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Ocular Pharmacokinetics of Dorzolamide and Brinzolamide Following Single and Multiple Topical Dosing: Implications for Effects on Ocular Blood Flow

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List of abbreviations :

AUC : Area under curve; C_{max} : Maximum concentration; T_{max} : Time at maximum concentration ; POAG : Primary open angle glaucoma ; IOP : Intraocular pressure ; OBF : Ocular blood flow ; CA : Carbonic anhydrase

ABSTRACT

Ophthalmic carbonic anhydrase inhibitors have been shown to improve retinal and optic nerve blood flow. However, the relative tissue distribution of commercially available carbonic anhydrase inhibitors to the optic nerve is not known. The objective of this study was to compare the ocular pharmacokinetics and tissue distribution profiles of dorzolamide and brinzolamide after single and multiple topical applications. Pigmented rabbits were treated with single or multiple topical administrations of 30 µl of Trusopt® (dorzolamide hydrochloride ophthalmic solution, 2 %) to one eye and 30 µl of Azopt® (brinzolamide ophthalmic suspension, 1 %) to the other eye. Rabbits were euthanized at 10 predetermined time intervals over a period of 24 hrs and ocular tissues and plasma samples were collected. For multiple dosing, rabbits were dosed twice a day with 8 hr interval between 2 doses and groups of rabbits were euthanized at 7, 14, and 21 days at 1 hr after the last dose and ocular tissues and plasma samples were collected. Drug levels in tissue samples were measured using LC-MS/MS. Pharmacokinetic parameters (C_{max}, T_{max}, and AUC_{0-24}) were estimated by noncompartmental analysis. After single dose, dorzolamide delivery (AUC_{0-24}) to the aqueous humor, anterior sclera, posterior sclera, anterior retina, posterior retina, anterior vitreous, and optic nerve was 2-, 7-, 2.6-, 1.4-, 1.9-, 1.2-, and 9-fold higher than brinzolamide. C_{max} was 2- to 5- fold higher for dorzolamide than brinzolamide in all ocular tissue. After multiple dosing, dorzolamide levels in aqueous humor, sclera, retina, vitreous humor, and optic nerve were higher than brinzolamide, but statistical significance was achieved only with aqueous humor, vitreous humor, and optic nerve. Upon multiple dosing, both drugs accumulated in all tissues except conjunctiva, where the drug levels were lower than those observed with single dosing. Dorzolamide levels in aqueous humor, anterior

vitreous, posterior vitreous, and optic nerve were 1.4-3.2-, 2.4-2.7-, 2.2-4.5-, and 2.4-5.2-fold higher than brinzolamide. No significant differences were found in the AUC of these two drugs in the cornea and conjunctiva after single as well as multiple dosing. Drug levels were significantly higher in anterior regions than posterior regions in sclera, retina, and vitreous for both drugs.

INTRODUCTION

Glaucoma is the most prevalent eye disease and a leading cause of blindness in Western industrialized world. Glaucoma is classified as primary when it is not associated with another disease and secondary when it results from another disease or drug treatment(Lee and Higginbotham, 2005). Glaucoma is further classified as open angle or angle closure glaucoma on the basis of anatomy of the anterior chamber. Primary open angle glaucoma (POAG) is the most prevalent glaucoma encountered in adults. POAG is progressive mutifactorial optic neuropathy that leads to loss of retinal ganglion cells (RGCs) and optic nerve damage (Siesky et al., 2008). The main etiological outcome of POAG is elevated intraocular pressure (IOP), a major risk factor for optic neuropathy.

Conventional treatment for POAG is the topical application of IOP lowering drugs. Beta-blockers and prostaglandin analogs are first line treatments in glaucoma, which reduce the IOP by decreasing aqueous humor formation and increasing aqueous humor non-conventional outflow, respectively. Second line treatment of choice for glaucoma is carbonic anhydrase (CA) inhibitors and alpha agonists (Lee and Higginbotham, 2005). Carbonic anhydrases are responsible for production of bicarbonate ion, which is secreted into posterior segment by ciliary body along with Na^+ as counter ion (Sugrue, 1996). Inhibition of carbonic anhydrases results in inhibition of bicarbonate ion production and reduction of IOP. Dorzolamide and brinzolamide are drugs of choice as topical CA inhibitors for glaucoma treatment.

Various preclinical as well as clinical reports suggested the involvement of reduced ocular blood flow (OBF) in the pathogenesis of POAG (Yoshida et al., ; Schmidt et al.,

1998; Harris et al., 1999; Bathija, 2000; Zhao and Cioffi, 2000). The reduced OBF may be primarily of vascular origin or a secondary effect of elevated IOP. Grunwald et al. showed that the optic nerve blood flow is reduced by 24 % in glaucoma patients (Grunwald et al., 1998). Some investigators have hypothesized that the death of RGCs and optic nerve head may be due to ischemia as result of elevated IOP or reduced oxygen supply because of reduced blood flow (Johnson et al., 2000; Gross et al., 2003; Kuehn et al., 2005; Rokicki et al., 2007). It has been shown that CAIs increase the cerebral blood supply after systemic administration (Okazawa et al., 2001) and ocular blood supply when administered topically (Martinez et al., 1999). To achieve an effect on OBF, topically administered CAIs must reach the inner ocular tissue such as retina and optic nerve in critical effective concentrations. Various literature reports have clearly shown the effect of dorzolamide on OBF (Harris et al., 1996; Martinez et al., 1999; Martinez and Sanchez, 2007; Martinez and Sanchez-Salorio, 2009). Topical application of dorzolamide results in significant improvement in blood flow to retina and optic nerve head(Harris et al., 1996; Martinez and Sanchez, 2007). However, very few studies have reported the effect of brinzolamide on OBF (Barnes et al., 2000; Kaup et al., 2004; Siesky et al., 2008). Further the reports from these studies are not conclusive. Barens et al. (Barnes et al., 2000) showed significant improvement in optic nerve head blood flow after topical application of brinzolamide in Dutch belted rabbits, whereas Kaup et al. (Kaup et al., 2004) showed no effect of brinzolamide on OBF. Recent clinical comparison of dorzolamide and brinzolamide on OBF in POAG patient showed an increase in retrobulbar blood flow with dorzolamide but not with brinzolamide (Martinez and Sanchez-Salorio, 2009).

In this study we have hypothesized that the effect of dorzolamide on OBF may be due to better delivery of dorzolamide to target tissues such as retina and optic nerve after topical application when compared with brinzolamide. To evaluate this hypothesis we have compared the ocular pharmacokinetics and tissue distribution of commercially available dorzolamide and brinzolamide formulations in Dutch belted (DB) rabbits after single and multiple topical dosing.

MATERIALS AND METHODS

Materials

The commercial formulation of Trusopt® was from Merck and Co. Inc. Trusopt contained dorzolamide hydrochloride, 2 % w/v, along with inactive ingredients including hydroxyethyl cellulose, mannitol, sodium citrate, sodium hydroxide, and water for injection. Benzalkonium chloride (0.0075 % w/v) was present as preservatives and the pH of the solution was about 5.6. The osmolarity range specified for the product was 260-330 mOsM. The commercial formulation of Azopt® was from Alcon Laboratories Inc. Azopt contained brinzolamide, 1 % w/v, as a sterile ophthalmic suspension along with inactive ingredients including mannitol, carbomer 974P, tyloxapol, edetate sodium, sodium chloride, hydrochloric acid, sodium hydroxide, and water for injection. Benzalkonium chloride (0.01 % w/v) was present as a preservatives. The pH of the solution was approximately 7.5 and the specified osmolarity was 300 mOsM. Timolol maleate (98%) was purchased from Sigma-Aldrich (St. Louis, MO). HPLC grade acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, NJ). All other chemicals and reagents used in this study were of analytical reagent grade.

Methods

Animals

Animal studies were conducted in accordance with ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and guidelines by animal care committee of University of Colorado Denver. Total 39 male Dutch Belted rabbits in the weight range of 1.8 to 3 kg were used in this study. Rabbits were housed under standard conditions with access to tap water and standard dry pellet rabbit feed *ad libitum.*

Single dose ocular pharmacokinetics

Thirty rabbits were used for ocular pharmacokinetic comparison of Trusopt® and Azopt® after single topical application. Animals were divided in to 10 groups (3 animals each). The rabbits were restrained in a rabbit restrainer and were allowed to stabilize for 10 min before dosing. Once the animal was stabilized in restrainer, drug solution was applied using a positive displacement pipette (Gilson 10-100µl) and sterile tips. Trusopt (2% Dorzolamide hydrochloride solution) was randomly applied to one eye and Azopt (1% Brinzolamide suspension) to the eye of each animal. The volume for the topical ocular dose was 30 µl/eye. To minimize the runoff of instilled dose, the eyelids were gently closed for a few seconds after dosing. The time of dose administered was recorded for each animal. At predetermined time intervals after dosing, blood samples were collected form marginal ear vein. Animals were euthanized by intravenous injection of sodium pentobarbitone (150mg/kg) into the marginal ear vein. Eyes were then enucleated using surgical accessories and snap frozen immediately in dry ice: isopentane bath and stored at -80 ^{0}C until dissection. The dry ice: isopentane bath was prepared in a stainless steel container and a ceramic tile was placed over the container and allowed to cool for 15 min. The eyes were removed from -80°C and placed in the dry ice container pending dissection.

Multiple dose ocular tissue distribution

Nine rabbits were used for comparison of ocular tissue distribution profiles of Trusopt® and Azopt® after multiple topical applications. Rabbits were divided into three groups (3 animals each). Rabbits received 30 µl of Trusopt® in right eye and 30 µl of Azopt® in left eye twice a day with 8 hr intervals between the doses. Group 1 received 14 doses over 7 days, group 2 received 21 doses over 14 days, and group 3 received 42 doses

over 21 days. Blood samples were collected from the marginal ear vein at 1 hr after the last dose. Immediately after blood collection, animals were euthanized by intravenous sodium pentobarbitone (150mg/kg) injection into the marginal ear vein. Eyes were then enucleated using surgical accessories and snap frozen immediately in dry ice: isopentane bath and stored at -80 ^{0}C until dissection.

Eye dissection and collection of various ocular tissues

Enucleated eye balls were dissected, while frozen, to isolate various ocular tissues. All dissection procedures were performed on a cooled ceramic tile to avoid thawing of the eye ball during dissection. After separation of anterior part, the reaming posterior globe was cut into two parts; at 1/3rd distance from the lens and 2/3rd from the posterior wall, and two parts of the retina, choroid, vitreous and sclera were separated. A new surgical blade was used for each eye. In order to prevent transfer of drugs between tissues of each eye, the surgical accessories were thoroughly rinsed with saline followed by methanol followed by saline and blotted dry after and between uses on each tissue. All the samples were weighed and stored at -80 ºC until further processing.

Tissue sample processing

Drug content in rabbit ocular tissues was estimated after extraction of drugs from tissues by double liquid-liquid extraction. Briefly, the ocular tissues were mixed with 500 µl of 0.1 M Tris-buffer (pH 8.5) and 5 µl of 20 µg/mL timolol (internal standard) in 4 ml glass tubes, vortexed for 10 min, and then homogenized using a hand homogenizer on an ice bath. Ethyl acetate (1.5 ml) was added to this homogenate and vortexed for 10 min on multitube vortexer (VWR LabShop, Batavia, IL). The organic layer was separated by

centrifugation at 3000 g for 10 min. 1.5 ml of ethyl acetate was added again to the aqueous layer to perform the double extraction and vortexed for 10 min and centrifuged at 3000 g for 10 min. The separated organic layers were combined and transferred into clean glass tubes and evaporated under nitrogen stream (Multi-Evap; Organomation, Berlin, MA) at 40 ºC. The residue after evaporation was reconstituted with 250 µl of acetonitrile: water (75: 25 v/v) and subjected to LC-MS/MS analysis. The liquid-liquid extraction method for extraction of dorzolamide and brinzolamide from rabbit ocular tissue was validated to determine the extraction recovery using three different concentrations (low, medium and high) to cover the entire range of expected concentrations of dorzolamide and brinzolamide in various tissues.

The aqueous humor and vitreous samples were analyzed directly after dilution, without liquid-liquid extraction. Briefly, the aqueous humor and vitreous samples were diluted 5- and 2.5- fold, respectively, with acetonitrile containing timolol as the internal standard, vortexed for 10 min and centrifuged at 10,000 g for 5 min. The supernatant (200 µl) was transferred into LC-MS/MS vials and subjected for analysis.

Calibration standards were prepared at 10 concentrations in appropriate blank rabbit ocular tissue by spiking known amount of analytes and internal standard. Quality control samples were prepared by spiking blank rabbit tissue with 3 different concentrations of analytes to cover the entire calibration range. Both calibration and quality control samples were processed on same day along with study samples.

LC-MS/MS analysis

Tissue levels of dorzolamide and brinzolamide in ocular tissue samples were measured by means of LC-MS/MS. An API-3000 triple quadrupole mass spectrometry (Applied Biosystems, Foster City, CA, USA) coupled with a PerkinElmer series-200 liquid chromatography (Perkin Elmer, Walthm, Massachusetts, USA) system was used for analysis. Analytes were separated on Zorbax extended C18 column (2.1 x 50 mm, 5 µm) using 5 mM ammonium formate in water (A) and acetonitrile (B) as mobile phase. A linear gradient elution at a flow rate of 0.3 ml/min with total run time of 6 min was as follows: 60% A (0– 0.8 min), 10% A (2.0– 4.0 min), and 60% A (5.0-6.0 min). Dorzolamide, brinzolamide, and timolol (internal standard) were analyzed in positive ionization mode with following multiple reaction monitoring (MRM) transitions: 325 → 199 (dorzolamide); 384 → 281 (brinzolamide); and 317 → 261 (timolol).

Pharmacokinetic and statistical Analysis

Pharmacokinetic analysis was performed by noncompartmental analysis using WinNonlin software (Version 1.5, Scientific Consulting, Inc.). Statistical comparisons between two experimental groups were performed using independent samples Student's t-test. The comparison of the mean between multiple ocular tissues of same group was performed using one-way ANOVA followed by the Tukey's post hoc analysis (SPSS, ver.11.5; SPSS, Chicago, IL). The results were considered statistically significant at $P < 0.05$.

RESULTS

LC-MS/MS method for dorzolamide and brinzolamide

A simple, selective, and sensitive LC-MS/MS method was developed for simultaneous analysis of brinzolamide and dorzolamide in rabbit ocular tissues. Timolol was chosen as an internal standard because its log P and pKa value are close to brinzolamide and dorzolamide. The representative chromatogram of analytes along with the internal standard is shown in Supplemental Figure 1. A simple liquid-liquid extraction method was developed for extraction of analytes from the rabbit ocular tissue. Both, the analytes and the internal standard have basic pKa's; therefore, basic pH was used during extraction to keep the analytes in the unionized form. Ethyl acetate was used as the organic solvent for the extraction because both analytes have good solubility in ethyl acetate. The method was validated for the extraction recovery at three different concentrations (low, medium, and high quality control samples), i.e., 25, 250, and 1250 ng/ml. The extraction recovery of internal standard was estimated at medium concentration. The mean extraction recoveries (mean ± S.D, N = 3) in rabbit eye tissues are shown in Table 1.

Single dose ocular pharmacokinetics

Concentration time profile of dorzolamide and brinzolamide following single topical administration in cornea, conjunctiva and aqueous humor are shown in Figure 1. Data for posterior segment tissues is shown in Figure 2. Dorzolamide showed significantly higher drug levels in aqueous humor, sclera, retina, vitreous, and optic nerve than brinzolamide for initial few hours. Both dorzolamide and brinzolamide rapidly distributed to inner ocular tissue following topical application. Maximum concentration of

dorzolamide in aqueous humor occurred within 30 min, whereas for brinzolamide it occurred after 45 min. Aqueous humor levels of dorzolamide at peak time was 1.8 fold higher than brinzolamide. Both dorzolamide and brinzolamide showed region-selective distribution in sclera, retina, and vitreous. Drug levels in the anterior part of sclera, retina, and vitreous were significantly higher than the posterior parts. Measurable drug levels were observed in anterior vitreous and retina for both drugs over the duration of 24 hrs. No drug levels were detected in posterior vitreous for brinzolamide at any time point, whereas dorzolamide was detected in posterior vitreous up to 3 hrs after dosing. Drug levels were not detected in the plasma for both dorzolamide and brinzolamide. Summary of pharmacokinetic parameters estimated by noncomapartmental analysis is shown in Table 2. The area under curve (AUC_{0-24} (μg.h/mL or g)) of dorzolamide was 2-fold higher in aqueous humor than brinzolamide, whereas there was no significant difference for AUC_{0-24} in cornea and conjunctiva. The AUC_{0-24} of dorzolamide was significantly higher than brinzolamide for all posterior segment tissues. The AUC_{0-t} of dorzolamide was 7-fold higher in anterior sclera, 3.5-fold higher in posterior sclera, 1.5-fold higher in anterior retina, 1.9-fold higher in posterior retina, 4-fold higher in anterior vitreous, and 9-fold higher in optic nerve when compared to brinzolamide. The peak concentration (C_{max}) was 2- to 5- fold higher for dorzolamide than brinzolamide in all ocular tissue. After single dose topical administration, dorzolamide showed significantly enhanced delivery to the posterior segment ocular tissue when compared with brinzolamide.

Distribution of dorzolamide and brinzolamide in ocular tissue following multiple topical dosing

The tissue distribution of dorzolamide and brinzolamide after multiple topical applications, in anterior segment ocular tissues and plasma on days 7, 14, and 21 are shown in Figure 3. Drug levels in conjunctiva and cornea were similar for both drugs at all three time points. Aqueous humor levels of dorzolamide were 1.4 to 3.2- fold higher than brinzolamide, with the levels being statistically different on days 7 and 21. Plasma dorzolamide levels were 1.3-1.9-fold higher than brinzolamide, with the levels being significantly different on days 7 and 14.

Distribution of dorzolamide and brinzolamide to sclera, retina, vitreous, and optic nerve after multiple topical dosing is shown in Figure 4. Both drugs exhibited regio-selective distribution in sclera, retina, and vitreous, with the drug levels in anterior sclera, retina, and vitreous being significantly higher than posterior sclera, retina, and vitreous, respectively. Between the two drugs, dorzolamide levels in anterior sclera and retina were higher than brinzolamide at all three time points, but the differences were not statistically significant. Dorzolamide levels were 1.4- to 1.6-fold higher in anterior sclera and 1.2- to 1.5-fold higher in anterior retina, when compared to brinzolamide. Dorzolamide levels were 1.1- to 1.6-fold higher in posterior sclera and 1.3- to 1.8-fold higher in posterior retina, when compared to brinzolamide. Dorzolamide concentration in both anterior and posterior vitreous and optic nerve were significantly higher than brinzolamide. Concentrations of dorzolamide were 2.4- to 2.7- fold higher in anterior vitreous and 2.2- to 4.5-fold higher in posterior vitreous, when compared to brinzolamide. Further, dorzolamide levels in optic nerve were 2.4- to 5.2-fold higher when compared with brinzolamide.

DISCUSSION

Our studies with commercially available formulations of dorzolamide and brinzolamide in pigmented rabbits indicated that dorzolamide delivery to the aqueous humor, vitreous humor, and optic nerve is significantly higher than brinzolamide both after single as well as multiple topical applications. Both drugs exhibited regio-selective distribution in sclera, retina and vitreous, with drug levels in anterior regions of these tissues being higher compared to posterior regions.

Topically applied carbonic anhydrase inhibitors enhance the retinal and optic nerve blood flow, in addition to reducing intraocular pressure (Martinez et al., 1999; Martinez and Sanchez-Salorio, 2009). In human subjects, dorzolamide treatment resulted in 3.8-fold higher peak systolic velocity and 6.7-fold higher end diastolic velocity in central retinal artery than brinzolamide treatment (Martinez and Sanchez-Salorio, 2009). Further, central retinal artery resistance index was reduced by dorzolamide, whereas brinzolamide did not have any effect on this index. To achieve their effect on ocular blood flow, topically applied carbonic anhydrase inhibitors must reach retina and optic nerve at therapeutically effective concentrations after topical application. The premise of this study was that the above differences between dorzolamide and and brinzolamide may be explained on the basis of delivery differences to target tissues such as retina and optic nerve.

In our study, dorzolamide showed enhanced delivery to the aqueous humor, vitreous humor, and optic nerve, when compared to brinzolamide, in both single and multiple topical dosing regimens (Figures 1-4), possibly due to differences in drug physicochemical properties such as lipophilicity and solubility. The amount of drug that crosses biological barriers to reach its target site depends on drug physicochemical properties including

lipophilicity, water solubility, and molecular size. Since dorzolamide (324 Da) and brinzolamide (383 Da) have similar molecular weights, differences in lipophilicity and water solubility might explain the enhanced drug delivery observed with dorzolamide.

The Log D of brinzolamide and dorzolamide at pH 7.4 are 6.6 and 1.72, respectively (DeSantis, 2000). Schoenwald et al. showed that the optimum log D for corneal permeability was between 2.0- 3.0, with further increase in lipophilcity resulting in lower corneal permeability (Schoenwald and Ward, 1978; Schoenwald and Huang, 1983).. Thus, an increase in drug lipophilicity beyond a certain point may not be beneficial for corneal transport, possibly due to a trade off in drug solubility. Higher lipophilicity of brinzolamide (Log P 6.6) may hinder its corneal permeability and entry into aqueous humor. Dorzolamide with a log D of 1.72 is within the optimum range of pH for corneal transport. Indeed, the apparent in vitro permeability of dorzolamide (10 µM dorzolamide solution in balanced salt solution) across excised rabbit cornea was 7.5-fold higher (1.5 x 10^{-6} cm/s) (Xiang et al., 2009), when compared to the highly lipophilic brinzolamide (AZOPT® 1% suspension) (0.20 x 10^{-6} cm/s) (Palma et al., 2009). However, normalization of brinzolamide in vitro permeability with soluble concentration (0.05 %) indicated that the actual permeability of brinzolamide is 4.0 x 10^{-6} cm/s, which is 2.66 fold higher than dorzolamide. Dosing strength of dorzolamide drop was 2 %, which is 2 fold higher than the brinzolamide eye drop (1%), and the soluble concentration for dorzolamide in the eye drop was 40 fold higher. Further, at physiological pH (pH 7.4), the aqueous solubility of dorzolamide is 0.67 %, which is 13.4 fold higher than the aqueous solubility of brinzolamide (0.05 %) (DeSantis, 2000). Thus, the major reason for higher delivery of dorzolamide to aqueous humor appears to be its higher concentration dependent flux across

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cornea than brinzolamide. Consistent with these differences in dosing strength and solubility, after single dose topical application, the C_{max} and AUC_{0-t} of dorzolamide in aqueous humor was 2-fold higher than brinzolamide (Table 2).

Although the C_{max} levels in cornea and conjunctiva in the single dose study were 1.9-2.4-fold higher for dorzolamide than brinzolamide, there was no significant difference in the exposure (AUC 0-t) of dorzolamide and brinzolamide to these surface tissues. Brinzolamide was administered as a suspension, which was potentially retained on the ocular surface for longer duration compared to solution. Indeed, Gupta et al. showed significant reduction in precorneal drainage rate for sparfloxacin nanosuspension when compared to sparfloxacin solution after topical application in a rabbit model (Gupta et al., 2010). Further, brinzolamide, due its highly lipophilic nature, may have entered cornea and conjunctiva well, but did not partition well out of the surface tissues into underlying fluid compartments.

After administration of nipradolol by topical, intracameral, and sub-Tenon routes, using autoradiography, it was shown that topically applied nipradolol reaches the choroid-retina via the conjunctival and transscleral pathway (Mizuno et al., 2009). Higher delivery of drug from conjunctiva and sclera into the posterior segment ocular tissues (retina, vitreous, and optic nerve) occurs when the drug is present at a higher concentration in the periocular space. In our single dose study, the C_{max} of dorzolamide was 1.9- fold higher in the conjunctiva and 5.8 fold higher in the anterior sclera than brinzolamide. This is consistent with the concentration dependent flux of dorzolamide to the posterior segment tissues via the conjunctival and transscleral pathway. In single dose study, dorzolamide showed significantly higher delivery (both AUC and C_{max}) to vitreous and optic nerve than

brinzolamide. Optic nerve levels of dorzolamide and brinzolamide were significantly higher than vitreous and posterior retina, which is consistent with a previously published report for betaxolol (Hollo et al., 2006). Dr. Maurice suggested that drug entry into the retrobulbar space via the conjunctival pathway contributes towards optic nerve drug delivery (Maurice, 2002).

Multiple dosing study was undertaken to compare the effect of repeated instillation on tissue accumulation and distribution profile of dorzolamide and brinzolamide. Comparison of drug levels in ocular tissue after multiple and single dose studies showed that drug levels in cornea, aqueous humor, retina, and vitreous were 1.6 to 90 fold higher after multiple dosing than single dosing (Table 3). Following multiple dosing, dorzolamide exhibited greater drug levels in all tissue except posterior sclera, where the levels were equal for both drugs. Multiple dosing was particularly beneficial in enhancing delivery of both drugs to the posterior regions of the back of the eye tissues including sclera, retina, and vitreous humor. However, multiple dosing resulted in a decline in drug levels in the conjunctiva when compared to single dosing, possibly due to enhanced drug clearance. We speculate that both dorzolamide and brinzolamide might enhance conjunctival blood flow with repeated topical dosing. Optic nerve, however, was not affected in terms of drug delivery between single and multiple doses. It should be noted that optic nerve may also receive the drug from systemic circulation and the drug levels in the plasma were higher with multiple dosing. Drug accumulation was also evident in cornea and aqueous humor for both drugs after multiple dosing. Although drug accumulation index was comparable between both drugs in anterior retina, posterior retina, and optic nerve, the accumulation index in anterior as well as posterior sclera was greater for brinzolamide. Based on our

dosing regimens and the half-lives extracted from the terminal phases in single dose studies, the estimated accumulation index with multiple dosing in various tissues is close to 1. Inspite of this, nearly all tissues except conjunctiva and optic nerve exhibited significant drug accumulation upon multiple dosing. The reasons for such accumulation are unclear at this stage. Either enhanced drug uptake or reduced clearance in several tissues upon multiple dosing might contribute to this effect.

Both dorzolamide and brinzolamide showed regio-selective distribution pattern in sclera, retina, and vitreous after single as well as multiple topical dosing (Figure 2 and 4), with drug levels in anterior parts of these tissues being significantly higher than the posterior parts. However, the extent of drug accumulation in the posterior segment was higher for dorzolamide than brinzolamide. Using autoradiography in albino rabbits, Mizuno et al. (Mizuno et al., 2009) showed regio-selective distribution of nipradiolol in the posterior segment after topical application. Nipradiolol levels in equatorial choroid-retina were 11.7 fold higher than posterior choroid-retina. Further, the nipradiolol levels in periocular tissue around equator region were 8.6 fold higher than periocular tissue near the optic nerve. Further, Hollo et al. also showed that after topical application of betaxolol in Cynomolgus monkey, drug levels in the anterior part were significantly higher than the posterior part of the back of the eye (Hollo et al., 2006). Such regional differences in tissue distribution following topical administration might be because the conjunctiva covers only the anterior part of the globe and makes an intimate contact with the sclera in this region, allowing ready exposure of drug to the sclera and underlying tissues in the anterior part of the globe. In addition, differential binding of brinzolamide and dorzolamide to the pigment in the choroid-RPE may also contribute to relative differences in their drug delivery. Single

dose did not result in detectable drug levels in plasma for both drugs, whereas multiple dosing resulted in significant drug levels in plasma for both drugs. Carbonic anhydrase (CA) inhibitors preferentially bind to CA in erythrocytes (Iester, 2008). After single topical application, a significant amount of drug binds to the CA in erythrocytes, potentially leaving the concentration of plasma below the quantification limits. With multiple dosing, CA in erythrocytes might get saturated, resulting in significant drug levels in the plasma.

In summary, dorzolamide showed higher delivery to the aqueous humor, vitreous, and optic nerve than brinzolamide after single as well as multiple topical administrations in pigmented rabbits. Higher tissue levels of dorzolamide in vitreous and optic nerve might be the reason for its greater effects on ocular blood flow in retina and optic nerve, when compared to brinzolamide. Further, the drug distribution within the ocular tissues sclera, retina, and vitreous is regio-selective for both brinzolamide and dorzolamide. Although drug levels were greater with dorzolamide with multiple dosing, drug accumulation index was >80 for both drugs in the posterior retina and there was significant accumulation in the posterior vitreous.

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Authorship Contributions

Participated in research design: Kadam, Ogidigben, and Kompella.

Conducted experiments: Kadam and Jadhav.

Contributed new reagents or analytic tools: Kadam and Jadhav.

Performed data analysis: Kadam and Kompella.

Wrote or contributed to the writing of the manuscript: Kadam, Ogidigben, and Kompella.

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Footnotes

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b) This work will be presented in part at the 2011 annual meeting of the Association for Research in Vision and Ophthalmology.

FIGURE LEGENDS

Figure 1. Dorzolamide showed better delivery to the cornea, conjunctiva, aqueous humor and optic nerve than brinzolamide after single topical application in pigmented rabbits. Plots show concentration versus time profiles in cornea, conjunctiva, aqueous humor, and optic nerve of pigmented rabbits after single topical ocular administration of Trusopt (dorzolamide HCl, 2.0%) or Azopt (brinzolamide suspension, 1.0 %). Data represent mean ± SD for N=3. * Significantly different from brinzolamide at $P \leq 0.05$.

Figure 2. Dorzolamide showed better delivery to the sclera, retina and vitreous than brinzolamide after single topical application in pigmented rabbits. Plots show concentration versus time profiles in sclera, retina, and vitreous of pigmented rabbits after single topical ocular administration of Trusopt (dorzolamide HCl, 2.0%) or Azopt (brinzolamide suspension, 1.0 %). Data represent mean ± SD for N=3. * Significantly different from brinzolamide at $P \leq 0.05$.

Figure 3. Dorzolamide showed better delivery to the aqueous humor and plasma than brinzolamide after multiple topical dosing in pigmented rabbits. Plots show tissue levels of dorzolamide and brinzolamide in cornea, conjunctiva, aqueous humor, and plasma after multiple topical ocular administration of Trusopt (dorzolamide HCl, 2.0%) or Azopt (brinzolamide suspension, 1.0 %). Animals were sacrificed at 1 hr following the last dose. Data represent mean ± SD for N=3. * Significantly different from brinzolamide at $P \leq 0.05$.

Figure 4. Dorzolamide showed better delivery to posterior segment eye tissues than brinzolamide after multiple topical dosing in pigmented rabbits. Plots show tissue levels of dorzolamide and brinzolamide in sclera, retina, vitreous, and optic nerve after multiple topical ocular administration of Trusopt (dorzolamide HCl, 2.0%) or Azopt (brinzolamide

suspension, 1.0 %). Animals were sacrificed at 1 hr following the last dose. Data represent mean ± SD for N=3. * Significantly different from brinzolamide (anterior) and †brinzolamide (posterior) at $P \leq 0.05$.

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Table 1. Mean extraction recovery of dorzolamide and brinzolamide in rabbit ocular tissues. Data are expressed as mean ± SD for N = 3. Extraction recovery was calculated as the ratio of the analyte peak area spiked before extraction to the analyte peak area of unextracted standard multiplied by 100.

Tissue Name	% Extraction Recovery of Dorzolamide		
	Low QC (25 ng/ml)	**Medium QC (250 ng/ml)**	**High QC (1250 ng/ml)**
Cornea	72.0 ± 3.1	68.28 ± 9.33	68.97 ± 3.95
Aqueous humor	76.1 ± 5.84	81.7 ± 6.06	71.69 ± 4.05
Conjunctiva	69.19 ± 6.91	70.02 ± 5.03	70.83 ± 1.19
Vitreous	74.28 ± 11.28	67.24 ± 4.31	77.39 ± 8.27
Retina	63.6 ± 3.38	70.67 ± 7.11	69.37 ± 2.54
Optic nerve	75.29 ± 10.47	68.64 ± 11.76	67.8 ± 3.60
Sclera	65.97 ± 8.03	78.89 ± 6.27	73.02 ± 9.45
	% Extraction Recovery of Brinzolamide		
Cornea	63.9 ± 4.75	67.32 ± 9.16	73.11 ± 3.67
Aqueous humor	73.05 ± 6.55	81.43 ± 7.96	79.96 ± 5.12
Conjunctiva	66.06 ± 4.27	70.83 ± 6.64	74.96 ± 2.75
Vitreous	70.7 ± 6.57	68.23 ± 7.09	75.48 ± 6.82
Retina	62.57 ± 7.6	73.08 ± 3.75	75.68 ± 3.61
Optic nerve	65.22 ± 5.07	71.71± 3.69	78.68 ± 4.66
Sclera	66.33 ± 6.97	80.97 ± 5.90	73.96 ± 7.45

Table 2. Pharmacokinetic parameters estimated for dorzolamide and brinzolamide after single topical ocular administration of Trusopt (2 %, dorzolamide HCl solution) and Azopt (1 %, brinzolamide suspension) to pigmented rabbit. Parameters were estimated using noncompartmental analysis (WinNonlin, version 1.5, Pharsight Inc.). C_{max} is the maximum observed drug concentration in a particular tissue; T_{max} is the time at which C_{max} occurs; $T_{1/2}$ is half life of drug in tissue; AUC_{0-24} is the area under the curve obtained by plotting the concentration-time data where‘t’ is the last time point at which drug concentration was measured; For brinzolamide no drug levels were detected in posterior vitreous.

Tissue	**Dorzolamide**				**Brinzolamide**			
	C_{max} (µg/g)	T_{max} (h)	$T_{1/2}$ (h)	AUC_{0-24} (µg.h/mL or g)	C_{max} (µg/g)	T_{max} (h)	$T_{1/2}$ (h)	AUC_{0-24} (µg.h/mL or g)
Cornea	36.61	0.25	1.08	51.21	19.27	0.50	1.71	50.88
Conjunctiva	68.74	0.25	0.720	49.55	28.80	0.25	1.01	37.94
Aqueous humor	1.92	0.50	7.06	6.159	0.97	0.75	6.16	3.14

Sclera (anterior)	47.18	0.50	0.559	36.13	8.17	0.25	0.929	5.22
Sclera (posterior)	4.92	0.25	1.09	3.46	2.70	0.25	0.134	1.34
Retina (anterior)	3.68	0.50	0.87	3.46	0.82	1.0	3.94	2.42
Retina (posterior)	0.091	0.50	1.23	0.095	0.059	0.50	0.812	0.051
Vitreous (anterior)	0.07	0.75	1.99	0.208	0.034	1.0	8.07	0.185
Vitreous (posterior)	0.0096	1.0	1.23	0.018	-	-	-	-
Optic Nerve	1.08	0.50	2.77	6.16	0.28	0.75	3.52	0.66

Table 3. Comparison of mean drug levels in ocular tissues after single and multiple topical dosing (42 doses). Drug levels were assessed at 1 hr following the last dose. In order to assess drug accumulation, ratios of drug levels between multiple and single dose regimens were calculated. Brinzolamide was not detected in the posterior vitreous after single dose administration.

Tissue	Dorzolamide			Brinzolamide		
	Single dose (µg/g)	Multiple dose (µg/g)	Multiple dose: Single dose Ratio	Single dose (µg/g)	Multiple dose (µg/g)	Multiple dose: Single dose Ratio
Cornea	10.31	21.4	2.08	7.99	14.1	1.76
Conjunctiva	10.10	5.89	0.60	6.77	4.57	0.68
Aqueous humor	1.49	2.35	1.58	0.530	1.48	2.80
Sclera (anterior)	9.12	7.67	0.85	1.57	4.20	2.60
Sclera (posterior)	0.460	0.56	1.21	0.140	0.57	4.07

Retina (anterior)	1.02	3.00	2.94	0.821	2.30	2.80
Retina (posterior)	0.023	2.10	90.13	0.015	1.30	86.7
Vitreous (anterior)	0.07	0.17	2.43	0.034	0.05	1.47
Vitreous (posterior)	0.0096	0.065	6.80	-	0.029	-
Optic Nerve	0.657	0.86	1.31	0.269	0.370	1.38

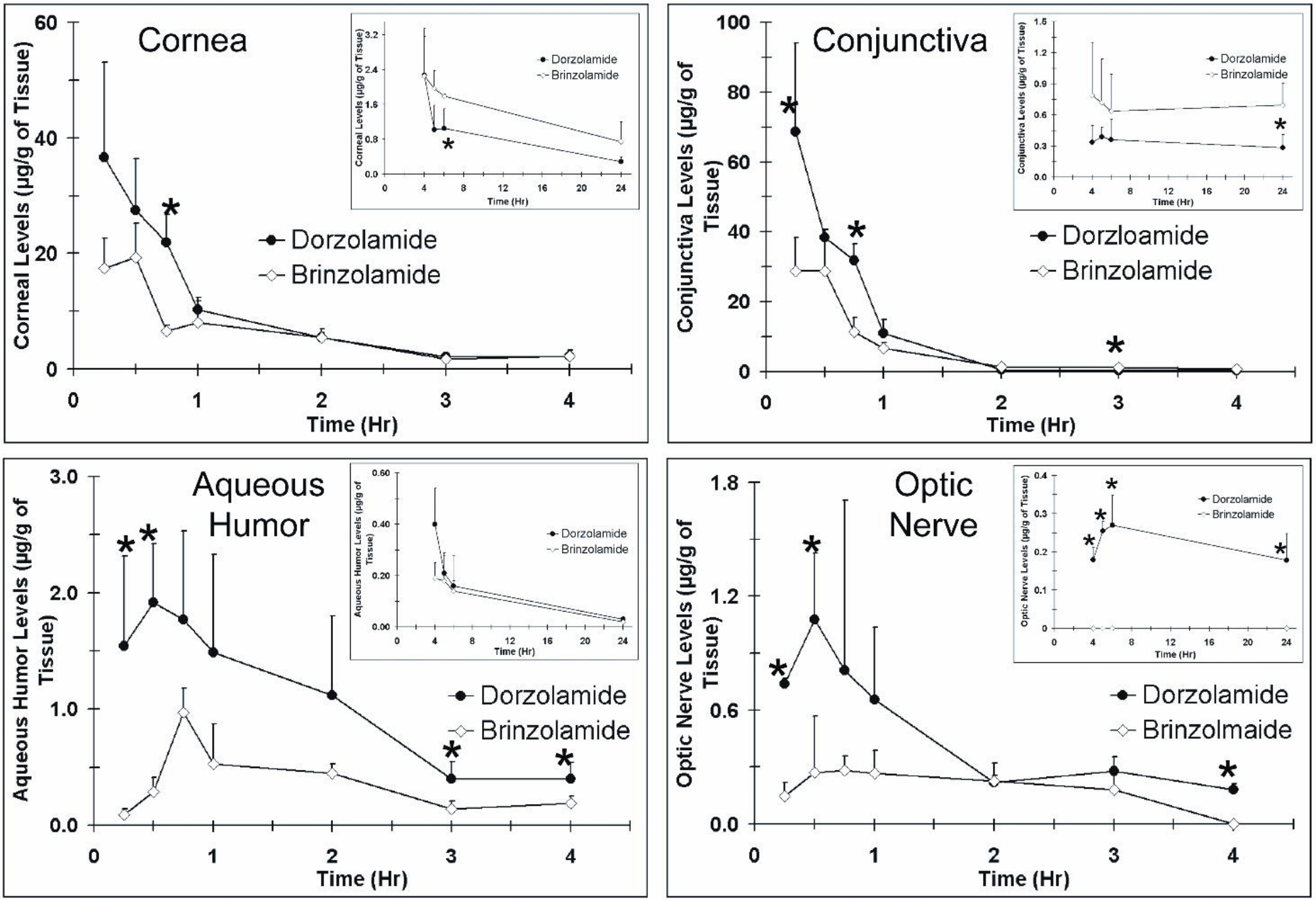
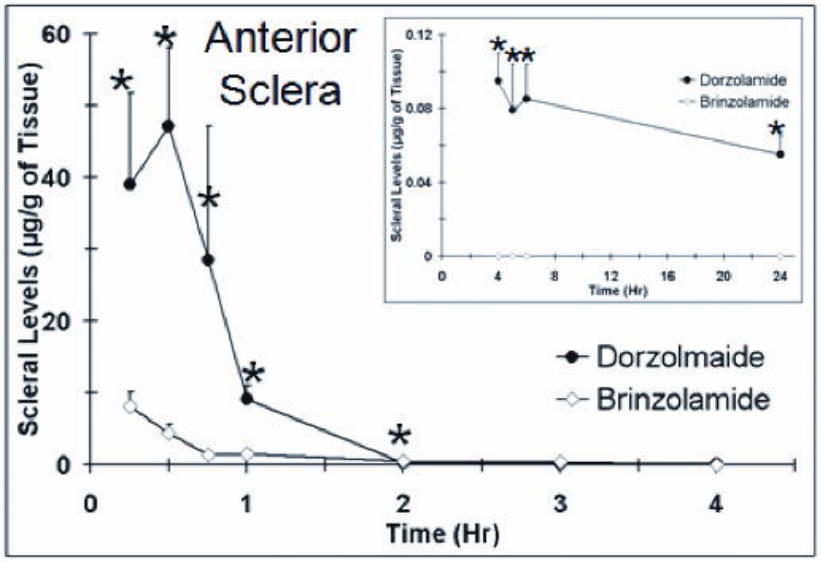
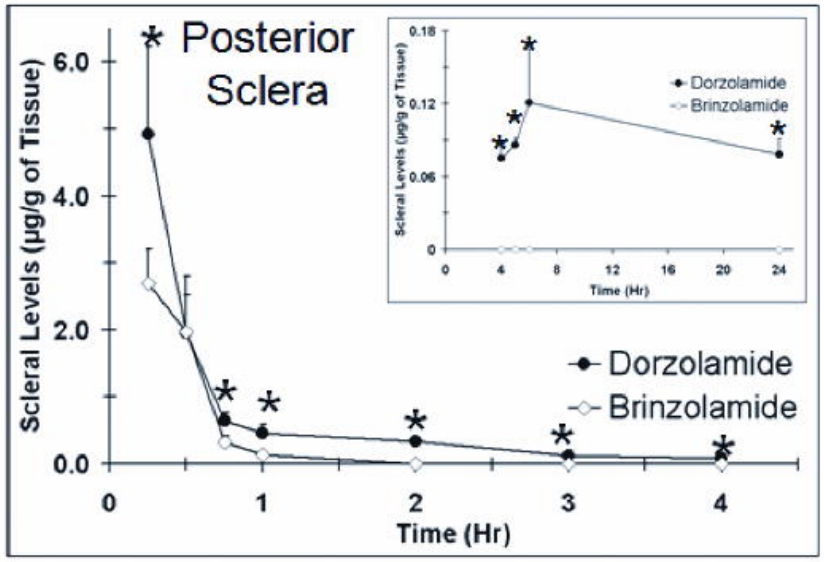
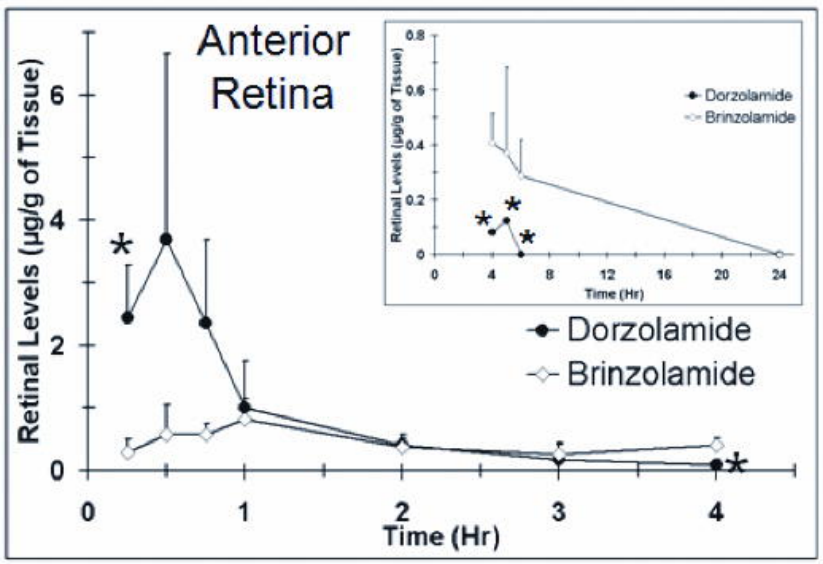
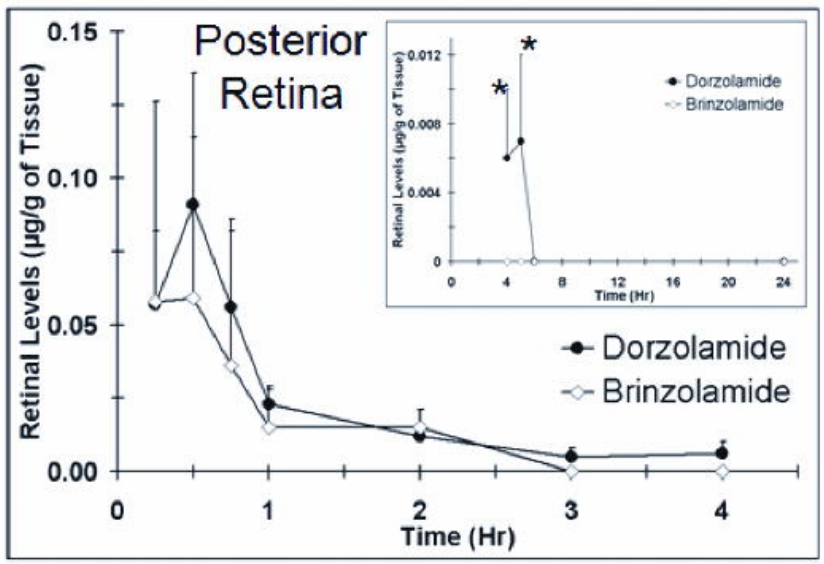

Cornea
Corneal Levels (µg/g of Tissue)
Time (Hr)
Dorzolamide
Brinzolamide
Conjunctiva
Conjunctiva Levels (µg/g of Tissue)
Dorzloamide
Aqueous Humor
Aqueous Humor Levels (µg/g of Tissue)
Optic Nerve
Optic Nerve Levels (µg/g of Tissue)
Brinzolmaide

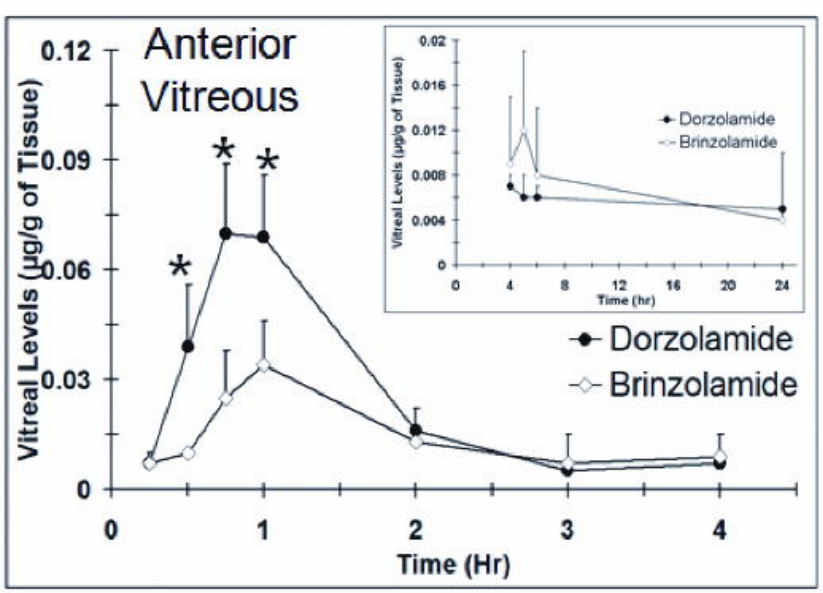
Figure 1


Anterior
Sclera
Scleral Levels (µg/g of Tissue)
Time (Hr)
Dorzolmaide
Brinzolamide


Posterior
Sclera
Scleral Levels (µg/g of Tissue)
Time (Hr)
Dorzolamide
Brinzolamide


Anterior
Retina
Retinal Levels (µg/g of Tissue)
Time (Hr)
Dorzolamide
Brinzolamide


Posterior
Retina
Retinal Levels (µg/g of Tissue)
Time (Hr)
Dorzolamide
Brinzolamide


Anterior
Vitreous
Vitreal Levels (µg/g of Tissue)
Time (Hr)
Dorzolamide
Brinzolamide

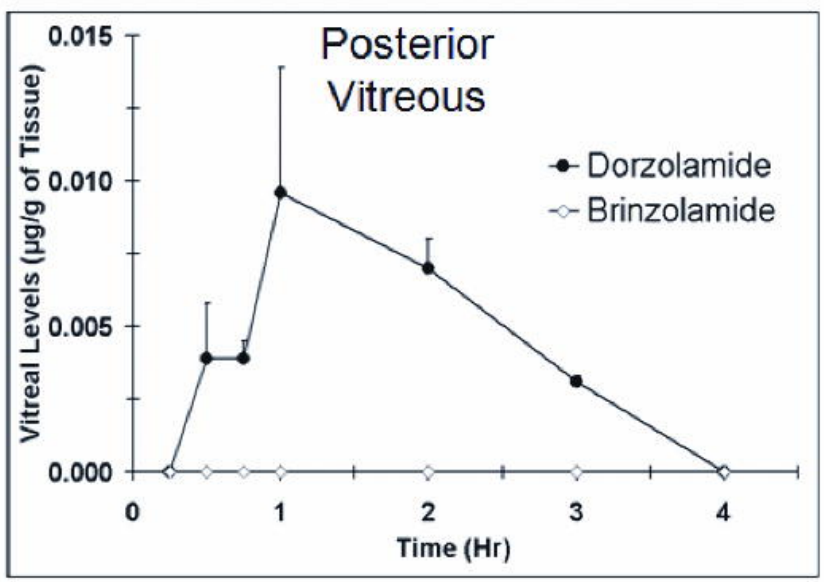

Posterior
Vitreous
Vitreal Levels (µg/g of Tissue)
Time (Hr)
Dorzolamide
Brinzolamide

Figure 2

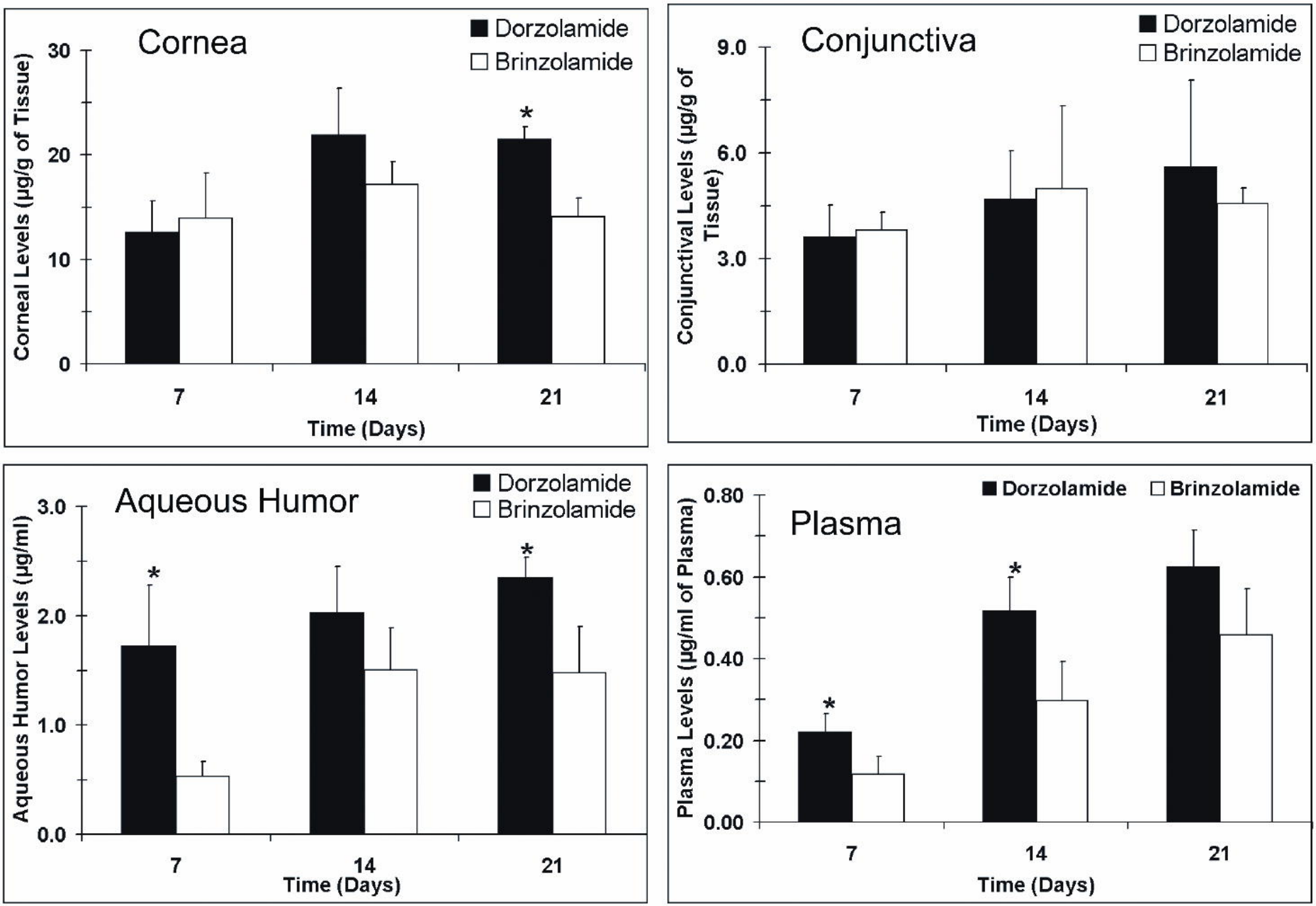

Cornea
Dorzolamide
Brinzolamide
Corneal Levels (µg/g of Tissue)
30
20
10
0
*
7
14
21
Time (Days)
Conjunctiva
Dorzolamide
Brinzolamide
Conjunctival Levels (µg/g of Tissue)
9.0
6.0
3.0
0.0
7
14
21
Time (Days)
Aqueous Humor
Dorzolamide
Brinzolamide
Aqueous Humor Levels (µg/ml)
3.0
2.0
1.0
0.0
*
*
7
14
21
Time (Days)
Plasma
Dorzolamide
Brinzolamide
Plasma Levels (µg/ml of Plasma)
0.80
0.60
0.40
0.20
0.00
*
*
7
14
21
Time (Days)

Figure 3

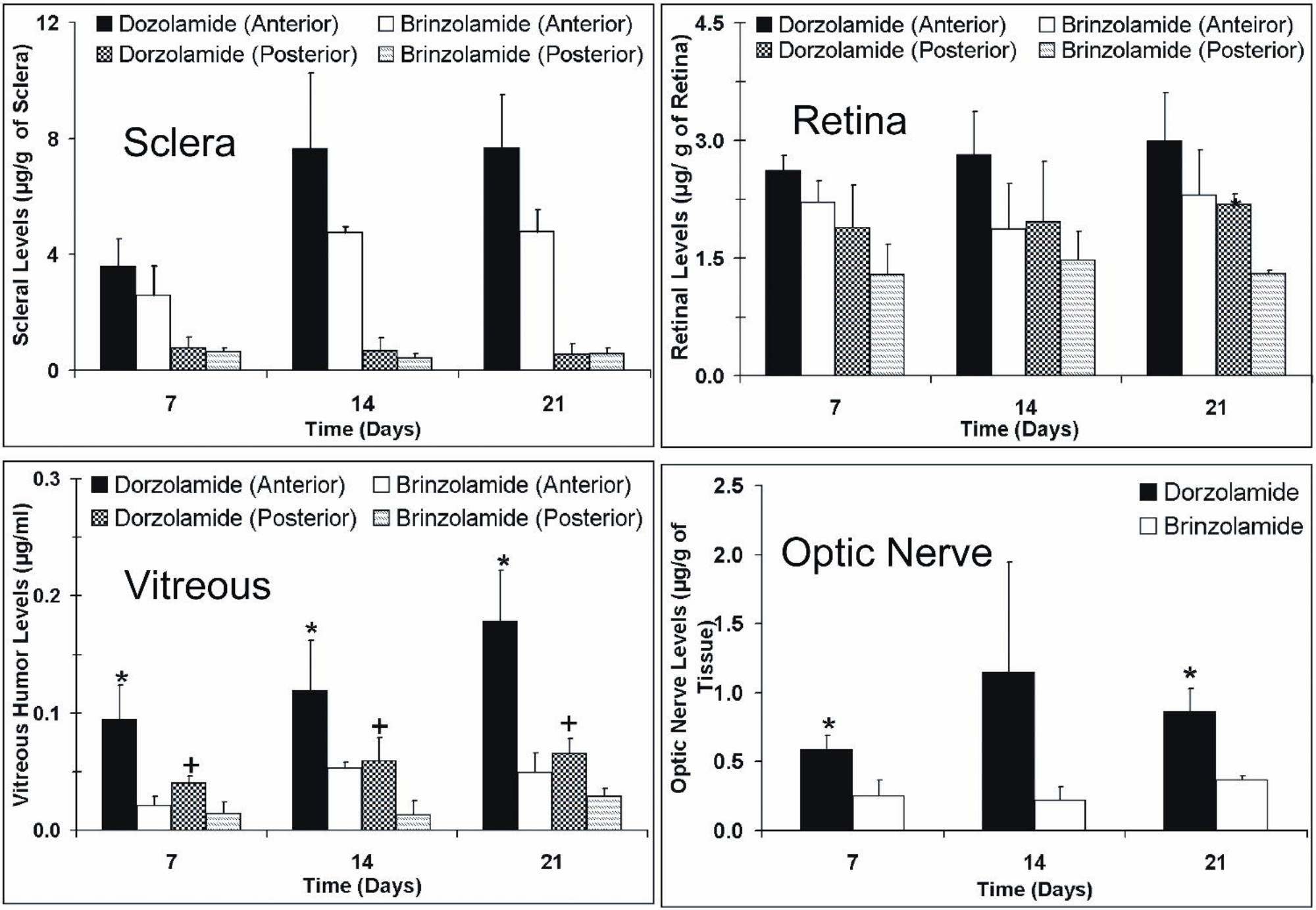

Sclera
Dozolamide (Anterior)
Brinzolamide (Anterior)
Dorzolamide (Posterior)
Brinzolamide (Posterior)
Scleral Levels (µg/g of Sclera)
12
8
4
0
7
14
21
Time (Days)
Retina
Dorzolamide (Anterior)
Brinzolamide (Anteiror)
Dorzolamide (Posterior)
Brinzolamide (Posterior)
Retinal Levels (µg/ g of Retina)
4.5
3.0
1.5
0.0
7
14
21
Time (Days)
Vitreous
Dorzolamide (Anterior)
Brinzolamide (Anterior)
Dorzolamide (Posterior)
Brinzolamide (Posterior)
Vitreous Humor Levels (µg/ml)
0.3
0.2
0.1
0.0
*
+
7
14
21
Time (Days)
Optic Nerve
Dorzolamide
Brinzolamide
Optic Nerve Levels (µg/g of Tissue)
2.5
2.0
1.5
1.0
0.5
0.0
*
7
14
21
Time (Days)

Figure 4