A Penetration and Safety Assay for Generic Ophthalmic Drugs

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Masters Report
Biomedical Engineering
University of Arizona
17 December 2010

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Abstract

Generic topical ophthalmic medications are poorly regulated by the Food and Drug Administration, resulting in an uncertainty of generics' efficacy and safety and unnecessarily placing patients at risk. In 1999, more than 200 documented cases of corneal damage as severe as corneal melting were linked to the use of a generic formulation of diclofenac, which was consequently pulled from the market. These devastating iatrogenic effects demonstrate the need for stricter testing of generic ophthalmic drugs prior to reaching the public. This report addresses this urgent need by proposing an *in vitro* model for simultaneously predicting corneal penetration and epithelial toxicity of topical ophthalmic formulations. Penetration and safety of ophthalmic medications have been studied separately, but until now, the development of an assay to accurately predict both penetration and safety in parallel has been overlooked. In this report, recent and ongoing research will be reviewed to (1) elucidate the complexities of corneal penetration and the effects of topical ophthalmic formulations on corneal penetration, and (2) identify important characteristics of existing models to incorporate in the proposed *in vitro* penetration and safety assay. Critical features of the model proposed here include a trephinated porcine cornea from tissue discards affixed in a Franz diffusion cell, permitting concurrent drug penetration and epithelial health monitoring. A robust, cost-effective penetration and safety assay such as this would provide drug companies with a valuable tool to eliminate chances of future iatrogenic effects due to topical ophthalmic drugs.

Introduction

Ten years ago, more than 200 cases of severe corneal damage, ranging from epithelial sloughing to full-blown corneal melting, were linked to the use of a generic topical ophthalmic drug, diclofenac (Flach, 2000; Congdon et al., 2001). Similar iatrogenic corneal events have also been reported, in addition to cases of diminished efficacy due to generic formulations (Jackson, 2010). These incidents highlight a major flaw in the regulation and testing of generic ophthalmic drugs by the Food and Drug Administration (FDA) and drug companies, respectively, which allows topical ophthalmic generic medications to be sold with limited screening. Specifically, inactive additives in topical ophthalmic formulations, which have been shown to vastly alter corneal permeability and epithelial integrity, are not strictly regulated. This significant deficiency lies in that the FDA does not require drug companies to demonstrate bioequivalence (absorption rates) or therapeutic equivalence (pharmacological effects) of a new generic ophthalmic formulation to the original innovator drug (Jackson, 2010). This regulatory hole is a serious problem that increasingly places patients' health at risk as more and more ophthalmic drugs come off patent protection and new generic formulations come to market in the next decade. Drug companies manufacturing and selling generic drugs are under extreme pressure to keep costs low to be competitive, so research and development of generic drug formulations will rarely exceed that which is required by the FDA. Therefore, lenient FDA regulations and inadequate drug company screening are significant impediments to ensuring generic efficacy and safety. To make generic ophthalmic drugs safer, a significant paradigm shift is urgently needed – either by stricter federal regulations or improved culture within drug companies that

enables enhanced screening of new drugs before they reach the public, or both. Here, we propose a solution to address the latter, with the development of a robust and cost effective *in vitro* efficacy and safety assay for improving generic ophthalmic drug screening.

This project's long-range goal is to improve the efficacy and safety of generic ophthalmic drugs, specifically to avert future occurrences of adverse corneal events due to generic formulations like the aforementioned diclofenac case. The objective of this report, as the next step towards obtaining this goal, is to lay the foundation for the development of a simple, robust and cost effective assay that accurately predicts efficacy and safety of new ophthalmic drug formulations. The central hypothesis is that drug penetration and epithelial health measurements acquired from a single robust *in vitro* assay, such as the one proposed within, will concurrently indicate a generic ophthalmic drug's efficacy and safety, respectively. This hypothesis is based upon (1) a review of literature demonstrating the effects of varying ophthalmic formulations on corneal penetration, (2) a review of *in vivo* and *in vitro* models for studying drug penetration into the eye, and (3) a review of techniques for studying and quantifying epithelial health. The rationale behind the proposed assay is that by providing a rapid, cost effective and robust tool for screening efficacy and toxicity of new ophthalmic formulations, drug companies will be better suited to evaluate the bioequivalence and safety of generics, resulting in safer generic ophthalmic medications.

The proposed model, designed to rapidly, robustly, and cost-effectively screen new generic formulations, is innovative for these reasons: (1) for the first time drug companies will have a

simple tool for simultaneously assessing generic ophthalmic drug efficacy and safety, and (2) robust and rapid *in vitro* screening will minimize animal testing by identifying comparable drug penetration and acceptable corneal health *ex vivo*, therefore reducing the number of *in vivo* animal studies and animal sacrifices.

This report aims to accomplish the following:

(1) emphasize the significance of generic drug formulations,

(2) elucidate the complexities of drug penetration into the eye,

(3) review prior art contributing to the design of the proposed model,

(4) provide a detailed explanation of the model, and

(5) recommend future work with the proposed model.

Background and Significance: Generic Ophthalmic Drugs and Ocular Drug Delivery

Recent Generic Ophthalmic Drug Recalls

The potential risks of generic ophthalmic formulations remain a serious concern among ophthalmologist (Jackson, 2010). While cheaper generic ophthalmic medications are attractive alternatives to more expensive brand-name drugs, there have been recent cases of drug recalls due to reduced generic efficacy and severe corneal damage (e.g., generic diclofenac). These recalls highlight the need for better generic screening, but also demonstrate the importance of closely monitoring patients if using generic formulations (Gallardo et al., 2006). Examples of generic ophthalmic recalls due to reduced efficacy and harmful side effects follow.

Prednisolone is an anti-inflammatory steroid commonly used to reduce swelling and irritation from eye injury or surgery. In 1999, several lots of the ophthalmic prednisolone formulation made by Alcon® Laboratories were recalled because the active ingredient was precipitating out of solution and clogging the dropper tip (Fiscella et al, 2001). This issue was easily detected because of dropper tip clogging, however slight variations in the suspensions due to precipitation or caking can lead to reduced concentrations of the active drug per dose, therefore reducing the amount of drug entering the eye. If only a sub-therapeutic level of the prednisolone is delivered, inadequate treatment of inflammation is likely.

A similar case of non-equivalency that led to a recall was with generic ophthalmic timolol. Timolol is a β-blocker indicated for reducing intra ocular pressure (IOP) to slow or prevent the progression of glaucoma. Clinicians noticed a trend of increasing IOPs in patients that had inadvertently switched to the generic brand, signifying a decrease in the generic drugs efficacy for pressure reduction (Weinreb et al., 2003).

Another generic formulation latanoprost, made by Sun Pharmaceuticals® and indicated for reducing IOP, was also recalled due to decreased efficacy as compared to the brand-name version. In this case, the formulation was found to have a higher pH than the brand-name, which caused a decrease in corneal penetration and decrease efficacy in IOP reduction (Cantor et al., 2008).

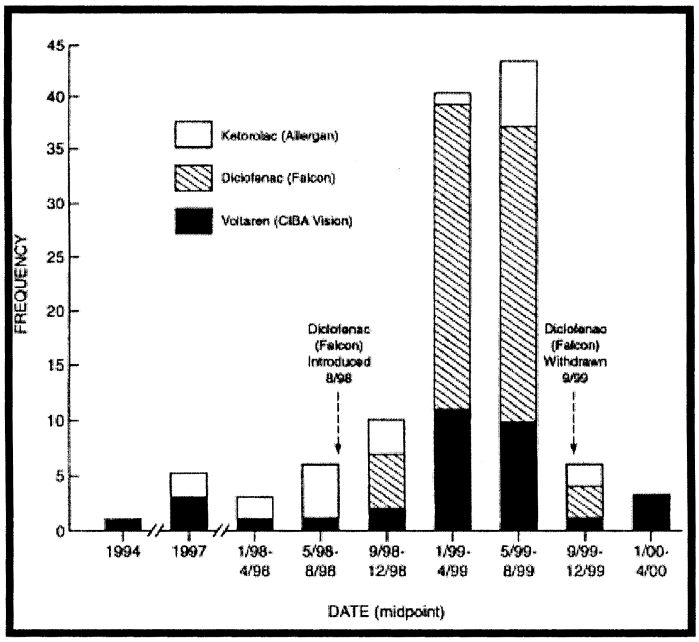


Figure 1: *Frequency of Adverse Cornea Events Linked to NSAID Use.* This histogram show the clear correlation between the generic diclofenac (striped bar) and corneal damage as compared to the brand-name (black bar) and another type of NSAID (white bar). (Used with permission: Congdon et al, 2001)

As emphasized in the *Introduction* of this report, a generic formulation of topical diclofenac, a nonsteroidal anti-inflammatory drug (NSAID), led to more than 200 cases of severe corneal disorders. Severity of these ranged from minor irritation of the corneal epithelium to corneal melting, in which epithelial cells slough off the cornea, exposing stroma collagen that then rapidly degrades. These severe iatrogenic effects prompted Falcon® Pharmaceuticals to immediately withdraw this generic formulation from the market. A study following this recall of generic diclofenac found a direct correlation between generic diclofenac use and adverse corneal events during the 13 months that the drug was available (Congdon et al, 2001). *Figure 1*, above, illustrates this obvious correlations by showing the frequency of corneal pathologies due to topical NSAID use from 1994 to 2000.

The exact mechanisms of the diclofenac incidents of corneal morbidity were never described, but studies following the recall identified the inactive ingredients in the formulation, rather than the active diclofenac, as the caused the breakdown of the corneal epithelium and subsequent severe corneal damage (Flach, 2000; Congdon et al., 2001). Unfortunately, horrific cases similar to the generic diclofenac disaster are probable to reoccur as the FDA is only strictly regulating the active ingredients in generic formulations, leaving the inactive ingredients, such as buffers and preservatives, unchecked.

Drug Development Process: Innovative New Drug versus Generic Drug

Competition from generic drug companies pushes drug costs down and is responsible for making previously expensive brand-name drugs more accessible to the public. The U.S. Drug Price Competition and Patent Term Act of 1984 (Hatch-Waxman Act) was a significant victory for both the consumer and generic drug companies vying to compete with a brand-name drug nearing patent expiration (Berndt et al., 2007). The Act specifically prohibits the FDA from requiring more than simple bioequivalence studies for generic drugs, expediting and reducing the cost of the approval process (Mossinghoff, 1999). Generic drugs are therefore allowed to bypass the expensive and time consuming pre-clinical testing and clinical trails that are required of innovator drugs. While this abbreviated path to generic drug approval benefits both the public (by making drugs cheaper) and the generic drug companies (by reducing testing costs), it introduces the regulatory gap that is chiefly responsible for the aforementioned generic diclofenac disaster.

To better understand the how generic ophthalmic medications have managed to reach the marketplace while being dangerous to patients, it is helpful to review the major difference in FDA requirements and drug development time and cost between innovative new drugs and generic drugs. For innovative new drugs, the FDA requires lengthy and expensive preclinical and clinical trials to ensure proper dosage, safety, and efficacy. The development process of a novel drug begins with discovery of a new compound and filing of a patent application to protect intellectual property. Preclinical testing *in vitro and in vivo* is completed in the laboratory to demonstrate that the new drug is safe enough for human trials, at which point an investigational new drug application (IND) is filed with the FDA. If approved, the drug moves into a Phase I clinical trial in which toxicity and safety are established in 20 to 50 healthy volunteers. Next, a Phase II trial aims to identify how well the drug works and to establish proper dosages in a group of 20 to 300 healthy volunteers and patients. If Phase II is successful, randomized testing in 300 to 3000 patients to demonstrate efficacy is conducted in the Phase III trial. If all testing has been successful to this point, the company files a New Drug Application (NDA) with the FDA, summarizing the data from the clinical trials. The FDA then decides whether to approve of reject the drug (Unger, 2010). This entire process for validating the efficacy and safety of the new drug may take as long as seven to 10 years (Mossinghoff, 1999) and cost between $500 million and $2 trillion (Adams & Brantner, 2006).

Alternatively, generic drugs have a much simpler path to approval. Generics are seen by the FDA as equivalents to original, innovator drugs, and therefore only require minimal bioequivalency testing for approval. No animal testing or full-scale human clinical trials are

required as bioequivalency testing is typically performed in a small sample of 24 – 36 healthy volunteers to establish comparable pharmacokinetics to the innovator drug. If successful, the drug company files an Abbreviated New Drug Application (ANDA) with the FDA. Surprisingly, no safety or efficacy testing is required. Systemic generic medications are easily tested for bioequivalency by simply measuring drug concentrations in blood and urine following administration of the drug. However, measuring generic topical ophthalmic bioequivalence is not as easy due to difficulty accessing the aqueous humor. Because non-invasive testing is not possible, generic ophthalmic medications are permitted to skip bioequivalency screening as long as the active drug is shown to be the same as the innovator (FDA: CFR 21-314, 2010).

While active drugs must be the same in a generic formulation as the brand-name version, the inactive ingredients may vary widely. Essentially, inactive ingredients such as preservatives, pH adjustors, antioxidants, thickening agents, buffers, and tonicity adjustors are not regulated extensively by the FDA. As the next section will highlight, these 'inactive' ingredients can actually play an enormous role in the behavior of topical ophthalmic medications.

Effects of Formulations on Corneal Penetration

The non-therapeutic ingredients in topical ophthalmic formulations can significantly modify the penetration efficiency of the active ingredient. Formulations may vary in pH adjusters, tonicity, preservative content, and other additives between different generic formulations and brand-name products.

As previously mentioned, β-blockers are commonly indicated in ophthalmology for decreasing IOP to slow the progression of or prevent glaucoma. The effect of various formulation additives on the penetration of four ophthalmic beta blockers was investigated by Ashton et al. (1991) in the cornea and conjunctiva of rabbits. Specifically, the group separately altered the pH, osmolarity, benzalkonium chloride and EDTA content of formulations for four β-blockers, atenolol, timolol, levobunolol, and betaxolol, and measured changes in corneal and conjunctival permeability for each. The results indicated that increasing the pH from 6.0 to 8.4 generally increased the penetration of the beta blockers for both the cornea and conjunctiva. Hypotonic (83mOsm/kg) formulations were found to predominantly increase corneal penetration and slightly increase conjunctival penetration of the β-blockers as compared to isotonic (284mOsm/kg) and hypertonic (583mOsm/kg) formulations. Benzalkonium chloride (BAK), a preservative, increased conjunctival penetration of all four beta blockers, but only improved the two least lipophilic β-blockers. Ethylenediaminetetraacetic acid (EDTA), a commonly used chelating agent that acts a preservative, either increased or decreased the corneal and conjunctival penetration with no correlation to concentration (Ashton et al., 1991).

Ophthalmic NSAIDs make up a generally safe class of mild analgesia and anti-inflammatory drugs indicated for reducing pain and inflammation in the eye. NSAIDs disrupt the synthesis of prostaglandin from arachidonic acid by inhibiting cyclooxygenase (COX-1 and COX-2) activity. In the eye, prostaglandins lead to vasodilation, increased vascular permeability, pupil constriction (miosis), and leakiness in the ocular-blood barrier. Ophthalmic NSAIDs are commonly used to maintain pupil dilation (mydriasis) during cataract surgery and have been shown to be very

effective in reducing pain and inflammation postoperatively. The high frequency of NSAIDs can also be attributed to their minimal adverse side effects as compared to corticosteroids (Nichols & Snyder, 1998). Formulations of NSAIDs are of particular interest because of their widespread use in ophthalmology, relatively low corneal penetration rate, and also the 1999 generic diclofenac disaster resulting in more than 200 cases of corneal melts. Corneal permeability has been shown to have a high dependency on formulation pH. Most NSAIDs are weak acids, which will lower the pH of the tear fluid and lead to decreased solubility (Reer et al., 1994). Reer et al. studied pH-adjusted diclofenac formulations and found that a formulation buffered in the pH range of 6.5 to 7.0 provided the best corneal penetration.

Fluoroquinolones are potent, synthetic antibiotics that are frequently used for prophylaxis and treatment against bacterial infections in the eye. When formulated as ophthalmic drops, they are used perioperatively as prophylaxis against the development of endophthalmitis. Also, fluoroquinolones are commonly prescribed to treat bacterial conjunctivitis. A group studied how the penetration of gatifloxacin (Zymar®), a newer fluoroquinolone, across the corneal changed depending on changes in the pH and preservative content (Rathore & Majumdar, 2006). Specifically, this group found that gatifloxacin penetrated nearly three times greater when optimized at a pH of 6.5, 0.01% (wt/vol) of BAK, and 0.01% (wt/vol) of EDTA.

In addition to the formulation's effect on penetration, permeability of the active drug across the cornea is also largely dependant on the drug's own characteristics. Because of the lipophilic nature of most physiological barriers, drug penetration across these membranes relies heavily

on the lipophilicity of the drug. However, increasing a drug's penetration across the cornea is not as simple as making the drug more lipophilic. Thirty years ago, Schoenwald et al. compared the corneal permeability of 11 steroids of varying lipophilicities (defined by their octanol-water partition coefficients) and demonstrated a parabolic relationship between corneal permeability and octanol-water partition coefficients, as depicted in *Figure 2,* below (Schoenwald et al., 1978).

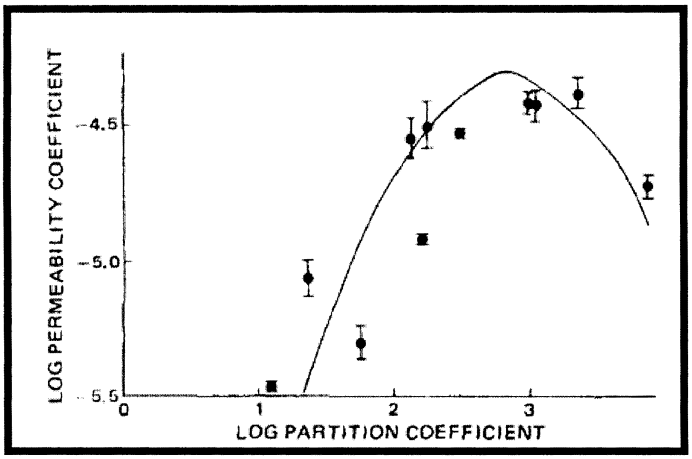


***Figure 2**: Corneal Permeability as a Function of Lipophilicity.* This log-log plot relates the permeability coefficients of 11 topical ophthalmic steroids to their octanol-water coefficients (larger octanol-water coefficient means more lipophilic). It is clear that corneal permeability increases with lipophilicity to a point, but then decreases. This parabolic relationship could be due to the lipid-aqueous-lipid nature of the cornea's structure (used with permission from: Schoenwald et al, 1978).

It is clear that additives to topical ophthalmic formulations play a significant role in drug penetration of the eye in addition to the active drug's inherent properties such as lipophilicity. While formulations can be adjusted to increase drug penetration and half life, additives may also introduce an increased risk of damaging the integrity of the corneal epithelium. With new generic formulations coming to market that have not been as fully scrutinized as their brand-name counterparts, there is uncertainty about the combined effects of the active and inactive

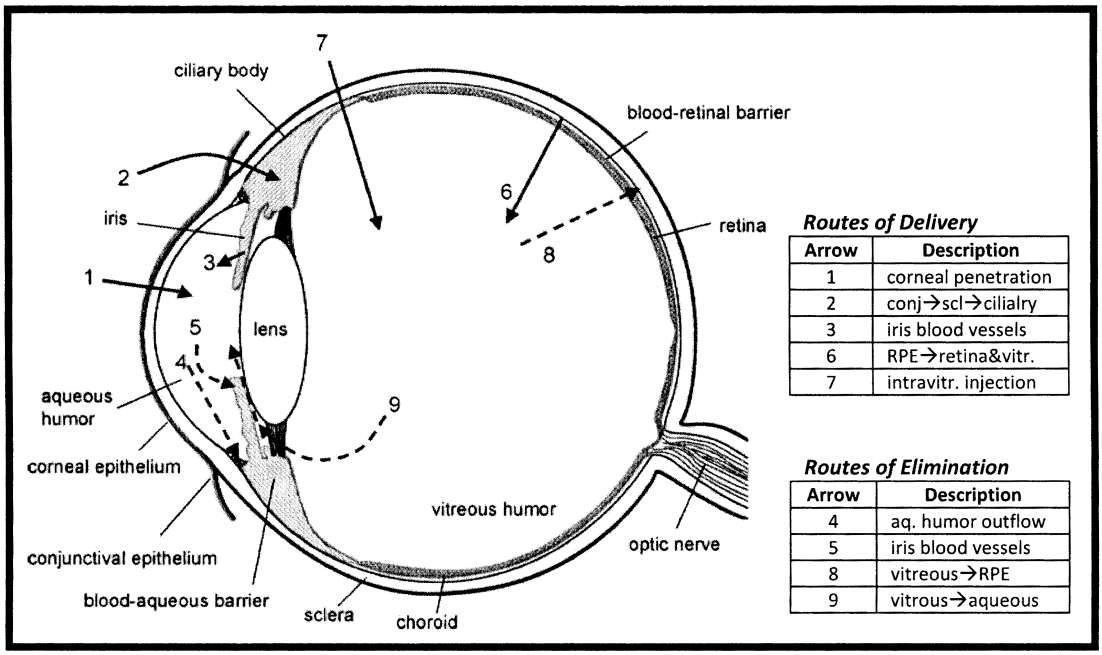
ingredients on penetration across the cornea and corneal epithelial toxicity. The corneal epithelium, which acts as the first line of defense against the penetration of exogenous agents, in addition to the other defense mechanisms in the eye that work to suppress drug penetration, are explored in the next section.

Anatomical and Physiological Barriers of the Eye

The eye is a well-sealed organ. Exogenous agents in systemic circulation are kept from entering the eye via the blood-aqueous barrier and the blood-retina barrier, limiting the utility of systemically administered therapeutics (i.e., oral or IV) for treating eye pathologies (Urtti, 2006). This makes topical solutions (eye drops) the principle dosage form for ophthalmic therapeutics. The efficacy of an ophthalmic drug, i.e., the maximum therapeutic effect, relies on the bioavailability of the drug within the target tissues of the eye. The bioavailability of a drug is primarily dependent on its ability to penetrate the efficient barriers of the eye, which is often a difficult task. This section reviews the nearly impervious barriers of the eye that restrict penetration of topical ophthalmic agents into the eye.

The most direct route of penetration into the eye is via intra-aqueous or intra-vitreous injection (*arrow 7*, *Figure 3*, below), in which 100% of the drug reaches either the anterior or posterior chamber (100% bioavailability). Direct injection into the eye is not the preferred administration method for many reasons, primarily because inserting a needle into the eye weakens epithelial integrity exposing the eye to infections. Secondly, intracameral infusions like these are not well tolerated by patients as they can be very painful (Olsen et al., 1995). While intra-vitreous injection of fluoroquinolone antibiotics may be necessary to access the difficult-to-reach

posterior segment in severe cases of endophthalmitis, the preferred route of administration for the *anterior* segment is topical instillation.



Routes of Delivery

Arrow	Description
1	corneal penetration
2	conj→scl→cilialry
3	iris blood vessels
6	RPE→retina&vitr.
7	intravitr. injection

Routes of Elimination

Arrow	Description
4	aq. humor outflow
5	iris blood vessels
8	vitreous→RPE
9	vitrous→aqueous

***Figure 3**: Drug Delivery and Barriers in the Eye.* This schematic of the eye depicts routes of drug delivery into the eye (solid arrows), the barriers that limit entry, and the routes of elimination (dotted arrows). For topically instilled ophthalmic drugs, trans-corneal penetration (1) is the most direct route of entry into the anterior segment even though drugs must pass through the tight junctions of the corneal epithelium (graphic used with permission: Hornof et al., 2005; table created by author).

The efficiency of the eye's protective barriers reflects the importance of the organ. Rapid tear clearance, tight corneal epithelial junctions, and the blood retina barrier (BRB) are always working to prevent dirt, microorganisms, and harmful chemicals from penetrating the globe and threatening visual acuity. However, in addition to blocking the harmful substances from entering, the barriers of the eye also limit entry of pharmaceutical agents. These natural barricades can minimize bioavailability of a drug within the globe, potentially leading to sub-therapeutic drug levels at the target.

The eyes are not conveniently located for topical application via drops, as the patient must tip their head back to position their eyes before instillation from a dropper bottle. Patients' manual dexterity varies widely, which can encumber the proper dosage from entering the tear volume due to spill over. Additionally, the natural blinking reflex (activated upon eye drop contact with the eye) can block or limit the total volume of the drop from contacting the eye. A typical eye dropper dispenses 30 to 50µL of solution per drop and the normal human tear volume (the total volume of tear fluid coating the eye) is only 7 to 10µL. Most of the excess volume is nearly immediately cleared, leaving behind as little as 5% of the instilled dose. Eye drop instillation will also cause increased tear secretion from the lacrimal gland which contributes to the flushing away of the drug out the lacrimal canal and down the nasolacrimal duct. Under normal physiological conditions, tears turnover at a rate of 16% per minute, which means nearly the entire dose is cleared from the surface of the eye within 5 minutes (Chrai et al, 1973). Drug penetration into the eye, which dictates a drug's therapeutic effect, principally depends on contact time with the cornea, meaning the rapidly diminishing drug concentration in the tear volume only has a narrow time-window to enter the eye.

The ultimate destination of topical ophthalmic drugs is the anterior chamber from which a drug has direct access to eye's anterior tissues that contact the aqueous humor (see *Figure 3*, above). While the cornea is the primary route of entry into the anterior segment, it acts predominantly as a barrier to drug diffusion and is considered the rate-limiting structure for drug absorption (Mitra, 2003). The cornea is a three-layered, 500µm thick transparent lens that

provides nearly 75% of the eye's refractive power. Five epithelial cell layers make up the anterior stratum of the cornea with the primary function of protecting the integrity of the cornea as any imperfection could reduce transparency and impede vision. The entire epithelium is 50μm thick and highly lipophilic, with zonulae occludentes (tight junctions) between cells that house pores no bigger than 60Å, effectively keeping out large and/or hydrophilic molecules. The integrity of the tight junctions, which can be quantified by measuring trans-epithelial resistance, is typically 12-16 kohm-cm^2, thwarting penetration of most compounds. The primary pathway for drug penetration through the epithelium is diffusion across the cell membranes, relying on drug-epithelial contact time, diffusivity, and cornea surface area of the (Mitra, 2003).

Once across the corneal epithelium, the drug molecules reach the highly hydrophilic, 450μm thick stroma layer consisting of approximately 200 lamellæ of hydrated collagen. Because this layer is primarily acellular (except sporadic keratocytes), it is mostly hydrophilic and lacks the tight junctions of the epithelium. Therefore, the stroma is more passable to hydrophilic drugs but less so to lipophilic molecules.

A penetrating drug will next hit the corneal endothelium, which is a single, 4 to 6 μm layer of squamous cells as the final barrier to the anterior chamber. The endothelium's primary role is to maintain the health and transparency of the avascular stroma with densely packed ion pumps and channels and aquaporins that work to move solutes and nutrients from the aqueous humor into the stroma while pumping water from the stroma to the aqueous humor (Verkman,

2008). While the endothelial cells' lipid membranes make this barrier lipophilic in nature, permeability has been found to rely primarily on molecular weight of a solute, allowing molecules under 70kDa to easily pass. Most topical ophthalmic drugs are small molecules of around 300 to 500Da (about 200 times less than the maximum), rendering the corneal endothelium highly porous to these compounds.

While the cornea is the primary route of drug penetration into the eye, it is not the only pathway (Ahmed & Patton, 1985). The remainder of the non-cornea surface of the eye is covered by conjunctiva, a three-layered, protective membrane that covers 17 to 20 times more surface area than the cornea (Mitra, 2003). The conjunctiva extends from the perimeter of the cornea covering the white of the eye (sclera) and continuously wraps around to coat the insides of the eyelids. Even with 20 times more surface area, conjunctival penetration into the anterior segment is poor due to proximity to the anterior chamber (*arrow 2*, *Figure 3*), and structure. Like the cornea, the conjunctiva carries a protective epithelial layer as its first line of defense. While not as robust as the cornea's epithelium (1.4 kohm-cm^2 vs. 12-16 kohm-cm^2), the conjunctival epithelium is effective in keeping most exogenous substances out. Below the conjunctival epithelium is the substantia propria, a layer filled with nerves, blood vessels and lymphatic vessels. Here, drug concentration faces major losses because of clearance to the blood stream or lymphatics.

Beneath the conjunctiva lies the sclera, which is made up primarily of irregularly structured collagen and elastin, providing the eye's white color. The sclera's primary inhibitor of drug penetration is its thickness, which ranges from 0.3 to 1.0mm (Olsen et al., 1995).

On a sub-cellular level, membranes of the epithelial cells of the cornea and conjunctiva house ion channels, exchangers, and transporters, aquaporins, and efflux pumps that can affect drug penetration. These trans-membrane proteins work together to keep the eye's tissues healthy, however they also play a significant role in the penetration kinetics of ophthalmic drugs. Specifically, ion pumps and channels are influence the movement of hydrophilic drugs based on their influence on water movement (Mitra, 2003). Efflux pumps, which are large proteins responsible for exporting exogenous agents, can move anionic and lipophilic drugs out of the eye (Karla et al., 2009).

Systemic delivery of drugs to the anterior segment is possible, but is relatively ineffective. As depicted in *Figure 3*, there is some transfer of drugs from systemic circulation into the anterior chamber via diffusion from blood vessels in the iris; however this is minimal (Hornof et al., 2005). Even with the effective corneal barriers, trans-corneal penetration remains the most direct path for topical ophthalmic drugs to the aqueous humor.

Pharmacokinetics and Pharmacodynamics of Ophthalmic Drugs

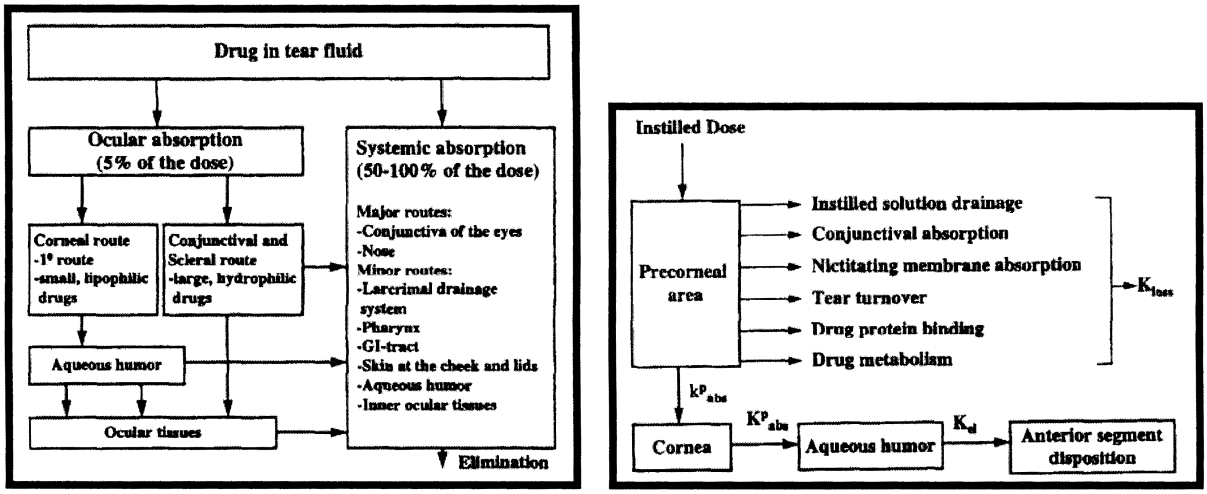


***Figure 4**: Compartmental Models of the Eye.* These block diagrams show typical pathways for a topically instilled ophthalmic medication and help explain a drug's pharmacokinetics. It is obvious that the major rate-limiting step in ocular drug delivery is drug loss from the tear volume (Used with permission: Worakul & Robinson, 1997).

The pharmacokinetics of a drug helps describe how and where a drug moves within the body after administration. This includes drug absorption, distribution, metabolism and excretion (ADME) from the body, which together describe how the body acts upon the drug. For intravenous and orally administered drugs, which are distributed via systemically, the rate limiting step is typically metabolism, or how fast the active drug is broken down into inactive metabolites. Alternatively, for topically instilled ophthalmic drugs which are distributed via the aqueous humor, the rate limiting step is absorption, from the tear volume across the cornea and into the anterior chamber. Compartmental models are often employed to explain the pharmacokinetics of ophthalmic drugs (Worakul & Robinson, 1997; Sakanaka et al., 2008). For IV or oral drugs, two-compartment models typically include the blood volume and the target tissue as the compartments with rate constants (denoted as *k*) to describe movement of the

drug into or out of each compartment. Pharmacokinetic models, such as those shown in *Figure 4* (above), help identify the variables to adjust in order to improve the penetration and bioavailability of a drug within the eye. For the *in vitro* model proposed in this report, the following compartments will be relevant: tear volume, cornea, and aqueous humor.

The pharmacodynamics of a drug helps describe how the drug interacts within the body to cause a therapeutic effect. The efficacy and potency of a drug can be extrapolated from dose-response curves which are generated by measuring a physiological response due to a given concentration of a drug. Such studies lend themselves primarily to *in vivo* testing where response can be directly measured (e.g. – extent of pupil dilation for mydriatic NSAIDs or IOP decrease for β-blockers). The proposed model *will not* be aimed at understanding the pharmacodynamics of generic drugs, but it is important to also consider how a drug will behave beyond corneal penetration.

Designing an *In Vitro* Model of the Cornea

An *in vitro* model of the cornea can provide valuable preliminary information about the effects of a drug composition or formulation on corneal penetration and toxicity. Many groups have studied corneal permeability to various drugs and formulations in both *in vivo* (Eremeev et al., 2006; Puustjärvi et al., 2006) and *in vitro* models (Burgalassi et al., 2004; Gratieri et al., 2010; Martín-Biosca et al., 2003) but few groups have attempted to better understand corneal epithelial toxicity (Dart, 2003). Currently, the published literature indicates that no research group is actively pursuing an *in vitro* model to study both corneal penetration and toxicity op

topical ophthalmic drugs in parallel. This section will review past and current research relevant to the development of the proposed penetration and toxicity model. The first goal, as an important step towards success of the model, is to demonstrate validity and robustness of model's ability to predict drug penetration. Once this has been accomplished, the model will then be expanded to meet the second goal of predicting drug toxicity. To help narrow the engineering design of the model, prior art was examined to identify the optimal techniques for testing corneal penetration and toxicity. The following subsections review literature to identify and describe the major components of the assay.

Assay's Design Criteria

Key engineering design criteria to consider while designing this penetration and safety assay for topical ophthalmic formulations include: physiological relevance (do the *in vitro* result accurately predict *in vivo* behavior?), robustness/reproducibility (does the assay provide accurate results with different users?), and cost effectiveness (is the assay cheap enough for drug companies to adopt into their research and development?). The ultimate goal of this research project is to design and assemble a robust, *in vitro* assay for studying (1) the penetration mechanisms and (2) the toxicity of ophthalmic therapeutics when applied topically to a model cornea. Based on a literature review of prior art, the following design features are important to consider for our model's design:

- Cornea substrate
- Diffusion chamber hardware
- Penetration measurement system
- Health and toxicity measurement system

This ultimate success of this proposed assay will be based on its ability to accurately and precisely predict satisfactory drug penetration and safety of topical ophthalmic drugs. While the assay's purpose is not to specifically identify exact pharmacokinetics or toxicity, it will provide researchers a 'go' or 'no-go' result that raises red-flags on potentially toxic formulations. As with any *in vitro* model, results must be later be confirmed with *in vivo* testing, however first-pass *in vitro* as performed by this model will significantly reduce the number of animals used. Validation of the model's accuracy and precision for identifying acceptable penetration rates and toxicity levels will be essential before implementation.

Corneal Substrate Selection

The key element to the proposed penetration and toxicity assay is the cornea substrate. While the most physiologically relevant corneal penetration models are *in vivo*, these live animal assays are very costly and not ideal quickly for a screening large numbers of compounds. Additionally, using live animals raises ethical questions that are circumvented by using *in vitro* surrogates. Therefore, due to the cost, time and ethical considerations the optimal cornea substrate for the proposed model must be *in vitro*.

Non-rodent *in vivo* animal models are typically closest substitute for human trials, but anatomical differences should be noted. For example, rabbits are commonly used as the animal model of choice for ophthalmic drug penetration and toxicity studies due to their large globe size while being a smaller and less expensive than using dogs or monkeys. However, anatomical differences are significant among species – rabbit anterior segment anatomy is vastly different than primates, which are closest in resemblance to humans (Short, 2008). Major differences

between rabbit and human eyes include (*rabbit vs. human*) cornea surface area (2.0cm^2 vs. 1.4cm^2), cornea thickness (0.40mm vs. 0.50mm), and aqueous humor volume (0.25-0.3mL vs. 0.1-0.25mL) (Worakul & Robinson, 1997).

Many research groups have attempted *in vitro* epithelial cell culture as an alternative to using live animals, such as rabbits, for penetration studies (Burgalassi et al., 2004; Toropainen et al., 2001). The most significant advantages of cell culture models are their low cost and that they do not require the sacrifice of animals (Burgalassi et al., 2004). However, these models have inherent deficiencies in that re-growing epithelial tight junctions to their normal physiological integrity is extremely difficult (Hornoff et al., 2005). Penetration results from epithelial cell culture models like these may be physiologically irrelevant because the epithelial tight junctions, the rate limiting barrier to penetration, are deficient. While recent improvements in cell culture models are promising, the closest *in vitro* surrogates to *in vivo* models are live, excised cornea with intact epithelium (Burgalassi et al., 2004).

Human cadaver corneas are not commonly used for penetration studies because of cost and difficulty of obtaining specimens with the corneal epithelial layer still alive. Therefore, excised animal corneas are ideal substitutes. Many groups have been successful using trephinated corneas from excised rabbit eyes (Valls et al., 2008) and pig eyes (Gratieri et al., 2010) to study ophthalmic drug penetration. Porcine eyes are typically discarded from slaughterhouses, making this a cheap and quickly obtainable tissue source for the proposed model.

Considering the various experimental models for corneal penetration studies reviewed above, the ideal surrogate for this assay is an excised porcine cornea. As pigs are ubiquitously raised and slaughtered for human consumption, discarded tissue, specifically the eyes, is readily available. Because drug penetration relies heavily on corneal epithelial health, it will be important to obtain the discarded eyes as quickly as possibly following animal death, meaning research laboratory proximity to slaughterhouse is critical. For the scope of the proposed study, the University of Arizona houses a Meat Sciences laboratory that slaughters pigs weekly, meaning direct access to live cornea tissue is achievable.

Additionally, it may be preferable to find an alternative to live animal cornea to reduce the assay's dependence on proximity to a slaughterhouse and/or availability of tissue discards. Potential solutions could include a robust cell cultured or synthetic surrogate of the cornea and should be explored (Minami et al., 1993).

Hardware Considerations

Following the cornea substrate, the second-most critical aspect of the model is the diffusion cell hardware, in which the trephinated cornea will be affixed to define two separate chambers. Primary considerations for hardware selection include validity of experimental setup (physiological relevance) and scalability (cost, reproducibility, ease of use).

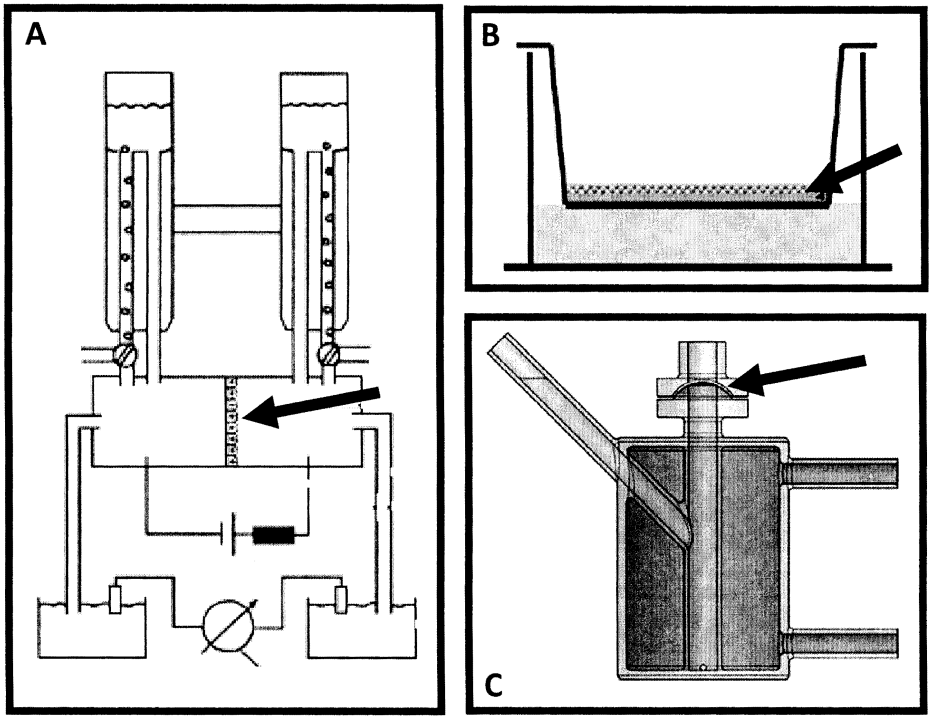


***Figure 5**: Diffusion Chamber Hardware Options: (A) Ussing Chamber* (Used with permission: Li et al., 2004). *(B) Snapwell* (Used with permission: Hornoff et al., 2005). *(C) Franz Cell* (drawn by author). These schematics depict three diffusion cells that have been used for corneal permeability testing (*not to scale).* Arrows point to the location of the cornea substrate for each apparatus.

In reviewing the literature describing drug penetration studies of the cornea, most groups used either an Ussing chamber (Ashton et al., 1991; Li et al., 2004), a Franz cell (Resch et al., 2010; Gratieri et al., 2010), or Snapwell plates (Burgalassi et al., 2004) as diffusion cell hardware as depicted in *Figure 8*, above. Each apparatus has advantages and disadvantages as compared to the others, such as cost, ease of use and physiological relevance, which are important factors in designing the proposed penetration and toxicity assay.

Li et al. (2004) describes how Ussing chambers can be used to measure trans-epithelial resistance during penetration studies to monitor epithelial tight junction integrity. A major disadvantage of an Ussing chambers is that they can be costly to set up due to the complexity

of glassware, chambers and equipment. Also, the Ussing chamber submerges both the epithelial and endothelial sides of the cornea in solution, making it difficult to administer test formulations and to access the epithelial layer for monitoring epithelial health.

Other groups have even used a very simple setup when examining cell-cultured epithelia layers with use of Snapwell plates (Burgalassi et al., 2004). Here, rabbit epithelial cells were grown on a porous support at the bottom of a small diameter well which was slightly submerged into a larger diameter well containing media, therefore separating the donor and acceptor chambers. An advantage of this diffusion cell over the other models is the multi-well design, allowing 12 or 24 simultaneous experiments while the Ussing chamber and Franz cell only accommodate a single experiment. While simple to use and cheap, the Snapwell plate lends itself specifically to cell culture models, and is therefore unacceptable for the proposed model due to the aforementioned reasons.

Gratieri et al. (2010) used a modified Franz cell to study as the basis for a model of excised porcine cornea for iontophoresis-enhanced penetration studies. The major advantages of a the Franz cell are cost, ease of use, and an air-substrate interface that more closely resemble physical conditions of the cornea. This setup is ideal for topical administration of test formulations and access to the corneal for epithelial health monitoring.

Major advantages of the Snapwell plate and Franz cell are low cost, physiological relevance, and ease of use as compared to the more expensive Ussing chamber. While the Ussing chamber

submerges both sides of the cornea, the Franz cell leaves an air-cornea interface on the epithelial side. The Franz cell is the preferred apparatus for the proposed model because of cost ($100s vs. $1000s), physiological relevance (air-epithelial interface vs. aqueous-epithelial interface), substrate compatibility (excised cornea tissue vs. cell culture), and access to the epithelial surface for toxicity monitoring.

Penetration Measurements

The first goal in assembling the proposed *in vitro* model is to validate corneal permeability. Simply, samples from the acceptor chamber of the diffusion cell can be sampled and analyzed for drug concentrations for the duration of an experiment. This data can then be used to calculate either the drug penetration rate or apparent permeability coefficient. The key to the precision and accuracy of penetration measurements is the sensitivity of the drug concentration measurement system.

One promising and fairly simple method for measuring drug penetration, as long as the sensitivity is shown to be adequate, is with a spectrophotometer. First, the wavelength at which the drug molecule absorbs the greatest should be identified. This is simply done by placing a small aliquot of high concentration drug solution into a cuvette and measuring its absorbance across the entire spectrum from ultraviolet to infrared light. The wavelength at which the drug absorbs the greatest is then selected and used to quantify concentration. Then, a set of concentration standards can be made by diluting the original drug solution into aliquots covering a range of known concentrations. By measuring the percentage of light absorption of each known standard at the previously identified wavelength, a linear relationship between

light absorption and drug concentration is obtained. This calibration curve is then used to extrapolate drug concentrations based on absorption data from experimental samples. Resch et al. (2010) used this straightforward method for quantifying ofloxacin concentration during a penetration study of amniotic membrane. Variations of this spectrophotometer method have demonstrated good precision and accuracy down to concentrations of 0.5µg/mL (Kanakapura & Rangachar, 2007).

A more sensitive and more expensive method for measuring concentration of a compound is via high performance liquid chromatography (HPLC). Santoro et al. (2006) demonstrated precision, accuracy and sensitivity of using HPLC to quantify concentrations of fluoroquinolone antibiotics down to 4.0µg/mL. In measuring beta blocker concentrations with HPLC, Aston et al. (1991) claimed a detection limit down to 5ng/ml (0.005µg/mL), which is 100 times more sensitive than the most sensitive spectrophotometric method.

The sensitivity of the concentration measurements is critical for the validity of this *in vitro* model. If the detection limit of the measurement system is too high, it would be impossible to accurately determine small changes in penetration rates due to changes in formulations, which is a critical aspect of the proposed assay. While sensitivity considerations point towards HPLC as the preferred measurements systems, cost and ease of use is also a factor which make spectrophotometry an attractive method also.

Corneal Health and Toxicity Measurement

Alternatives to toxicity screening in live animals are desirable (Herzinger et al., 1995). In developing new topical ophthalmics, the Draize test (which has been used since the 1940s) is often employed to screen for potential toxicity in rabbits (Dart, 2003). In this test, the new drug formulation is applied to rabbits' eyes, and over the next 14 days the rabbits are observed for changes in corneal opacity, iris inflammation, conjunctival redness, chemosis, and discharge to identify toxicity. At the conclusions of the test, the rabbits are typically euthanized. While this method is effective in screening for immediate and severe toxicity, it does not serve utility to identify the subtle changes in epithelial health that could indicate potential epithelial sloughing or corneal melts (like the generic diclofenac case). Alternatively, an *in vitro* assay, like the one proposed here, could provide a more precise method for monitoring slight changes in corneal health. Additionally, an *in vitro* assay would not require the sacrifice of animals for toxicity studies and could therefore be more ethical and less expensive.

The effect of the drug on epithelial health is a critical element of the proposed model, as breakdown of this protective barrier can be catastrophic for the health of the cornea. It will be essential to not simply identify whether the drugs kill the epithelial cells, but to monitor small changes in cell health over the duration of the experiment, which is significantly more complex. Changes in epithelial metabolic activity is a good indicator of a changes in cell health, meaning measuring concentrations of metabolic compounds like NADH or ATP could be used.

Among the few groups that do study corneal epithelial toxicity, most of these use cell culture models as their substrate (Dart, 2003; Geerling et al., 2001; Poon et al., 2001). These techniques are typically endpoint assays, ending in cell sacrifice and meaning these techniques would not be suitable for non-invasively monitoring cell health. In studying the toxicity of tear substitutes on epithelial cells, Geerling et al. (2001) used cultured human corneal epithelial cells *in vitro* to assess toxicity due to varying concentrations as compared to control in a viability assay and an ATP assay. The viability assay simply used fluorescent live-dead staining and by counting live and dead cells. The ATP assay used a luminescence technique to determine cellular ATP content (Geerling et al., 2001). Poon et al. (2001) also used these techniques to evaluate toxicity of serum eyedrops *in vitro* on a cell cultured epithelial model.

While viability assays and ATP assays are effective endpoint indicators of toxicity in cell culture, these techniques would not be suitable for our model. Specifically, to monitor the health of excised cornea throughout the course of an experiment, a non-invasive technique will be needed.

To avoid the problems of the endpoints assays described above, a significantly more complex method for determining cell health is to monitor the concentration of NADH in the corneal epithelial layer, which is typically 45 μm thick consisting of five cell layers. Measurement of NADH levels can be indicative of the metabolic activity of the cells and therefore health. Piston et al. demonstrated a two-photon microscopy method for measuring changes in NADH autofluorescence due to epithelial poisoning from cyanide (Piston et al., 1995). A simpler

microscopy method could instead target changes in the regular hexagonal cell structure of the squamous cells that make up the outermost epithelial cell layer. Changes in squamous cell morphology could indicate toxicity (Dart, 2003).

Trans-epithelial resistance is often measured in penetration studies to monitor the integrity epithelial tight junctions during penetration studies (Toropainen et al., 2001; Li et al., 2004). In this simple, non-imaging technique, electrical resistance across the cornea can be monitored with electrodes on either side of the membrane. By applying a known voltage (*V*) and measuring the changes in current (*I*), the resistance (*R*) is calculated using Ohm's Law: $R = \frac{V}{I}$. Alternatively, resistance can also be measured by inputting a known current and measuring change in voltage. Larger resistances indicate tighter junctions between epithelial cells and smaller resistances indicate looser junctions. While this method is easy to use, it may not provide information sensitive enough to correlate changes in resistance to epithelial toxicity and will need to be explored.

Description of Proposed Model

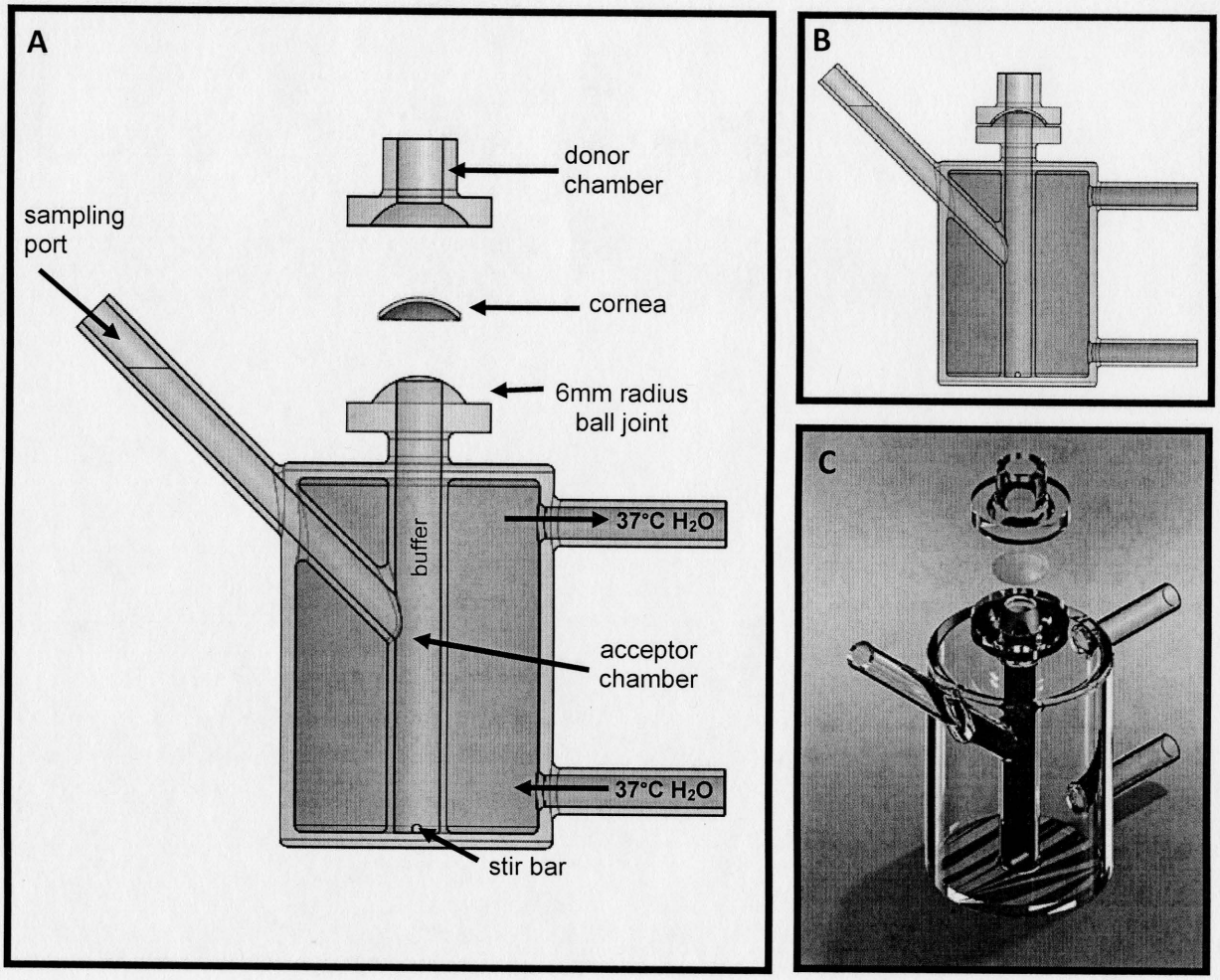


***Figure 6**: (A) Schematic of the Proposed Model.* This schematic depicts the key components of the proposed model. The excised cornea will be sandwiched between the concave surface of the donor chamber and the convex surface of the acceptor chamber, which will be clamped together to ensure a tight seal. The acceptor chamber will be surrounded by a warm water jacket to keep the system at physiological temperature and to keep the epithelial and endothelial cells alive for the duration of the experiment. *(B) Cross-section of Collapsed Model. (C) Graphical Rendition of the Proposed Model.*

Based on the reviewed prior art, the proposed model will be comprised of the following key components:

1. Modified Franz Cell diffusion chamber (glass with spherical joint and warm water jacket)
2. Live excised porcine cornea (from slaughterhouse, trephinated)

3. Aqueous humor surrogate (tbd)
4. Drug concentration quantification (spectrophotometry or HPLC)
5. Epithelial health quantification (tbd)

As seen in *Figure 6*, above, the proposed model consists of a modified Franz cell housing a trephinated porcine cornea. The cornea is clamped between the spherical surfaces of the donor and acceptor chambers with the epithelial side exposed. The acceptor chamber is filled with the surrogate aqueous humor (contacting the endothelial side of the cornea) homogenized with a magnetic stir bar and the warm water jacket is supplied by a heat bath at 37°C. After applying the drug formulation to the epithelial surface, samples of from the acceptor chamber are obtained over the duration of the experiment to determine the drug flux across the cornea.

The apparent permeability coefficient (P_{app}), in cm/min, is the most frequently used term for representing a drug's permeability through the cornea. This coefficient can be calculated with: $P_{app} = \frac{\partial Q}{\partial t SAC_0}$, where $\delta Q/\delta t$ is the permeation rate (mol/sec), *SA* is the surface area (cm^2), and C_0 is the initial concentration (mol/ml) in the donor compartment (Valls et al., 2008). The permeation rate ($\delta Q/\delta t$) is determined by the concentration-time curve generated by measuring the amount of drug penetrating the cornea over time.

The diameter of the diffusion cell's orifice will determine the exposed surface area (*SA*) of the cornea through which test drugs will permeate. For example, with an outer corneal radius (*r*) of

6mm, and an orifice radius (a) of 2.5mm, the total surface area of the exposed corneal 'dome' will be: $SA = 2\pi r\left(r - \sqrt{r^2 - a^2}\right)$, which comes to 0.206cm^2. The typical surface of a human cornea is 1.04cm^2 (Worakul & Robinson, 1997), meaning the model will represent 20% of a human cornea and should be considered when interpreting data.

The anterior chamber of the human eye typically houses 0.1 to 0.25mL of aqueous humor (Worakul & Robinson, 1997). The model's acceptor chamber, which represents the anterior segment of the eye, is 5mL in the current design, meaning the volume will be 20 to 50 times larger. This will only present a problem if the drug concentration measurement system (spectrophotometer or HPLC) is not sensitive enough to detect small concentrations. A simple modification can be made to reduce this volume if better sensitivity is needed.

Histology can be used to confirm the epithelial integrity after experimentation, but trans-epithelial resistance can be monitored to indicate changes in the tight junction integrity. Resistance can be measured by placing an electrode on either side of the cornea, as previously described. Consistent corneal thickness for the duration of an experiment demonstrates the health of the endothelial cells, which regulate the corneal stroma hydration. A pechymeter, which is used clinical for cornea thickness measurements, can be used to for corneal thickness monitoring.

To mimic the aqueous humor, many groups use a buffer that simulates the physiological constituents of aqueous humor (Valls et al., 2008; Burgalassi et al., 2004; Toropainen et al.,

2001). Specifically groups have used glutathione bicarbonate Ringer solution at a pH 6.5 (Burgalassi et al., 2004), artificial tear solution with added glutathione (0.13g/L) and adenosine (0.09g/L) (Valls et al., 2008), balanced salt solution (Toropainen et al., 2001), and 25mM HEPES buffer solution (Gratieri et al. 2010). For the proposed model, these will have to be evaluated to identify which keeps the excised cornea healthiest for the duration of testing (4 to 6 hours) while not interfering with the absorption spectra (concentration measurements) of tested drugs.

The proposed model will be successful if it meets the following criteria:

- Predictive of corneal penetration
- Predictive of epithelial health
- Robust and reproducible
- Cost effective

As with any *in vitro* model, *in vivo* testing will still need to be completed to confirm the validity of the *in vitro* results. Since the goal of the proposed assay is to weed out as many insufficiently penetrating or toxic formulations *in vitro*, the number animals used for testing will be minimized.

Conclusion

The development of a simple, robust, and cost effecting assay for enhanced screening of ophthalmic generics is urgently needed. The reviewed literature points towards the feasibility

of assembling such a model that can robustly screen ophthalmics for penetration and safety. The gap in FDA regulation of generic ophthalmic formulations places the public at unnecessary risk, which could be alleviated if drug companies self-imposed stricter research and development methods. However, because drug companies are businesses and must make money to survive, the only way for non-FDA required screening to be implemented is to make the proposed assay cost effective.

The novelty of the proposed model is its ability to simultaneously evaluate penetration and toxicity of topical ophthalmic formulations, which, based upon a thorough literature review, has not been previously demonstrated. Many groups have assembled ocular penetration models for the purpose of better understanding and improving penetration kinetics into the eye and some groups have even developed methods to quantify epithelial health. By combining the best qualities of these two methods, the proposed model will be a powerful tool for rapidly screening new ophthalmic formulations, resulting in reduced animal studies and safer and more efficacious drugs. Additionally, if the proposed model proves to be successful in predicting penetration and safety of generic ophthalmic formulations, it is likely to also serve utility as a tool for screening *novel formulations* in pre-clinical testing in addition to generic formulations.

The logical next step the project's progression is to identify specific aims for researching drug penetration and toxicity with the proposed model as part of the author's future Ph.D. work. Future specific aims could include:

Specific Aim 1: Evaluate the differences in corneal penetration between commercially available brand-name and generic topical ophthalmic medications.

Working Hypothesis: Variations between additives in brand-name and generic formulations alter drug penetration kinetics, resulting in varying efficacy.

Specific Aim 2: Develop a sensitive measurement system for quantifying subtle changes in epithelial health that are precursors to corneal toxicity.

Working Hypothesis: Minor variations in mitochondrial NADH levels or squamous cell morphology are indicative of corneal toxicity.

Even after validating the model for accuracy in predicting a the penetration and toxicity of a generic formulation, the success of the proposed assay will ultimately be determined by drug companies' willingness to adopt an extra layer of screening that is not required by the FDA. Predominantly, the assay must be cost effective for drug companies to subscribe to this extra research expense. Arguments in support of the assay's cost effectiveness could include the (1) reduction of costly animals testing and (2) reduced risk of costly drug recalls. The current rendition of the assay as depicted and described within this report, may not be the least expensive incarnation of the assay due to the need for live excised porcine corneas and single-run hardware, so future modifications will have to be made.

As ophthalmic NSAIDs, fluoroquinolones, and β-blockers come off patent protection in the coming years, many generic formulations will be vying to compete for market share. If efficacy and safety screening of generic ophthalmic drugs does not improve soon, repeats of the 1999

diclofenac disaster are possible. Implementation of an efficacy and safety assay, like the one proposed in this report, into the screening process of generic ophthalmic drugs will eliminate uncertainty and protect the public's health.

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