?setid=d868575c-7eea-483e-ac35-151398bfa01e&type=display)
Figure 6-7. Molecular structure of voriconazole with molecular weight equal to 349.311g/mol. (Photo courtesy of https://drfungus.org/knowledge-base/voriconazole/)

Figure 6-8. Multiple examples of plastic on glass tissue grinder. (Photo courtesy of https://commons.wikimedia.org/wiki/File:Tissue_glass_teflon_Dounce_homogenizer-02.jpg)
Figure 6-9. Multiple examples of glass on glass tissue grinders. (Photo courtesy of https://en.wikipedia.org/wiki/Dounce_homogenizer)

Figure 6-10. Box-and-whiskers plot of the areas under the curve comparing plastic on glass tissue grinder and glass on glass tissue grinder. (Photo courtesy of author)
CHAPTER 7
CONCLUSION

Results indicate that by using iontophoresis, 100,000IU/mL nystatin was able to be successfully electromigrated into corneal tissue and reach detectable levels. While drug levels did not reach 100% yield, this proof of concept opened the door for assays with other topical medications, specifically voriconazole, a more clinically relevant drug in veterinary ophthalmology. Initially, 1% voriconazole appeared to be electromigrated into corneal tissue successfully. Upon further evaluation, the drug concentrations did not represent a statistically significant difference when compared to topical control groups (ie groups allowed to passively diffuse into corneal tissue). This shows that before being utilized in veterinary medicine, more research into an appropriate iontophoretic protocol needs to be performed. Lastly, glass on glass pulverizers seem to be superior in extracting 1% voriconazole from tissue as compared to plastic on glass pulverizers.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

James William Herrmann was born in Houston, Texas. His father, Kohle, is a veterinary ophthalmologist and his mother, Mary, is an acting coach and ballet teacher. He completed his undergraduate degree in May 2008, earning a Bachelor of Arts in business. After completing his pre-requisites for veterinary school, he began his education at Texas A & M’s College of Veterinary Medicine. He earned his doctorate of veterinary medicine in 2014. Shortly thereafter, he began a rotating internship at Auburn University from 2014-2015. From 2015-2016, he completed an ophthalmology specialty internship at the University of Illinois at Champagne-Urbana. After his specialty internship, he began his training in the combined master’s degree and residency program in Comparative Ophthalmology at the University of Florida College of Veterinary Medicine.