Section A-Research paper

TO STUDY THE OCULAR PHARMACOKINETIC PROPERTIES OF BRINZOLAMIDE O.5% OPHTHALMIC SOLUTION ON RABBIT OCULAR BLOOD FLOW

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ABSTRACT

Glaucoma is an ocular disease condition that is more frequent in older persons and causes high intraocular pressure, posing a major danger to vision if not treated. Because the marketed brinzolamide has disadvantages, the present optimal formulation was developed with our preservatives. The current investigation was aimed to ensure the safety and effectiveness of created formulation for ocular medication administration. The prepared 0.5% Brinzolamide ophthalmic solution 0.5% was tested in New Zealand White rabbits well tolerated and reduced intraocular pressure (IOP) 11.11 mmHg compared with saline and placebo control samples. Additionally, a significant increase in the area under change in IOP from baseline (ΔIOP) *vs*. time curve and a longer mean residence time (MRT) were also observed for the test formulation compared to the commercially available Brinzolamide 1% and $p < 0.001$. The T/R ratio was calculated for the pharmacokinetic parameters of Cmax, Tmax, AUC0-t and MRT cumulatively for left and right eyes. The blood concentration versus time data shown the similar exposure for both test and reference formulations. The T/R ratio of blood for Cmax, Tmax, AUC_{0-t} and MRT was found to be within the acceptable bioequivalence range which shows the equivalence of Test formulation with that of Reference formulation.

Keywords: 0.5% Brinzolamide, Intraocular pressure, Invivo study, Cmax, AUC_{0-t}.

INTRODUCTION [1-3]

The eye is probably the most important sensory organ of the body. It is responsible for sight and also visual cues for maintaining balance. The eye is also an immune-privileged site and is derived from the forebrain during embryological development, it retains the challenges of the blood–brain barrier for drug delivery in the form of the blood–retina barrier among others[2]. The anatomy and structure of the eye are very complex, and it is divided into two parts, i.e., the Anterior segment and the posterior segment of the eye; the portion front to the lens is called an anterior segment, whereas the portion behind the lens of the eye is referred to as the posterior segment. The anterior segment of the eye consists of the cornea, pupil, iris, ciliary body, conjunctiva, anterior chamber, lens, and aqueous humor; moreover, the posterior segment of the eye contains vitreous humor, sclera, choroid, and retina[1].

The diseases of the anterior segment of the human eye generally include corneal infections

and disorders like pterygium, Fuchs' dystrophy, dry eyes, corneal neovascularization, and autoimmune disorders, e.g., cataracts. However, other types of diseases are referred to as disorders of the posterior segment such as glaucoma, cytomegalovirus retinitis, age-related macular degeneration, Diabetic retinopathy, Retinitis pigmentosa (RP), Proliferative vitreoretinopathy and Uveitis[1]. For managing diseases in the anterior segment of the eye, topical administration is the most common route. Drug transport via corneal/non-corneal routes involves several intricate biological processes; consequently, the bioavailability of topically applied drugs is poor in the internal tissues of the eye.

The main barriers for ocular drug delivery are

(1) Elimination from lachrymal fluid (pre-corneal barrier): most of the instilled volume is either drained from the conjunctival sac into the nasolacrimal duct or cleared from the precorneal area, resulting in poor bioavailability of drugs.

(2) Corneal barrier: anatomically, the corneal barrier is due mainly to intercellular tight junctions (zonula occludens) which completely surround the superficial epithelial cells, serving as a selective barrier for small molecules and completely prevent the diffusion of macromolecules via the paracellular route. Corneal stroma, instead, is a highly hydrophilic tissue with an open structure that allows the diffusion of hydrophilic drugs up to 500 kDa size, while it is a rate-limiting barrier for most lipophilic drugs. The corneal endothelium is responsible for maintaining normal corneal hydration, and it has been estimated that drugs with molecular dimensions up to about 20 nm can diffuse. The drug transport across the corneal epithelium is essential via paracellular or transcellular routes. The hydrophilic drugs penetrate primarily through the paracellular pathway, while lipophilic drugs prefer the transcellular route. Lipophilicity, solubility, molecular size, charge, and degree of ionization also affect the cornea's route and rate of penetration. Particulate material in the nanometer range has been reported to follow the endocytic pathway depending on the matrix's optimized lipophilic–hydrophilic properties and reduced particle size.

(3) Non-corneal absorption: topically applied ocular drugs may be absorbed through the bulbar conjunctiva and the underlying sclera into the uveal tract and vitreous humor, which results in drug loss at desired site[3].

Glaucoma is a multifactorial optic neuropathy and is the second leading cause of blindness worldwide. A major risk factor is increased IOP in the eye when the ratio between aqueous humor formation (inflow) and its outflow is unbalanced. Lowering IOP via various pharmaceuticals and/or surgical techniques is currently the mainstay of glaucoma treatment. The topical route is the one most commonly used, owing to its suitability for chronic administration. Drug bioavailability can be improved by the delivery system, also decreasing the dosage. Treatment options are 'inflow inhibitors' (beta-antagonists, carbonic anhydrase inhibitors) and 'outflow enhancers' (alpha agonists, prostaglandin analogs, and miotic agents). Innovative 'inflow' (hydroxysteroid dehydrogenase-1 inhibitors; melatonin) and 'outflow' agents (dopamine, serotonin, adenosine agonists, cannabinoids) are currently under study[3].

EXPERIMENTAL[4-15]

The objective of this study was to evaluate the tissue distribution of Brinzolamide in different ocular tissues along with plasma and blood after single dose of topical ophthalmic application of Brinzolamide ophthalmic solution 0.5% in New Zealand White rabbits.

In vivo pharmacokinetic study[10-12]

Experimental design:

An open label, balanced, randomized, three-period, three- treatment, three-sequence, single-dose crossover study design in which six healthy White Rabbits were received one treatment (product) each with a wash out period of 7 days such that all products are tested in all the six healthy White Rabbits during the study.

***In vivo* study protocol**

Subject selection

Six healthy White Rabbits with body weight range of 1.9 to 2.5 Kgs will be selected through physical examination.

Study Compliance

The study is being conducted in accordance with the standard operating procedures & The recommendation of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines for laboratory animal facility and approval by Institutional Animal Ethics Committee (IAEC) protocol.

Veterinary Care

During study if any animal gets injured, ill or moribund, care taken as per currents veterinary practices. If required, for humane reasons animals will be euthanized as per standard procedures. The objective of the study will be considered before any decision and event of unlikely situations.

Safety Precautions

All necessary safety measures will be taken care during the conduct of study. When required, personnel involved in study must wear gloves, head cap and facemask in addition to protective clothing to ensure adequate personnel health and safety.

TABLE NO: 1. Test Item Information

Name of the Test Item	Group I: Normal control Group II: Ophthalmic drops 0.1% as formulation test solution Group III: Ophthalmic drops 0.25% as formulation test solution Group IV: Ophthalmic drops 0.5% as formulation test solutions. Group V: Standard marketed product – Azopt suspension 1%.
Storage Conditions	Room temperature

Table No: 2. Test System & Test system management

Animal Species	Rabbit (*Oryctolagus cuniculus)*
Strain	New Zealand White
Justification for selection of species	Rabbit is one of the recommended species of species for evaluating ophthalmic preparations
Body weight range	1.9 to 2.5 Kgs (At treatment)
Age at treatment	2-4 months

Sex	Male & Female animals
Number of groups	05
Number of animals	08 animals per group
Total number of animals	32

Housing

Individual animal will be housed in a standard stainless-steel cage having facilities for holding pelleted food and drinking water. The water will be provided in polycarbonate water bottles fitted with rubber cork and a stainless-steel sipping tube.

Diet

The animals will be fed *ad libitum* with laboratory animal feed throughout the experimental period. The feed is analyzed periodically for any microbial contamination.

Water

Reverse osmosis, UV sterilized drinking water will be provided *ad libitum* throughout the experimental period. The water is analyzed periodically for any microbial contamination.

Acclimatization

The animals will be acclimatized for a minimum period of five days to laboratory conditions. Animals will be observed for clinical signs once daily during acclimatization period. Veterinary examination will be performed before selecting the animals and only healthy and active animals will be used in the study.

Animal Identification

During acclimatization and treatment period, each animal will be identified by ear marking with animal number written on the inner ear lobe using permanent marker pen.

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SW500 Imported with 100 probes, extra probe base - Manual, Printer with power supply and cable- Carry case and User Manual

Experimental Procedure[16-18]

Study Design

The animals categorized into 5 groups with 8 animals in each group. G1 is considered as the normal control group, groups G2 to G4 are applied with ocular test formulations and G5 are applied with ocular Reference formulations. The animals categorized and randomized based on the baseline IOP measurements and body weights.

After the instillation predetermined concentration (50 µl) of Reference and Test item formulations, the IOP measurements will be made at 0, 0.5, 1, 2, 4, 6, 8, 10, 12, 14, 16 and 24 h. The recordings of IOP will be measured for both the eyes by using tonometer. Animals of the all-groups instillations of test & Reference formulations will be single time a day for both the eyes.

Table No: 3 Experimental Procedure

S. NO.	Group	Formulation	Dose administration	Number of animals
1	G1	Normal Control	-	08
2	G2	TF 1	Single dose	08
3	G3	TF 2	Single dose	08
4	G4	TF 3	Single dose	08
5	G5	RF 4	Single dose	08

All the animals will be observed for IOP for both the eyes. The body weights of the animals will be measured prior to the instillation of test formulations and at the end of the study. Animals will be observed for any clinical signs of toxicity at the sites of instillations.

Determination of Ocular Tissue Distribution Study of Brinzolamide Ophthalmic Solution 0.5% after topical Ophthalmic Application in New Zealand White Rabbits

Study Objective

The objective of this study was to evaluate the tissue distribution of Brinzolamide in different ocular tissues along with plasma and blood after single dose of topical ophthalmic application of Brinzolamide ophthalmic solution 0.5% in New Zealand White rabbits.

Materials and Methods

Table No: 4 Test item details

Name of Test Item	Brinzolamide Ophthalmic Solution 0.5%
Appearance	White semi-translucent solution filled in white preservative free bottles
Storage condition	Room Temperature

Table No: 5 Reference Item Details

Name of Test Item	AZOPT – Brinzolamide Ophthalmic Solution 1%
Appearance	White opaque homogenous suspension
Storage condition	Room Temperature

Test System Details

Justification for the selection of test system

Rabbit was chosen as the test system because this species is commonly used for assessing the pharmacokinetics and tissue distribution of test compounds like ocular formulations.

Table No: 6 Test System details

Species	*Oryctolagus* cuniculus (Rabbit)
Strain	New Zealand White
Age at the time of dosing	2-3 month
Body weight at the time of dosing	Group -1: 1787.61 – 2101.13 g Group -2 : 1800.81 – 2159.96 g
Sex	Male
Number of animals/ groups	6
Number of groups	2

Total number of animals	6
Acclimatization	Prior to acclimatization, a physical health examination was performed on all animals by a veterinarian. Healthy animals were acclimatized to the experimental room for 6 days for Group-I and Group-II.

Administration of Test Item Formulation

Rabbits were kept in rabbit holder 15 minutes before the ocular instillation of the formulation.

Each rabbit received 50µL of Test or Reference formulation of Brinzolamide ophthalmic solution 0.5% into both left and right eyes. The formulation was applied with a calibrated adjustable micropipette fitted with disposable tips into the lower conjunctival sac of the eye by pulling the lower lid away from the eyeball. After instillation, the upper and the lower lids were held together for a few seconds to avoid rapid removal of the eye drop from the ocular surface.

Sample Collection

At each sample collection time point, the blood was collected in pre-labeled K2-EDTA coated tubes. For each animal, two aliquots were prepared for each blood sample. One aliquot was used as such for the bioanalysis. Another aliquot was centrifuged at 3000rpm for 10 minutes at 4°C and the blood sample was separated in the pre-labelled vials.

After blood collection, the rabbit was euthanized by an overdose of thiopentole sodium (100mg/kg i.v.). Immediate after the sacrifice, conjunctiva and eye lids were collected from both the eyes and washed with the normal saline to remove the excess adhering test formulation. Then, both the eye balls from each rabbit were enucleated and washed with the normal saline to remove the excess adhering test formulation. Eye balls were snap frozen in dry ice. The ocular tissues were collected on ice tray to delay the thawing. To open the eye ball, an incision was made at the corneal limbus with a scalpel blade. The ocular tissues (iris and ciliary body) were collected in individual pre-labelled, pre-weighed vials. To separate the retina/choroid tissue from the underlying sclera, scalpel blade was used.

A new surgical blade was used for each eye. To prevent transfer of drugs between tissues of each eye, the surgical accessories were rinsed thoroughly with saline followed by methanol followed by saline and blotted dry after and between uses on each tissue. All of the tissue samples were weighed and stored at -80°C until further processing. blood samples were kept at -80°C till analysis.

Experimental Procedures- *Bioanalysis* Phase

The tissues were homogenised using the Fast Prep instrument. Homogenizing buffer Tris HCl pH 8.5 was added to the tissues in the ratio of 1:10 (Ciliary body and Iris,). Lysing matrix D was added to the tissues containing homogenising buffer and spun at speed of 4 M/S for 4 cycles. After homogenization, the samples were centrifuged at 4000 rpm for 5 min and the supernatant was transferred to fresh Eppendorf tubes. The supernatant was analyzed using LC-MS/MS. The estimated concentrations of Brinzolamide in tissue homogenates (ng/mL) were converted to ng/gm using the following formulae:

Concentration (ng/g) = (Concentration in aliquot (ng/mL)/ Volume of aliquot analyzed (mL)) *Total volume of homogenate (mL) / Weight of tissue homogenized (g)

Clinical signs and mortality/viability

In Group-1 and Group-2, all the rabbits were administered with test and reference drug product of Brinzolamide ophthalmic solution 0.5% in both left and right eyes. All the animals were observed for clinical signs of toxicity, eye irritation and mortality. No clinical signs of toxicity, eye irritation and mortality were found during the study period.

Determination of Pharmacokinetic Parameters:

Pharmacokinetic parameters such as peak plasma concentration (C_{max}), time at which C_{max} occurred (T_{max}), area under the curve (AUC), elimination rate constant (K_{el}), biological half-life ($t_{½}$), are calculated in each case using the data by Kinetica^{TM} 2000 software (Inna Phase Corporation, U.S.A) using non-compartmental approach.

Statistical analysis: The results will be expresses as the Mean ± SEM. The results obtain from the present study will be analyze using One-way ANOVA followed by Dunnett's test. Data will compute for statistical analysis using Graph Pad Prism Software. Differences between the data will be consider significant at $P<0.05$.

Results and Discussion

In-vivo Studies

Ocular Tolerance Studies

The mean Brinzolamide concentrations data for individual matrix are given in below table.

Table No:7 Concentrations Data in different Matrices

Matrices	Group	Concentration (ng/g) at time points*						
		0.25hr	0.5hr	1 hr	2 hr	4 hr	8 hr	12 hr
Ciliary Body	G1	14234± 4423	27152 ± 13536	30328 ± 6632	16195 ± 6111	8509 ± 2866	7259 ± 1717	3840 ± 1100
	G2	21782 ± 3327	35245 ± 13272	31680 ± 10274	51373 ± 18146	8094 ± 3990	7278 ± 2834	6761 ± 1454
Iris	G1	9454 ± 4742	33986 ± 12159	38658 ± 4351	22495 ± 7007	9422 ± 5407	6872 ± 1920	5469 ± 1158
	G2	16224 ± 3987	31169 ± 10905	24383 ± 5822	28761 ± 10175	10129 ± 4319	7534 ± 2139	6017 ± 656
Blood	G1	227 ± 87	1433 ± 458	2107 ± 107	1713 ± 324	2159 ± 176	3560 ± 1029	3482 ± 104
	G2	236 ± 58	703 ± 222	1278 ± 186	2477 ± 538	3443 ± 535	4458 ± 512	3463 ± 502

Concentration data is represented as Mean ± SEM.

G1= Test formulation treatment, G2= Reference formulation treatment, hr=hour

Concentration data for ocular tissues is in ng/g and is a mean of 3 left eye and 3 right eye tissue concentration data (n=6) at each time point.

Concentration data for Blood is in ng/mL and is a mean of 3 rabbit data (n=3) at each time point.

Pharmacokinetic Studies

Table No:8 Pharmacokinetic Studies of Test Formulation 0.5%

Pharmacokinetic Parameters	Test Formulation 0.5%			
	C_{max}	T_{max}	AUC_{0-t}	MRT
Units	ng /gm	h	ng.h /gm	h
Ciliary body	49211.9	0.7	124397.2	3.4
Iris	40767.8	0.7	171992.2	3.2

Blood	3560.1	7.0	32698.8	6.05

Table No:9 Pharmacokinetic Studies of Reference Formulation 1%

Pharmacokinetic Parameters	Reference Formulation 1%			
	C_{max}	T_{max}	AUC_{0-t}	MRT
Units	ng /gm	h	ng.h /gm	h
Ciliary body	51373.3	1.0	200002.4	2.9
Iris	40169.1	0.4	153373.4	3.0
Blood	4457.8	7.0	40513.0	5.7

The T/R ratio of blood for Cmax, Tmax, AUC_{0-t} and MRT are found to be within the acceptable bioequivalence range (0.70 – 1.00) which shows the equivalence of Test formulation with that of Reference formulation.

The T/R ratio of Cmax for most of the ocular tissues are within the acceptable equivalence range showing the similar maximum concentration for both test and reference formulations. However, the T/R ratio of Tmax, AUC_{0-t} and MRT are not in acceptable range for most of the tissues. This variation could be due to the staggered sampling and the less sample size.

Figure No:1 Brinzolamide concentration in Ciliary body with different time points

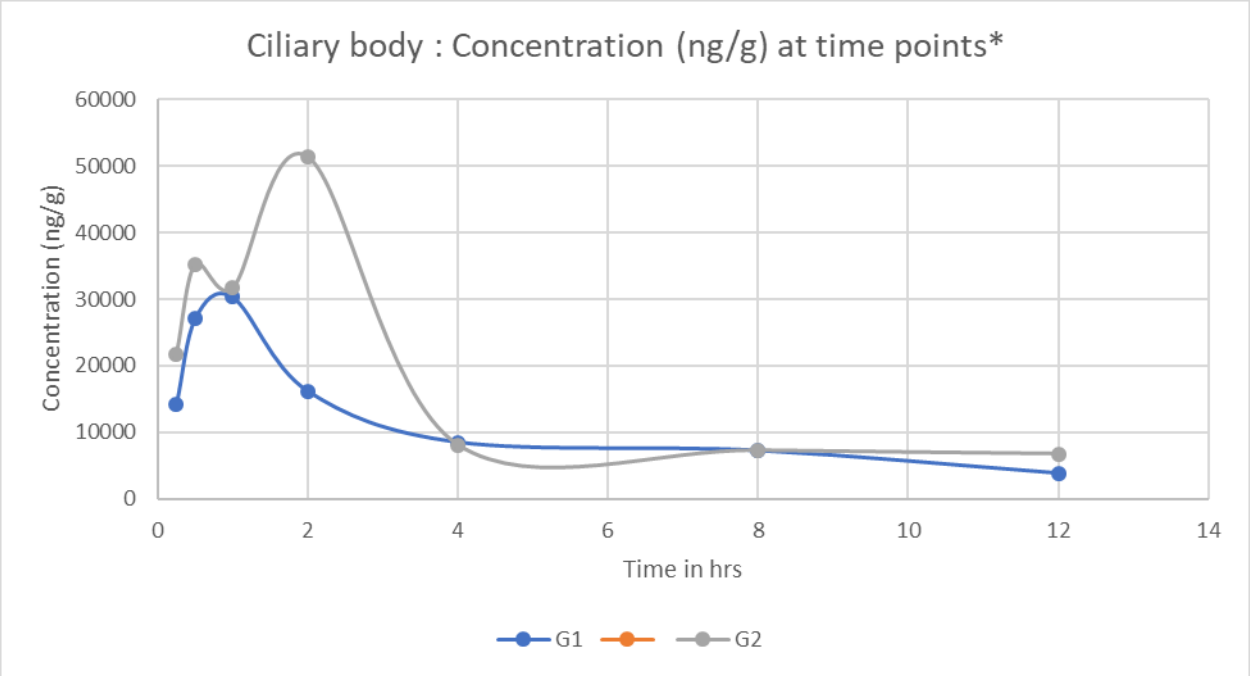


Figure No:2 Brinzolamide concentration in Iris with different time points

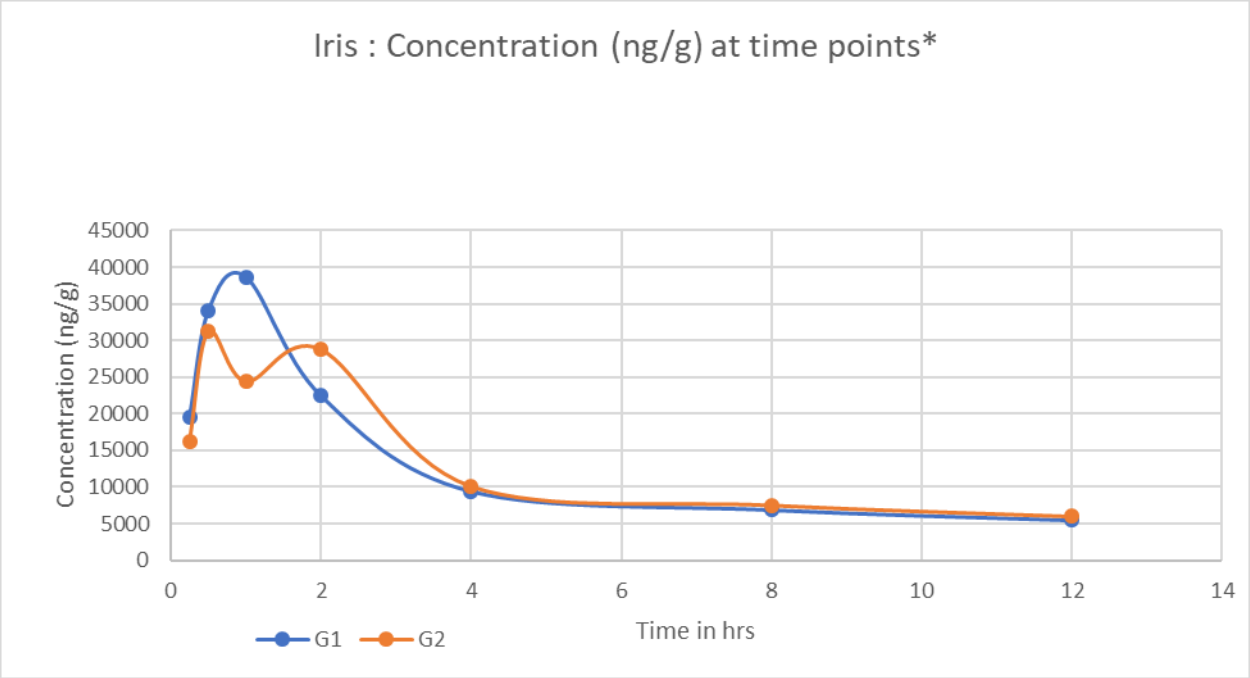
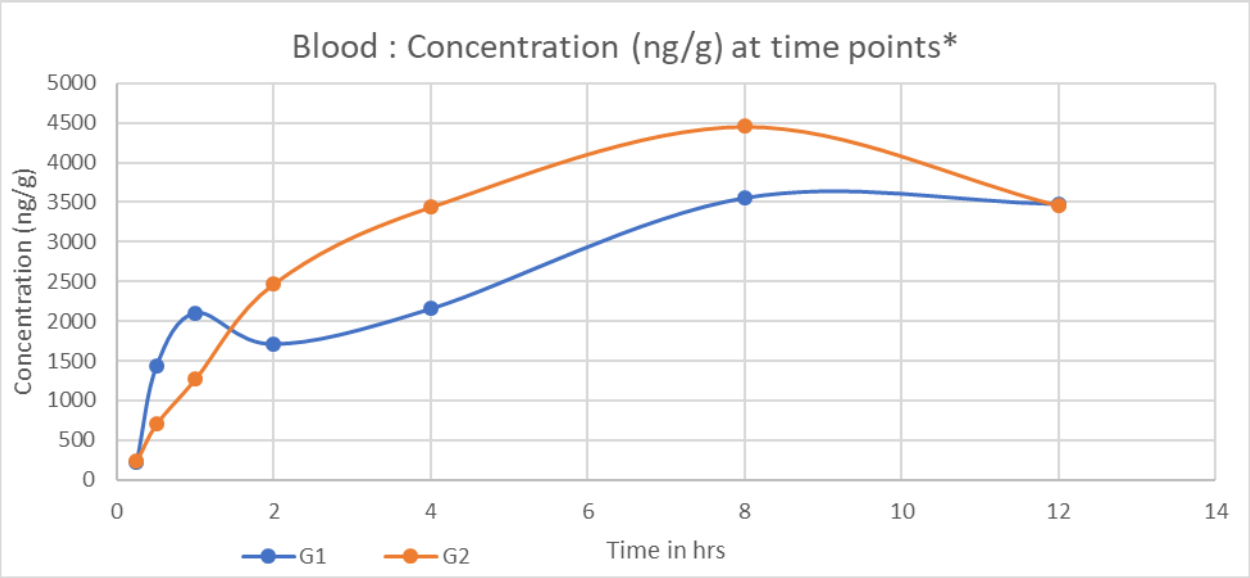


Figure No:3 Brinzolamide concentration in Blood at different Time Points



Conclusion

The blood concentration versus time data shown the similar exposure for both test and reference formulations. The T/R ratio of blood for Cmax, Tmax, AUC_{0-t} and MRT was found to be within the acceptable bioequivalence range which shows the equivalence of Test formulation with that of Reference formulation. The T/R ratio of Cmax for most of the ocular tissues are within the acceptable bioequivalence range showing the equivalence in maximum concentration for both test and reference formulations.

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