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Optimization of the *In Vitro* Transcorneal Release and the *In Vivo* IOP-Lowering Effects of Latanoprost Ophthalmic Gel Formulations Using Azone™ as a Penetration Enhancer and Carbopol-974® as a Mucoadhesive

Mohsen. Afouna[1]*, Ashraf B.A. Naim[2]
1.Department of Pharmaceutics & Pharmaceutical Technology, College of Pharmacy, Al-Azhar University, Nasr City, Cairo, Egypt
2.Department of Pharmacology & Toxicology, College of Pharmacy, King Abdulaziz University, Jeddah, Saudi Arabia

ABSTRACT

The objectives of this study were to maximize; ***a)*** the *in vitro* transcorneal release, ***b)*** the IOP-lowering effect and, ***c)*** the duration of action, of Latanoprost acid (LAT) ophthalmic gels. The *in vitro* transcorneal release of LAT from a 1st set of gel formulations that containing different concentrations of Azone™ (as enhancer) with fixed concentration of C-974® (as mucoadhesive) were studied. Formulation that showed greatest permeability parameters at lowest Azone™ concentration was selected for preparation of a 2nd set of ocular gels containing various C-974® concentrations. The *in vitro* transcorneal release was assessed, and the best C-974® concentration required for preparation of formulations that can be conceded as ideal ophthalmic LAT gels have been pinpointed and scaled up for *in vivo* IOP-lowering efficacy study using TONO-PEN™ AVIA tonometer in rabbits for 4-consecutive days. Various test formulations have showed significant but varied augmentations in both, *in vitro* and *in vivo* results. Formulations (GAZ-4) & GC-4 have shown the highest therapeutic IOP lowering effects; i.e., (7.8±1.8), (6.5±2.1), respectively. Particularly noteworthy with both formulations the IOP base-line didn't re-established after 24 hours, and their durations of action in the single-dose study were 47±2.25, and 48±1.5, respectively. The *in vitro* release, onset, magnitude & duration of action of action of LAT gels have been enhanced and extended for up to 2-day with two gel formulations. Nonetheless, the success in developing a novel ophthalmic formulation depended for great extent upon the crucial net outcomes of a very sensitive interplay/balance between the drug and additives.

Keywords: Azone; Corneal transport; Ocular delivery; Ocular enhancers; Carbopol-974; Glaucoma; IOP lowering effect; Mucoadhesive

*Corresponding Author Email: mafouna@pharma.asu.edu.eg
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INTRODUCTION

Glaucoma is a progressive optic neuropathy affecting more than 70 million individuals worldwide and it represents a major cause for irreversible blindness[1]. In November 2004, WHO demonstrated that glaucoma is responsible for approximately 4.5 million blind; i.e., ~12% of the total burden of world blindness. It has been reported that glaucoma is the second leading cause of blindness globally[2]. One of the most important risk factors for progression of such disease is the increased IOP. High IOP can result in retinal ganglion cell loss and optic nerve atrophy leading to irreversible blindness. Ocular drugs are usually administered as aqueous eyedrops. Latanoprost (LAT) is a selective FP prostanoid receptor agonist with good therapeutic index in the eye. LAT ((+)-isopropyl (Z)-7-[(1R,2R,3R,5S(3,5-dihydroxy-2-[(3R)-3-hydroxy-5-phenylpentyl]cyclopentyl-[5-heptenoate) is an ester prodrug analogue of prostaglangin F2α that has been investigated for potential treatment of primary open-angle glaucoma and ocular hypertension[3,4]. LAT is hydrolyzed by esterases in the cornea after its ophthalmic installation and reduces both normal and elevated intraocular pressure (IOP) by increasing uveoscleral outflow[5,6]. It is the active ingredient of Xalatan® eye drops (Pfizer Limit Ramsgate Road, Sandwich, Kent, CT13 9NJ).

Amongst the main obstacles encountered with ocular drug delivery in a therapeutically effective concentration from ophthalmic deliver systems (ODS) are, ***i)*** the very short average residence time of the administered dose, particularly ophthalmic solutions (5-25 minutes)[7,8] ***ii)*** the extensive pre-corneal loss because of the fast tear drainage & solvent evaporation and alteration of drug thermodynamics, ***iii)*** the high possibility of excessive loss of drug through the nasolacrimal drainage that may cause systemic effects &/or side effects[9]. ***iv)*** the very limited accommodation capacity of the eye; 10-30 µl for blinking & non-blinking human eye respectively, and ***v)*** the inherent physiological involuntary defense mechanism of the eye (blinking & tearing). Subsequently, the ocular bioefficacy of a topically applied ocular drug drops is dramatically very low, only 1-10%[10,11]. The corneal membrane consists of three essential layers; i.e., epithelium, stroma and endothelium. On the one hand, the epithelium layer is lipophilic contains approximately 100-fold greater amount of lipid material per unit weight than stroma. Therefore, it represents the main barrier for the hydrophilic (i.e., poorly soluble) drug molecules. On the other hand, the stroma which is a hydrophilic, gel-like structure represents a moderate barrier for hydrophobic drug molecules. Albeit, the lipophilic nature of the endothelium, it does not serve as a barrier for both lipophilic & lyophobic drug molecules

because of its histological nature of being thin, single-layer structure with a relatively larger paracellular junctions. Therefore, it is more easily crossed through transcellular, pore-cellular and paracellular transport pathways[12]. Accordingly, it is favorable for the drug to have biphasic partitioning aptitude to successfully cross the corneal membrane[13-17].

Therefore, ocular therapy is a unique challenge when it comes to delivery of a drug with pharmacologically effective level. Studies have shown that the outmost layer (i.e., the epithelium penetration) is commonly the rate-limiting step to the transcorneal transport of, particularly with high hydrophilic drug molecules. Thus, drug molecules must have sufficient lipophilicity to be able to penetrate this barrier[18] with restricted/little or no difficulties. Amongst the approaches employed to bypass epithelium barrier is to embrace a suitable viscosity improving agent (VIA) to prolong the contact-time of drug with the absorbing surfaces, and a corneal penetration enhancer to expedite the trans-corneal transport. Nevertheless, increasing the viscosity of the aqueous ophthalmic drops vehicle to the lower viscosity range (5–25 cps) have in most cases limited or insufficient increase in contact time with the corneal absorbing tissues[19,20] and leads to a quantifiable decline in the diffusion of drug molecules. Another formulation-related approaches to maximize the therapeutic efficacy of ocular drugs include using of; ***a)*** extended release dosage form with water-soluble polymer[21,22], and ***b)*** preparation of lipophilic ion-pair from a drug and additives[23,24].

Carbopol® polymers are very efficient viscosity improving agent (thickeners), suspending agents, and stabilizers at low concentrations (0.1-3.0 wt%). All Carbopol® polymers have high molecular weight, cross-linked polyacrylic acid polymers. The main differences amongst these polymers are; ***a)*** the crosslinker type, ***b)*** density and ***c)*** solvent utilized to prepare the polymer[25,26]. Different Carbopol® grades are generally used as thickeners (viscosity improving agents) and mucoadhesives and bioadhesives in preparation of wide-variety of pharmaceutical dosage forms including solid, semi-solid dosage forms (ophthalmic and cutaneous gels), emulsions, suspensions, liquids (with a wide-range of viscosities and rheological characteristics), nasal, rectal, intestinal, buccal, vaginal, and in tablets formulation[27,28]. However, certain better mucoadhesiveness of Carbopols such as Carpobol® (C-974®) were advantageous in comparison with the C-971® [29].

One of the very significant approaches expedite the absorption of ocular drugs is the incorporation of an ocular penetration enhancer(s)[30,-32]. Regardless of the occasional drawbacks of some enhancers such as irritation, reversible morphological, and changes in the corneal membrane[33,34] an inert, safe, none allergenic non-irritant, shorten the onset of action, physically

& chemically compatible with the drug and other additives, and cosmetically acceptable, potent with the minimum concentration with both hydrophilic (in particular) & lipophilic drugs are essential requirements for ideal ocular enhancer. Without going into details, Azone (1-dodecylazacycloheptan-2-one) permeation enhancer meets the aforesaid requirements of ideal enhancer to varied extents[35,36]. The corneal penetration of hydrophilic compounds (acetazolamide, cimetidine, guanethidine, and sulfacetamide) was enhanced by at least 20-fold at 0.1% Azone[37]. It has been reported for the first time, presence of Azone that is apparently not toxic but is effective in delivering immunologically active concentrations of cyclosporine following topical application to the cornea[35,38,39].

Hitherto a significant question still exists; to what magnitude the information available about Azone can be universalized. To date, the vast majority of research done has been carried out using *in vitro* models and/or in animals without ensuring the relevance of these studies concerning to human *in vivo*. Further, it is exceptionally difficult to correlating the huge pile of results of these studies because of differences in methodology, inter/intra-laboratory variability, animal model and selection of drugs. Therefore, one of the important indirect objectives for this study is to draw the attention of interested researchers to the uniqueness of Azone as very potent, safe, nonirritant, and effective in very low concentrations as a permeation enhancer. Therefore, Azone could provide an attractive opportunity to preclinical and/or human studies[35,41-42]. Albeit the aforementioned technical, physiological and physic-chemical discrepancies in emerging ophthalmic delivery system, the task of having extended, therapeutically effective, safe and stable ophthalmic formulations of hydrophilic drugs has been growing & attractive research area. The present study is devoted to develop and characterize ODS that are convenient, extended and possibly controlled drug release and increased therapeutic effect. Therefore, it was necessary to; ***i)*** design, prepare and quantitatively determine the *in vitro* permeability parameters of wide variety of LAT gel formulations containing combinations between various concentrations of C-974® (as a mucoadhesive) and Azone (as a corneal penetration enhancer), and ***ii)*** to *in vivo* evaluate the augment and magnitude of the IOP lowering effect of LAT ocular formulations in management of glaucoma.

MATERIALS AND METHODS

Animals:

Rabbits and corneas of adult male New Zealand albino rabbits weighing 3.0-4.0 Kg/each were used throughout this study. The animals were provided by the King Fahd Medical Research

Center, Jeddah, Saudi Arabia. Animal use was approved by the Institutional Review Board for Animal Research/ Studies who ensured the care and use of animals conformed to the Declaration of Helsinki and the Guiding Principle in Care and Use of Animals (DHEW publication NIH 80-23) & fulfilled with the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised in 1985).

Drug and Chemicals

Latanoprost, azone, sodium octane sulfate, acetonitrile, acetonitrile, , and benzalkonium chloride were purchased from Sigma Aldrich Chemical Co., St. Louis. Carpobol-974® (C-974®) was obtained Lubrizol Advanced Materials, Inc. 9911 Brecksville Road, Cleveland, Ohio. Sodium chloride and hydrochloric acid were obtained from Spectrum Chemical Co., Gardena, CA. C-943 and sorbitol were provided by Fisher Scientific Co., Fair Lawn, NJ. Analytical grades of disodium edetate dihydrate (EDTA) were purchased from Merck (Germany). All other chemicals used in this study were commercially available compounds of special reagent or analytical/HPLC grade and they are used as received without any further modifications.

Equipment

A PermeGear Flow Type Franz diffusion system of vertical cells, PermeGear, Inc., Hellertown, PA USA, Water auto-sampler HPLC system with chime station, variable wave length UV detector, Water Associates, Inc., Milford, MA, USA. HPLC column-(RX-C8, 25-cm x 4.6 mm, 5µm) was obtained from ChromTech, ChromTech International AB, Hägersten, Sweden. Thermostatically controlled water bath, water bath shaker, sonicator, hot-plate/stirrer, pH meter were obtained from Fisher Scientific Co., Fair Lawn, NJ. USA. Touch less TONO-PEN™ AVIA tonometer®, Reichert, Inc., NY, and Millipore filter paper, (0.45µm, HA), were obtained from Millipore corporation, Bedford, MA, USA.

HPLC Assay of LAT

High-performance liquid chromatography (HPLC) method was employed using a Kromasil RX-C-18 column maintained at room temperature, with a variable UV detector wavelength of 210-nm. The mobile phase consisted of a mixture of acetonitrile and 0.05 M potassium phosphate buffer (70:30 v/v), pH 3.0 and flow rate of 0.5 ml/min. The mobile phase was then purified by filtration under vacuum using a 0.45µm filter and degassed by sonication for up to 20 minutes. Samples of 1-ml were used for quantitative analysis of their LAT contents throughout the analysis procedures[43].

Preparation of LAT Ophthalmic Gel Formulations

Compositions of the test ophthalmic gel formulations are shown in Tables (1 & 2). On one hand,

Table 1 shows the composition of LAT ophthalmic gel formulations that were prepared with various concentrations of Azone™ as a transcorneal release enhancer with a fixed concentration (1.5%) of C-974® as a mucoadhesive to identify the lowest concentration of Azone that induces the greatest permeability parameters. Table 2, on the other hand shows the composition of LAT ophthalmic gel formulations that were prepared with fixed (lowest) concentration of Azone (0.5%) that induces the greatest permeability parameters; with a varied concentrations of C-974® to pinpoint & scale-up the best formulation(s) amongst the two test sets of LAT ophthalmic gel formulation for further in vivo IOP-lowering efficacy studies.

Table 1: Composition of the Second Set of LAT Test Ophthalmic Gel Formulations Containing Different Concentrations of Azone™ as Penetration Enhancer and Fixed Concentration (1.5%) of C-974® as Mucoadhesive.

Formulation	LAT (μg/ml)	Azone (%)	C-974® (%)	EDTA (%)	BENZ-Cl (%)
*GAZ-$0_{Control}$	5	0.0	1.5	0.1	0.03
*GAZ-1	5	0.125	1.5	0.1	0.03
*GAZ-2	5	0.250	1.5	0.1	0.03
*GAZ-3	5	0.375	1.5	0.1	0.03
*GAZ-4	5	0.500	1.5	0.1	0.03
*GAZ-5	5	0.625	1.5	0.1	0.03

*Isotonic test gel formulations were first prepared and the isotonicity was maintained using sorbitol when necessary.

Table 2: Composition of the First set of LAT Test Ophthalmic Gel Formulations Containing Different Concentrations of C-974® as Mucoadhesive with Fixed Concentration (0.5%) of Azone™ as Enhancer.

Formulation	LAT (μg/ml)	Azone$ (%)	C-974® (%)	EDTA (%)	BENZ-Cl (%)
°GC-$0_{Control}$	5	0.5	0.0	0.1	0.03
*GC-1	5	0.5	0.5	0.1	0.03
*GC-2	5	0.5	1.0	0.1	0.03
*GC-3	5	0.5	1.5	0.1	0.03
*GC-4	5	0.5	2.0	0.1	0.03
*GC-5	5	0.5	2.5	0.1	0.03

*Isotonic test gel formulations were first prepared and the isotonicity was maintained using sorbitol when necessary.

Isotonic negative control solution (0.0% C-943®) was first prepared and the isotonicity was maintained using 0.9% saline.

§This set formulations were prepared using the formulation of LAT that gave the best *in vitro* permeability parameters using Azone 0.375%].$

The tonicities of the gel formulations were adjusted with sorbitol and the final pH values of all formulations were adjusted to be 6.9; i.e., the pH value of the commercially available LAT; Xalatan® eyedrops with 0.1N HCl[44]. Ingredients of each formulation were prepared, mixed and sterilized by filtration using 0.22µm Millipore filters, while C-974® sterilized by autoclaving at 121°C for 30 minutes has been added to rest of incorporated formulation ingredients under aseptic conditions. Isobel & Stanley have reported that little to no change in the viscosity or pH upon repeatedly subjecting a Carbopol® polymer gel to autoclaving at 121°C for 30 minutes[45].

In Vitro Corneal Permeability Studies

The animals were sacrificed by administering an overdose of a sodium pentobarbitone solution via the marginal ear vein. Then the corneas were meticulously excised and mounted in a penetration chamber. Each fresh cornea was then rinsed with normal saline, and gently mounted with its epithelial surface facing the donor half-cell medium using a small pinch clip over a receiver half-cell containing the receiver fluid and stirred gently with magnetic stirrers (about 600 rpm). The permeation assembly was carried out using a full-equipped modified Franz vertical transcorneal diffusion system employing finite dose technique. Samples from the receiver compartment were carefully withdrawn at predetermined time intervals and replaced immediately with equal volumes of fresh pre-heated degassed medium[15,46,47]. Thereafter, the LAT permeability parameters for each test formulation were calculated. All the experiments were conducted in 3-6 replicates at 34°C. The concentration in the donor half-cell has been determined at the end of each experiment. All samples were analyzed for test compounds by HPLC methods. The apparent permeability coefficients (P_{app}) of the test compounds will be calculated using the following equation:

$$P_{app} = \frac{\Delta Q}{\Delta t \times A\, C_0 \times 60}\, cm.sec^{-1}$$

Where $\Delta Q/\Delta t$ is the steady-state flux across the cornea (µg.cm^{-2}.hr^{-1}), A is the available corneal surface area (cm^2) for diffusion, and C_0 is the initial drug concentration (µg.ml^{-1}) in the donor compartment at $t = 0$. Flux per unit surface area ($1/A \times (\Delta Q/\Delta t)$ was calculated from the slope of the linear portion of the cumulative amount permeated per unit surface area versus time plot. The results of experiments performed in triplicates have been presented as mean ± SD The significance of any statistical differences between the compounds in the amount permeated at each time point and the mean values were calculated by one-way analysis of variance (ANOVA)

using SPSS 16.0.1 software, (SPSS Inc., Chicago, IL, USA) and the criterion for statistical significance was $p < 0.05$.

In vivo IOP measurement

In vivo IOP lowering effects of LAT-gel formulations

The *in vivo* studies were executed on normotensive un-anaesthetized albino rabbits weighing 3.0-4.0 kg. The rabbits were individually caged at controlled temperature, with a 12/12-hr light/dark to mimic the normal life-cycle, and fed on a regular diet, with free access to water. The necessary number of the experimental animal was randomly divided into groups of 10-rabbits/formulation. Formulations that showed the highest permeability parameters (Tables 3 & 4), designated as GAZ-3, GAZ-4, and GAZ-4 from the 1st set along with formulations encoded GC-1, GC-2, GC-4 along with the recognized reference standard were used for the *in vivo* IOP lowering efficacy studies. It should be noted that formulation GAZ-4 is identical in compositions with GC-3. Each formulation has been moderately shaken with electric shaker/vortex for about 20-30 seconds to ensure dosage uniformity, accuracy and to ease the withdrawal and dosing procedures. Thereafter, a dose of 50µl from each test formulation was administered once a day for 4-sucssive days onto the conjunctival sac using a positive displacing pipette to each individual rabbit in each animal group. For IOP measurements, TONO-PEN™ AVIA tonometer (Reichert Inc., Depew, NY, USA), was used to measure the baseline as well as the *in vivo* IOP lowering effects after predetermined time intervals; i.e., after the first 3-hr (the reported average onset of action), and after additional 24-hr. Acute eye irritation/corrosion and ocular irritation potential of the gel formulations were tested. The irritation test was carried out in accordance to the Organization for Economic Cooperation and Development (OECD/OCED); test No. 405 guideline 405[48].

Extended In vivo IOP lowering effects of promising LAT-gel formulations

Importantly, the IOP for each animal has been recorded at the end of each day throughout the previous experiments. Formulations that will exhibit promising IOP lowering efficacies will be subjected further studies to determine their level and extent of actions; i.e., from the time of dosing till re-establishing the IOP base-line. For this experiment, the experimental animals will be randomly divided into equal groups (10-rabbits/group). The animals will be handled and housed, hospitalized exactly as stated above. Then the animals will be treated as described above with a single-dose of each formulation. The IOP lowering effects will be measured at appropriate time intervals that will accurately describe the outcomes.

RESULTS AND DISCUSSION

In Vitro Corneal Permeability of LAT-Gel Formulations

The first set of LAT gel formulations containing different concentration of Azone as transcorneal penetration enhancer and fixed concentration (1.5%) of C-974® as a mucoadhesive & viscosity improving agent (crosslinkage thickener) were prepared. Formulation GAZ-$0_{Control}$ was void of Azone to serve as a negative control (Table-1). Figure-1 shows the cumulative amounts of LAT (µg/ml) represented as mean ± SD % of total LAT released from the test formulations into the receiver compartment of the diffusion cell as a function of time; hr (n=3). Table 3 shows the calculated permeability parameters for the first set of LAT formulations including, mean steady-state flux (J_{ss}); µg.cm^{-2}.sec^{-1}, *lop* P_{app}, and the enhancement factor (EF) calculated as $P_{app\text{-}Test\ s}$ /P_{app}-$_{control}$. The data in Table 3 also revealed that the transport characteristics of LAT through excised fresh corneal membrane significantly ($p<0.01$) increased with the increased concentration of Azone up to concentration 0.5%. However, higher concentrations (i.e., 0.625%) didn't show any significant difference in with the overall permeability parameters. Figure 2 reveals a good linear (R= 0.9418) and direct relationship between the apparent permeability coefficient (P_{app}; cm.sec^{-1}) for this set of LAT ophthalmic gel formulations and the % of the added permeation enhancer (Azone). In other words, the temporal pattern of LAT release from the test formulations appears to be a single-valued function of the concentration of Azone; i.e., concentration dependent. This could be related but not limited to the assumption that the enhancement of drug permeation by Azone is basically due to its ability to reversibly increasing the fluidity of the intercellular lipid bilayers of the corneal membrane. Thereby, it lessens the diffusional resistance of the corneal epithelial layer to drug; i.e., increasing the drug diffusivity and partitioning. Different but related scenario is that, Azone evokes its effect as permeation enhancer via increasing the drug solubility as well as the thermodynamic activity of the system and/or changing the ratio between ionized and unionized drug molecules in favor of the later[16,36,49]. Moreover, the enhancements factor (EF) of was also found to be a function in the concentration of Azone. Accordingly formulations GAZ-3, GAZ-4, GAZ-5 along with the reference standard Xalatan™ 0.005% eye drops will be selected for additional *in vivo* IOP lowering effect experiments.

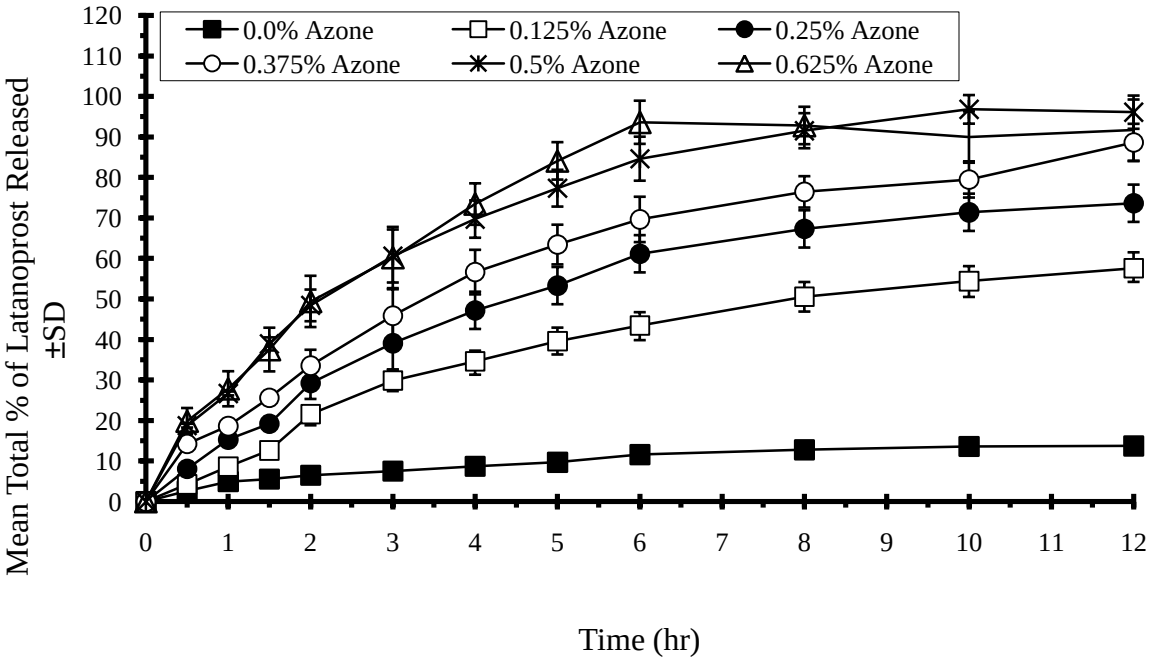

Figure 1: Mean total % of LAT delivered into the receiver chamber of an automated Franz' diffusion system from LAT eye gels containing different concentrations of Azone corneal penetration enhancer and fixed concentration (1.5%) of C-974® as a mucoadhesive across freshly excised rabbit's cornea (n=3±SD).

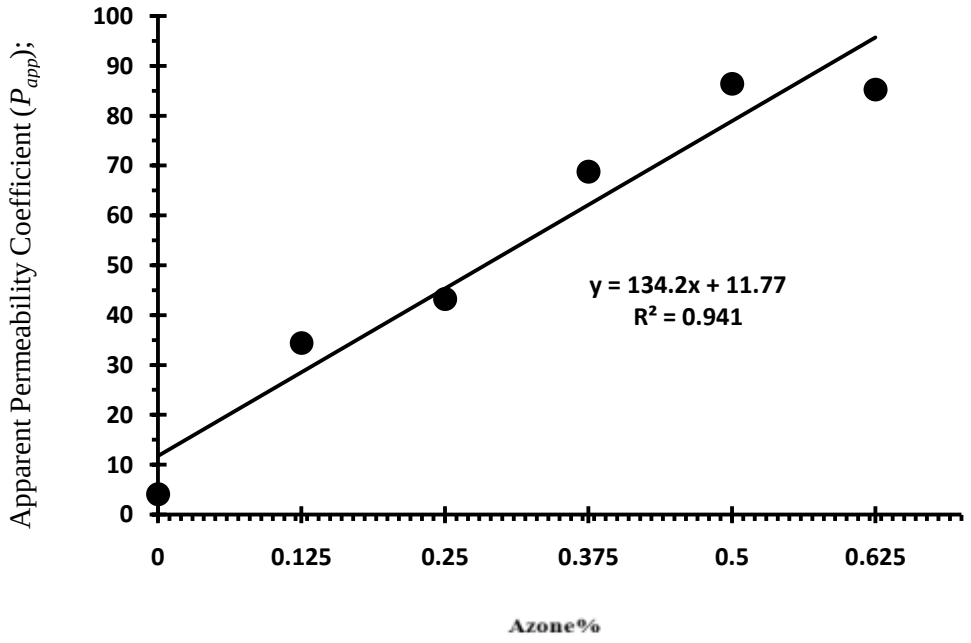

Figure 2: Relationship between the concentration of Azone™ as a transcorneal permeation enhancer and the *in vitro* apparent permeability coefficient of LAT ophthalmic gel formulations across freshly excised rabbit's cornea (n=3±SD).

Table 3: Effects of Different Concentrations of Azone™ upon the Permeability Parameters of LAT Formulations Containing Fixed Concentration 1.5% C-974® as Mucoadhesive through Freshly Excised Rabbits Cornea.

Formulation Code	Mean Steady-State Flux (J_{ss}) µg.cm^{-2}.sec^{-1}).	Log P_{app}	(EF) P_{app} Test /$P_{app\text{-control}}$
GAZ-$0_{Control}$	19.12±3.39	-5.39	1
GAZ-1	50.10± 5.70	-4.46	8.61
GAZ-2	79.37±7.10	-4.36	4.76
GAZ-3	77.80±9.76	-4.16	10.56
GAZ-4	86.10±7.30	-4.06	21.09
GAZ-5	79.87±12.09	-4.07	20.84

Table 4: Effects of Different Concentrations of C-974® upon the Permeability Parameters of LAT Formulations Containing Fixed Concentration 0.5% Azone™ as Penetration Enhancer through Freshly Excised Rabbits Corneal Membrane.

Formulation Code	Mean Steady-State Flux (J_{ss}) µg.cm^{-2}.sec^{-1}).	Log P_{app}	(EF) P_{app} Test /$P_{app\text{-control}}$
GC-$0_{Control}$	19.12±4.40	-4.01	1
GC-1	57.10±7.700	-4.03	0.96
GC-2	63.37±13.10	-4.05	0.91
GC-3	86.10±7.30	-4.06	0.88
GC-4	59.10±7.70	-4.36	0.44
GC-5	61.37±13.10	-4.63	0.24

In preparation of a pharmaceutical drug product, it is highly recommended to use the lowest possible number of additives with the lowest effective concentration. Therefore, a 2nd set of LAT gel formulations containing a fixed concentration (0.5%) of Azone (lowest concentration that induced the highest permeability parameters in the 1st set of LAT ophthalmic gel formulation) as transcorneal penetration enhancer along with varied concentrations of C-974® as a mucoadhesive & viscosity improving agent (crosslinker/thickener) were carefully prepared. Formulation GC-$0_{Control}$ was void of C-974® (a simple eye drops) to serve as a negative control (Table-2). Figure-3 shows the cumulative amounts of LAT (µg/ml) represented as mean ± SD % of total LAT released from the test formulations into the receiver compartment of the diffusion cell as a function of time; hr (n=3). Table 4 shows the calculated permeability parameters for the 2nd set of LAT formulations including, mean steady-state flux (J_{ss}); µg.cm^{-2}.sec^{-1}, *lop* P_{app}, and the enhancement factor (EF) calculated as $P_{app\text{-}Test\ s}$ /P_{app}-control. The data in Table 4 also revealed that the transport characteristics of LAT through excised fresh corneal membrane significantly ($p<0.01$) decreased with the increased concentration of C-974®.

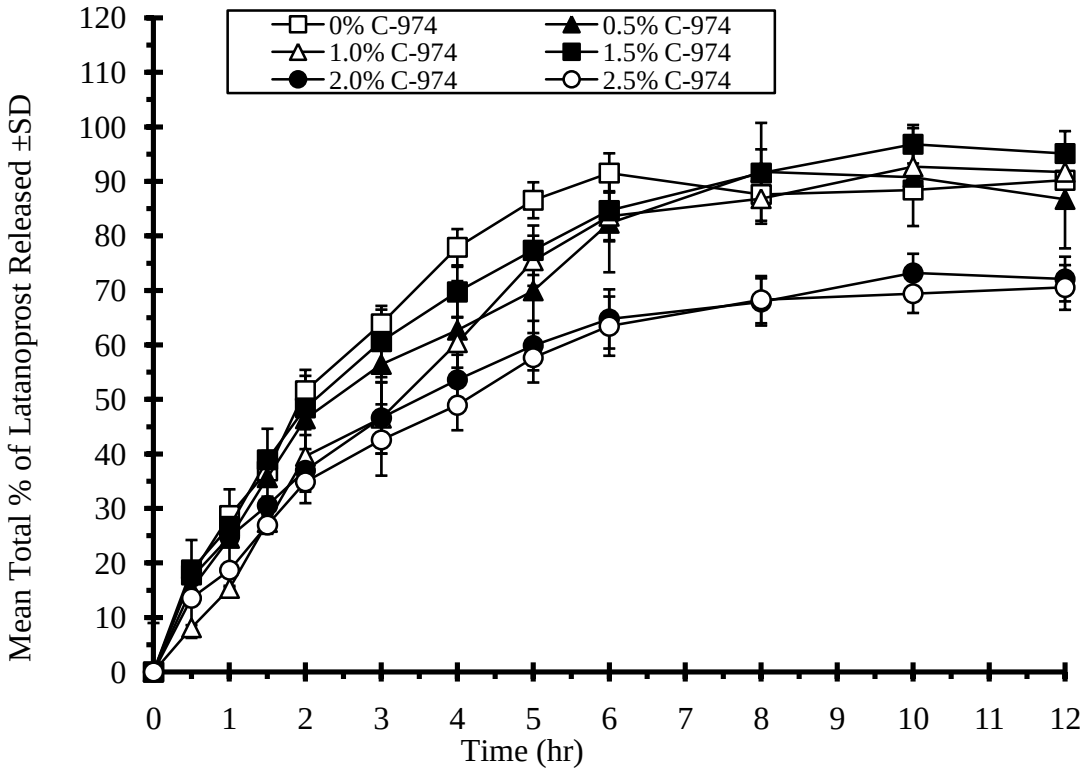

Figure 3: Mean total % of LAT delivered into the receiver chamber of an automated Franz diffusion system from LAT eye gels containing different concentrations of C-974® as a mucoadhesive and fixed concentration (0.5%) of Azone as a corneal penetration enhancer across freshly excised rabbit's cornea (n=3±SD).

Figure 4 reveals almost-linear (R= 0.8178) but reversible relationship between the apparent permeability coefficient (P_{app}; cm.sec^{-1}) for this set of LAT ophthalmic gel formulations and the % of the added C-974® as a mucoadhesive. Even though, this cannot be generalized because the exact correlation between viscosity of the vehicle and transcorneal penetration is difficult to be established as it is generally not the rate-limiting step in the corneal absorption process. In addition to the fact that release of a penetrant from the vehicle of formulation is governed by numerous factors related to the physicochemical properties of the drug, vehicle, extent of their mutual affinity (if exists) and to the portioning of the drug from that vehicle to the absorbing surface[50]. However, the viscosity of the formulation will affect the drug diffusion. Formulations GC-3 (which is identical with formulation GAZ-4) and GC-4 have shown significantly ($p>0.01$) higher permeability parameters than formulation GC-5. Meanwhile, the *in vitro* corneal permeability parameters of formulations GC-$0_{Control}$ (void of C-974® ; eye drops), GC-1 (containing 0.5% C-974®) and GC-2 (containing 1.0% C-974®) have shown LAT release faster than those of the rest of all test formulations of the 2nd set, particularly during the first ~6,

8 & 10 hr, respectively (depending upon the C-974® concentration) . Formulation GC-$0_{Control}$ has showed the fastest onset and the shortest duration of action (~18-20-hr) which is shorter than/but comparable with that observed with the reference standard Xalatan® up to (~21-22 hr). Moreover, formulations designated GC-1, GC-2, GC-3 & GC-4 have shown reasonable thixotropic phenomena; i.e., Non-Newtonian fluids/semisolid feature in which viscosity decrease with the time of shearing, and the subsequent recovery of viscosity after cessation of shearing[51.52], hysteresis loop, the best rheological characteristics for ophthalmic gels including physical appearance, flowability, spreadability, texture, uniformity and elegancy, (unpublished data). Therefore, these formulations have been scaled up for further the *in vivo* IOP lowering effects.

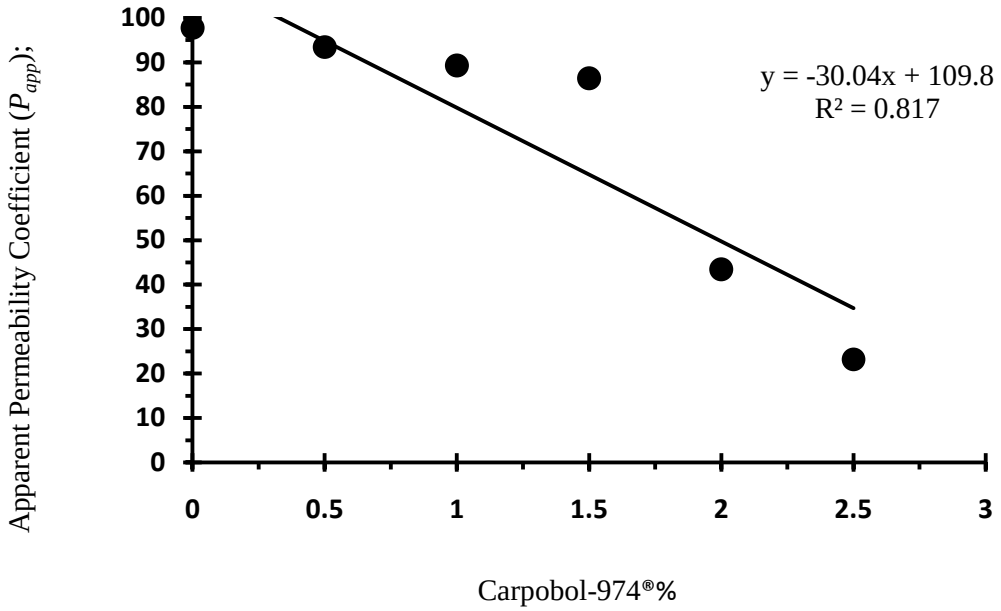

Figure 4: Relationship between the concentration of Carbopol® as a mucoadhesive and the in vitro apparent permeability coefficient of LAT ophthalmic gel formulations across freshly excised rabbit's cornea (n=3±SD).

In vivo IOP Measurement

***In vivo* IOP Lowering Effects of LAT Ophthalmic Gels**

As it has been illustrated the current study was conducted to examine the hypothesis that different concentration of very essential formulation-related factors; i.e., ***a)*** Azone as a corneal penetration enhancer, and ***b)*** C-974® as a mucoadhesive that already have shown the above broadly varied of permeability parameters (Tables 3 & 4 respectively) upon the *in vivo* IOP in

management of glaucoma. The average IOP base line of the normotensive rabbit (23±2) was measured and recorded prior to administration of each dose. Figure (5) shows the ΔIOP for the

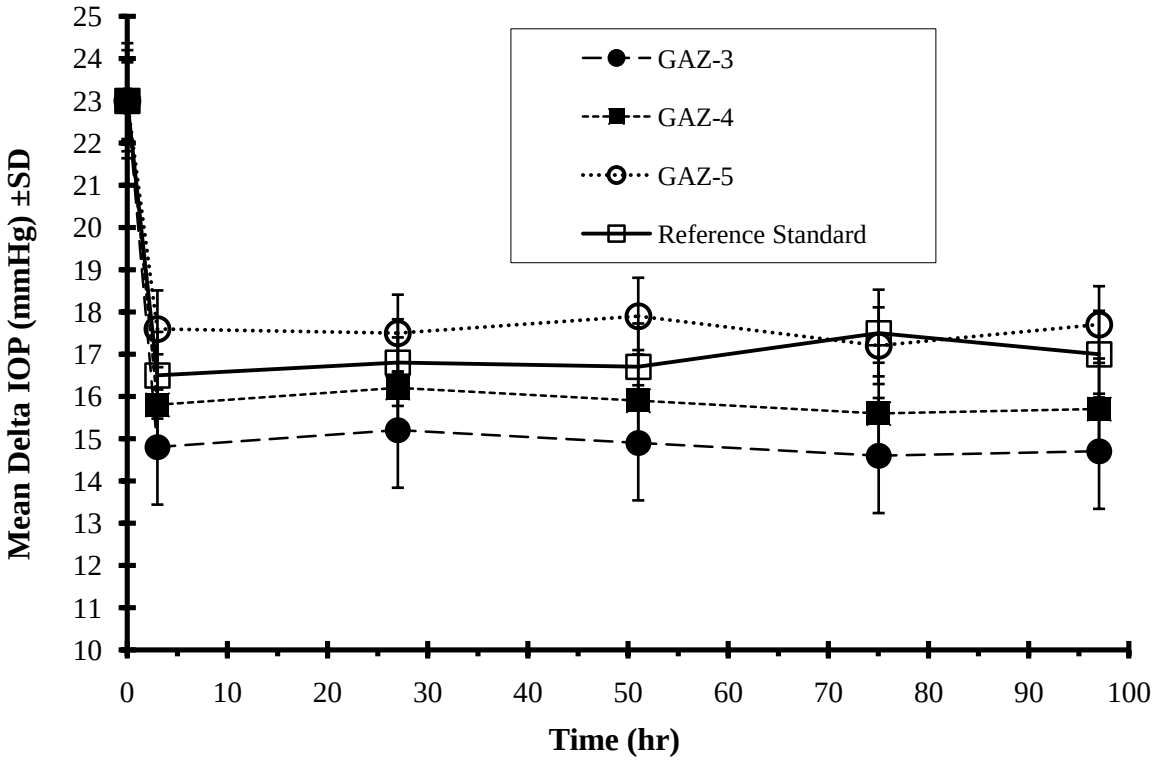

Figure 5: Mean IOP lowering effects expressed as the difference from the base line (23+3.0 mmHg) for scaled up LAT ophthalmic gels containing different concentrations of Azone as a corneal penetration enhancer compared to the reference standard (Xalatan®) in normotensive New Zealand Rabbits (n=3±SD).

scaled up ophthalmic gel formulations of the first set; i.e., GAZ-3, GAZ-4 and GAZ-5 along with ΔIOP for LAT commercial ophthalmic solution (Xalatan®) applied topically once/day for four successive days. The maximum ΔIOP measurements for the tested formulations; GAZ-3, GAZ-4, GAZ-5 & (Xalatan®) were (7.8±1.8), (6.2±2.0), (5.5±1.7), and (5.6±2.0) mmHg, respectively. The mean ΔIOP ±SD values and the onset of actions have been achieved within the time range of 1.5-3.5 hours, in direct relation with the concentrations of corneal penetration enhancer (Azone) as well as the permeability parameters. The higher of Azone concentration the shorter of the onset time up to 0.5%. Nonetheless, formulation GAZ-3 containing 0.5% of Azone has shown comparable onset of action but significantly higher ΔIOP than these with formulation GAZ-5 containing 0.625.0% and the commercial reference standard Xalatan®. The assumption that more compact gel network assembly and/or fusion complex could be formed with higher concentrations of the mucoadhesive, crosslinker; (C-974®) could be a reasonable explanation for

these results. This assumption may in turn lead to the extended duration of action and possibly reduced LAT release from and diffusion though such compacted gel[53]. The likelihood of ocular irritation due to administration of the test gel formulation or one of its ingredients was assessed in New Zealand albino rabbits according to OECD, 2002[48]. Upon inspection, no signs of ophthalmic irritation (i.e., tearing, redness, inflammation, and/or swelling) have been recorded after with used test gel formulation or any of its constituents at the used concentration during the time course of the experiments.

Figure (6) shows the *in vivo* ΔIOP measurements for the scaled up ophthalmic gel formulations designated GC-1, GC-2, (GC-3/GAZ-4), GC-4 of the 2nd set, and the reference standard (RS) Xalatan®. The maximum ΔIOP measurements for the formulations of this set were (6.0±2.2), (7.8±1.8), (6.5±2.1), (4.8±1.7) and (6.5±1.5) mmHg, respectively. The onset of action range for these formulations was 1-4 hrs. Such wide-range of the onset of action is likely because this set of formulations encompasses formulations that contain wide-variety of C-974® mucoadhesive (0-2.5%). Formulations with lower concentrations of C-974® exhibited shorter onset of action than those containing higher concentration. The duration of action was found to be a function of the mucoadhesive concentration. In other terms, the higher the concentration of C-974®, the longer the duration of action. Moreover, it's obvious that concentrations of C-974® greater than (1.5%) significantly ($p>0.05$) extended the duration of action, but reduced the efficacy with delayed the onset of action. Taking onto the role of the added penetration enhancer (0.5%) in increasing the bioefficacy, presence of C-974® as mucoadhesive is naturally functioning to increase the contact period with ocular absorbing surfaces; i.e., providing more time for the drug to be delivered, which in turn will further increase the ΔIOP. Evidently, the *in vitro* release and the *in vivo* pharmacodynamics for both sets of LAT ophthalmic gel formulations is largely depends upon the combined effect of two of the furthermost essential additives to an ocular drug delivery system; i.e., mucoadhesives, thickener and enhancers, because of their enormous positive/competitive and in some cases could be transitive contributions for variable extents to the pharmacotherapy as well as to the overall outcomes in developing an ocular drug delivery system.

Extended In vivo IOP lowering effects of promising LAT-gel formulations

The IOP for each animal has been recorded at the end of each day throughout the previous *in vivo* experiments. Formulations (GAZ-4/GC-3) and GC-4 have showed very encouraging IOP lowering efficacies, *particularly noteworthy both didn't re-establish the IOP base-line after 24 hours*. Therefore, the goal of this study was to study the hypothesis that the ocular administration

of these two formulations would be of duration of action longer than that of the rest of the tested formulations. Obviously, Figure (7) shows that formulations designated GAZ-4/GC-3 & GC-4, have induced significantly higher therapeutic IOP lowering effects than that of the reference standard Xalatan®; i.e., (7.8±1.8) and (6.5±2.1) respectively. In figure (7) the *in vivo* ΔIOP lowering effects that have been recorded after the first 3 & 24-hr, then each two-hours till the 40th hr, and finally on hourly bases until the IOP re-establishing the base line (23 ±2) to exactly determine the duration of actions of the two formulations. The average IOP lowering effects for formulations GC-3/GAZ-4 and GC-4 & were lasted for 47±2.25, and 48±1.5, respectively. The IOP lowering effects for the two ophthalmic gel formulations remained eventually unchanged during the duration of actions. This relatively high steady level of the IOP lowering effects and magnitudes could be partially explained by the relatively rigid nature of the channels of C-974® gel micro-matrix characterized by a very high macro-viscosity and regions of water-thin micro-viscosities. The presence of these channels could help increasing the initial release rate as well as the *in vivo* IOP lowering effects of LAT-C-974® containing gels[29,52,53]. Moreover, the complex net outcomes of the interplay between the variables in tested ophthalmic gel formulations; i.e., enhancement factors (EF), Azone (enhancer), C-974® (mucoadhesive and thickener) and ΔIOP measurements could explain these findings. These complex net outcomes of the interplay between these variables might be taken into account, because the *in vitro* release experiments usually designed to maintain the drug formulation in immanent direct contact with corneal epithelium layer throughout its entire pre-designed experimental time course. However, this is not the scenario in the *in vivo* experiment or in management of patients with glaucoma, where the ocular therapeutic efficacy of an applied dose affected (negatively or positively) by the previously explained variables in addition to the physiological, physic-chemical properties of the drug and the formulations vehicles. Regardless of all kinds of co-/inter/lack of--relation and its magnitudes between variables scrutinized in this study, the results unambiguously revealed that, for ocular drug delivery system, the correlation between *in vitro* release data and the *in vivo* efficacy is evidently complex and controlled by massive number of disputed, overlapped, combined, integrated, competitive and in some cases contradictive factors that should be taken into our calculus prior to developing an ocular drug delivery system, as well as, extrapolating or generalizing the *in vitro* studies outcomes to the clinical situations.

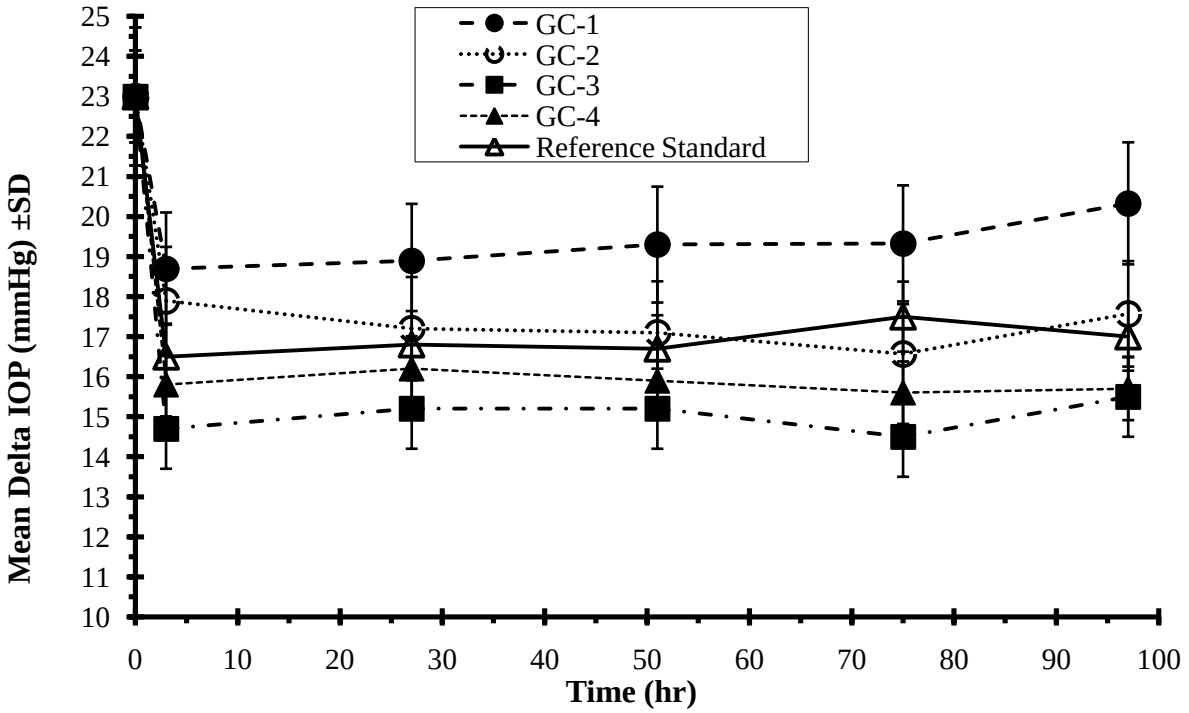

Figure 6: Mean IOP lowering effects expressed as the difference from the base line (23+2.0 mmHg) for rationally scaled-up LAT ophthalmic gels containing different concentration of C-974® as a mucoadhesive compared to the reference standard (Xalatan®) in normotensive New Zealand Rabbits (n=3±SD).

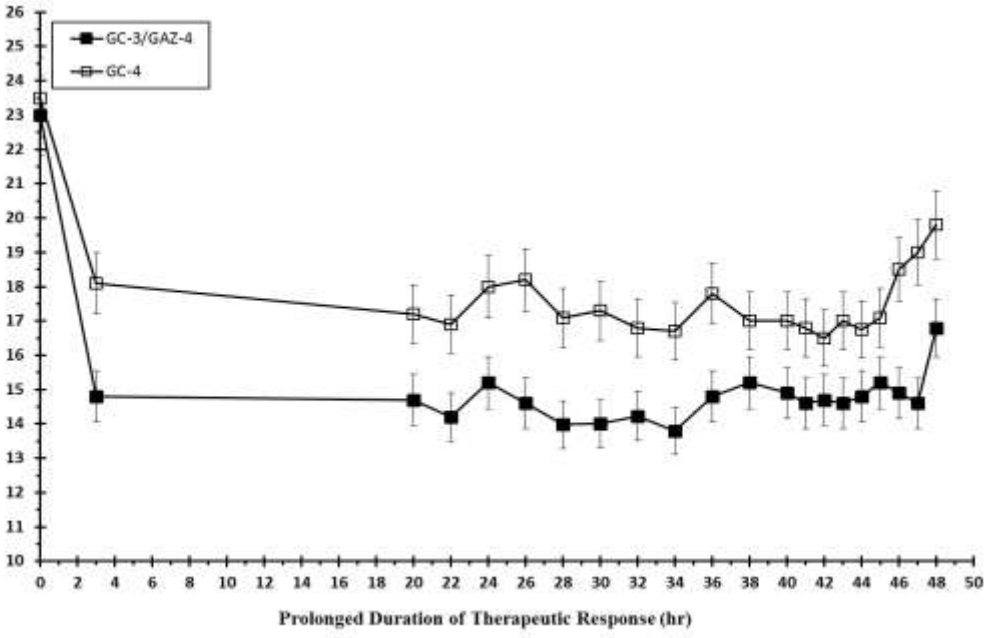

Figure 7: Mean extended duration of action of the two-scale-up LAT ophthalmic gel formulations (i.e.; GC-3/GAZ-4 & GC-4) in normotensive New Zealand Rabbits (n=3±SD).

CONCLUSION

the *in vitro* corneal drug transport, onset of action, augmenting IOP lowering effect, increasing the extent of LAT therapeutic efficacy for the test formulations largely depend upon the net outcomes of the interplay between; ***1)*** the prolonged residence time of LAT in conjunctival cavity caused by the mucoadhesive & crosslinker (C-974®), ***2)*** the accelerated drug transport by the penetration enhancer (Azone), and ***3)*** the reduced diffusivity of the drug throughout the vehicles of gel formulations resulting from the increased viscosity by the thickener, ***4)*** The rheological and physicochemical characteristics of the formulation and drug, in addition to ***5)*** the inherent unique physiological and anatomical constraints of the eye represent further vital element that restricts successful development of ocular delivery systems. It is clear from the foregoing complex discussions that a plethora of efforts have been and continue to be devoted to improving delivery of drugs into the different ocular layers/tissues. It is therefore erratic that only a very few systems have actually been marketed or innovated into human clinical trials so far. Interested research scientists need only consider the mantra of this study including onset of action, efficacy level, duration of action, drug targeting and patient compliance as a start to recognize why the challenge is still monumental.

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