Maximization 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 I. Afouna[a*], Hatem R. Roshdy[a], Hany M. Ibrahim[a], Ashraf B. Naim[b] and Adnan El-Marzoqi[c]

[a]Department of Pharmaceutics & Pharmaceutical Technology, College of Pharmacy, Al-Azhar University, Nasr City, Cairo, Egypt
[b]Department of Pharmacology & Toxicology, College of Pharmacy, King Abdulaziz University, Jeddah, Saudi Arabia
[c]Department of Ophthalmology, College of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia

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ABSTRACT

The objective of this study was to maximize the *in vitro* transcorneal release, the intraocular pressure (IOP) lowering effect and the duration of action, of the Latanoprost acid (LAT) ophthalmic gels. The *in vitro* transcorneal release of LAT from a first set of gel formulations containing different concentrations of Azone (as enhancer) with a fixed concentration of C-974® (as mucoadhesive) was studied. The formulation that showed the greatest permeability at the lowest Azone concentration was selected for the preparation of a second set of ocular gels containing different C-974® concentrations. Their *in vitro* permeabilities were evaluated, and the C-974® concentration yielding the greatest *in vitro* permeability was chosen. The *in vivo* IOP-lowering efficacy study for the scaled-up formulations from both sets of the test formulations was performed using a Tono-pen Avia® tonometer in rabbits for 4 consecutive days. To determine the duration of action, the most effective formulations were used for a single-dose study, and the IOP was measured at predetermined intervals until the IOP base-line was reestablished. The majority of the tested formulations showed significant but varied augmentations in both the *in vitro* and *in vivo* permeability results. The formulations GAZ-4 and GC-4 showed the greatest IOP lowering effects, i.e., 7.8±1.8 mmHg and 6.5±2.1 mm Hg, respectively. It is particularly noteworthy that for both formulations the IOP lowering effect continued for 24 hours. Their duration of action in the single-dose study were 47±2.25 hours and 48±1.5 hours, respectively. It was concluded that the *in vitro* release, onset, magnitude and duration of action of the LAT gels were increased and extended for up to 2 days for the two gel formulations.

KEY WORDS: Azone, corneal transport, ocular delivery, ocular enhancers, Carbopol-974®, glaucoma, IOP lowering effect, mucoadhesive, thixotropy, intra ocular pressure

INTRODUCTION

Glaucoma is a progressive optic neuropathy affecting more than 70 million individuals worldwide and is a major cause for irreversible blindness (1). In November 2004, the World Health Organization reported that glaucoma is responsible for approximately 4.5 million blind people, approximately 12% of the total burden of world blindness. It has been reported that glaucoma is the second leading cause of

*Corresponding author: Mohsen I. Afouna, Department of Pharmaceutics & Pharmaceutical Technology, College of Pharmacy, Al-Azhar University, Nasr City, Cairo, Egypt, E-mail: mafouna@pharma.asu.edu.eg, afounamohsen@yahoo.com

blindness globally (2). One of the most important risk factors for the progression of glaucoma is increased Intra Ocular Pressure (IOP). Elevated IOP can result in retinal ganglion cell loss and optic nerve atrophy leading to irreversible blindness.

Ocular drugs are usually administered as aqueous eye drops. Latanoprost is a selective FP prostanoid receptor agonist with good therapeutic index in the eye. Latanoprost ((+)-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 studied for potential treatment of primary open-angle glaucoma and ocular hypertension (3, 4). It is hydrolyzed by esterases in the cornea reducing both normal and elevated IOP by increasing the uveoscleral outflow (5, 6). It is the active ingredient in Xalatan® eye drops.

The main difficulties for ocular drug delivery in a therapeutically effective concentration from ophthalmic delivery systems (ODS) are (a) the very short average residence time of the administered dose, particularly ophthalmic solutions (5-25 minutes) (7, 8), (b) the extensive pre-corneal loss because of the fast tear drainage and solvent evaporation and alteration of drug pharmacodynamics, (c) the high possibility of excessive loss of drug through the nasolacrimal drainage that may cause systemic effects and/or side effects (9), (d) the very limited accommodation capacity of the eye, that is 10-30 μl for blinking and non-blinking human eye, respectively and (e) the inherent physiological involuntary defense mechanism of the eye (blinking and tearing). Subsequently, the ocular bio-efficacy of topically applied ocular drug drops is very low, only 1-10% (10, 11).

The corneal membrane consists of three essential layers, i.e., the epithelium, stroma and the endothelium. The epithelium layer is lipophilic and contains approximately 100-fold greater amount of lipid material per unit weight than the stroma. Therefore, it represents the main barrier for the hydrophilic (i.e., poorly lipid soluble) drug molecules. On the other hand, the stroma which is a hydrophilic, gel-like structure represents a moderate barrier for hydrophobic drug molecules. Despite the lipophilic nature of the endothelium it does not serve as a barrier for either lipophilic or lyophobic drug molecules because it has a thin, single-layer structure with relatively large paracellular junctions. Therefore it is more easily crossed through trans-cellular, pore-cellular and para-cellular transport pathways (12). The drug molecule should therefore ideally have biphasic partitioning properties to be able to successfully cross the corneal membrane (13-17).

Ocular therapy presents a unique challenge when it comes to delivery of a drug with pharmacologically effective levels. Studies have shown that the outmost layer (i.e., the epithelium penetration) is commonly the rate-limiting step to the transcorneal transport of hydrophilic drug molecules. Thus, drug molecules must have sufficient lipophilicity to be able to penetrate this barrier (18). One approach used to bypass the epithelium barrier is to incorporate a suitable viscosity improving agent (VIA) to prolong the contact-time of the 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 to a viscosity range of 5–25 mPa. provides in most cases a limited or insufficient increase in the contact time with the corneal absorbing tissues (19, 20) and may even lead to a quantifiable decline in the diffusion of the drug molecule. Other formulation approaches for maximizing the therapeutic efficacy of ocular drugs include using (a) extended release dosage forms with water-soluble polymers (21, 22) and (b) the preparation of a lipophilic ion-pair from the drug molecule and the excipients (23, 24).

Carbopol® polymers are very efficient viscosity improving (thickeners) and suspending agents, as well as, stabilizers at low concentrations (0.1-3.0 wt %). All Carbopol® polymers are high molecular weight, cross-linked polyacrylic acid polymers. The main differences between these polymers are (a) the crosslinker type, (b) density and (c) solvent used to prepare the polymer (25, 26). Different Carbopol® grades are generally used as thickeners (viscosity improving agents), mucoadhesives or bioadhesives for the preparation of a 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, the mucoadhesiveness differ and for example Carpobol® C-974® has a greater mucoadhesiveness compared with C-971® (29).

A common approach to expedite the absorption of ocular drugs is the incorporation of an ocular penetration enhancer(s) (30-32). The limitations of some enhancers include irritation and reversible morphological changes in the corneal membrane (33, 34). An ideal ocular permeability enhancer must be inert, safe, non-allergenic non-irritant, shorten the onset of action, physically and chemically compatible with the drug molecule and other excipients, and cosmetically acceptable, potent with the minimum concentration with both hydrophilic (in particular) and lipophilic drugs. Azone (1-dodecylazacycloheptan-2-one) meets the aforesaid requirements of an ideal enhancer (35, 36). The corneal penetration of hydrophilic compounds acetazolamide, cimetidine, guanethidine, and sulfacetamide increased by at least 20-fold using 0.1% Azone (37). Azone was reported to be effective in delivering immunologically active concentrations of cyclosporine following topical application to the cornea (35, 38, 39).

To date, the vast majority of research carried out using Azone has been in *in vitro* models and/or in animals without ensuring the relevance of these studies to humans through *in vivo* studies. Further, it is difficult to correlate the data from 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 to the uniqueness of Azone as a very potent, safe, nonirritant, and effective permeation enhancer. Azone could provide an attractive opportunity for incorporation into formulations used in preclinical and/or human studies (35, 41, 42).

The objective of this study was to develop and characterize ocular delivery systems that would be convenient, provide extended, possibly controlled drug release and increased therapeutic effect. Therefore, it was necessary to (a) design, prepare and quantitatively determine the *in vitro* permeability of different LAT gel formulations containing combinations of various concentrations of C-974® (as a mucoadhesive) and Azone (as a corneal penetration enhancer), and (b) to evaluate *in vivo* the increase and magnitude of the IOP lowering effect of LAT ocular formulations in the management of glaucoma.

MATERIALS AND METHODS

Animals

Rabbits and corneas of adult male New Zealand albino rabbits weighing approximately 3.0-4.0 kilograms each were used throughout this study. The animals were provided by the King Fahd Medical Research Center, Jeddah, Saudi Arabia. The animals used were approved by the Institutional Review Board for Animal Research/Studies who ensured the care and use of animals conformed with the Declaration of Helsinki and the Guiding Principle in Care and Use of Animals (DHEW publication NIH 80-23) and met the "Principles of Laboratory

Animal Care" (NIH publication #85-23, revised 1985).

Drug and Chemicals

Latanoprost free acid (LAT), Azone, sodium octane sulfonate, acetonitrile and benzalkonium chloride were purchased from Sigma Aldrich Chemical Co., St. Louis, MO). Carpobol-974® (C-974®) was obtained from Lubrizol Advanced Materials, Inc. 9911 Brecksville Road, Cleveland, OH. 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 KGaA (Germany). All other chemicals used in this study were commercially available compounds of special reagent or analytical/HPLC grade and were used as received.

Equipment

A PermeGear Flow Type Franz diffusion system with vertical cells (PermeGear, Inc., Hellertown, PA USA), a water auto-sampler HPLC system with chime station, variable wave length UV detector, (Water Associates, Inc., Milford, MA, USA), and a HPLC column-(RX-C8, 25-cm x 4.6 mm, 5µm) (ChromTech International AB, Hägersten, Sweden) were used for the experiments. A thermostatically controlled water bath, water bath shaker, sonicator, hot- plate/stirrer, and pH meter were obtained from Fisher Scientific Co., Fair Lawn, NJ. USA. A Touchless Tono-Pen® Avia tonometer made by Reichert, Inc., NY, and Millipore filter paper, (0.45µm, HA), were obtained from Millipore corporation, Bedford, MA, USA.

HPLC Assay of LAT

A high performance liquid chromatography (HPLC) method was employed using a Kromasil RX- C-18 column maintained at room temperature, with a variable UV detector set at a 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. An injection of 1.0 microliter taken from samples of 1 ml was used for the quantitative analysis of LAT content (43). Analyses of both negative control (LAT-free formulation) and positive control (LAT-containing formulations) showed that none of the additives used including Azone interfered in the quantitative specific assay methodology for LAT contents in all tested formulations.

Preparation of LAT ophthalmic gel formulations

Compositions of the test ophthalmic gel formulations are shown in Tables 1 and 2. Table 1 shows the composition of the 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 aiming to identify the lowest concentration of Azone that induced the greatest permeability.

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, shows the composition of the LAT ophthalmic gel formulations that were prepared with a fixed (lowest) concentration of Azone (0.5%) that induced the greatest permeability, with varying concentrations of C-974® to identify and scale-up the best formulation(s) amongst the two test sets of LAT ophthalmic gel formulation for use in further *in vivo* IOP lowering efficacy studies. The tonicities of the gel formulations were adjusted with sorbitol and the final pH values of all formulations were adjusted to 6.9, i.e., the pH value of the commercially available Latanoprost Xalatan® eyedrops with 0.1N HCL (44).

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

The ingredients of each formulation were prepared, mixed and sterilized by filtration as aqueous solutions using 0.22µm Millipore filters. C-974® in its dry powder form was subjected to autoclaving at 121°C for 30 minutes and was incorporated into the formulation under aseptic conditions. Isobel and Stanley have reported that there was little or 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 excised and mounted in a penetration chamber. Each fresh cornea was then rinsed with normal saline water and gently mounted with the 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 modified, fully equipped Franz vertical transcor-neal diffusion system employing a finite dose technique. Samples from the receiver compartment were drawn carefully at predetermined time intervals and replaced immediately with equal volumes of fresh pre-heated degassed medium (15, 46, 47). Then the LAT permeability parameters for each test formulation were calculated. All the experiments were replicated 3 times at 34°C. The concentration in the donor half-cell was determined at the end of each experiment. All samples were analyzed for LAT using HPLC. The apparent permeability coefficients (P_{app}) of the test compounds were calculated using Equation 1:

$$P_{app} = \frac{\Delta Q}{\Delta t + AC_0 \text{ x } 60 \text{ x } 60} = per cm.\ per sec \qquad \text{Eq 1}$$

Where, $\Delta Q/\Delta t$ is the steady-state flux across the cornea (mg.cm $^{-2}$.hr^{-1}), A is the available corneal surface area (cm^2) for diffusion and C_0 is the initial drug concentration (mg.ml^{-1}) in the donor compartment at t = 0. Flux per unit surface area $1/A$ x $\Delta Q/\Delta t$ was calculated from the slope of the linear portion of the cumulative amount permeated per unit surface area versus time. The significance of any statistical differences between the compounds in the amount permeated at each time point and the mean values were calculated using a 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 performed on normotensive un-anaesthetized albino rabbits weighing 3.0-4.0 kilograms. The rabbits were individually caged at a controlled temperature and kept on 12/12-hour light/dark to mimic a normal life-cycle, fed a regular diet with free access to water. The required number of the experimental animals were randomly divided into groups of 10-rabbits per formulation.

The formulations that showed the highest permeability parameters (Tables 3 and 4), that is, GAZ-3, GAZ-4 (identical to GC3), and GAZ-4 from the first set together with the formulations GC-1, GC-2, GC-4 and the recognized reference standard were used for the *in vivo* IOP lowering efficacy studies. Each formulation was moderately shaken with an electric/vortex shaker for about 20-30 seconds to ensure dosage uniformity, accuracy and to facilitate the withdrawal and dosing procedure. Thereafter, a dose of 50 µl from each test formulation was administered once a day for 4 successive days onto the conjunctival sac using a positive displacing pipette to each individual rabbit in each animal group. A Tono-Pen® Avia tonometer was used for measuring the IOP baseline, as well as, the *in vivo* IOP lowering effects after predetermined time intervals i.e., after the first 3 hours (the reported average onset of action) and then after each additional 24 hours. Acute eye irritation/ corrosion and ocular irritation potential of the gel formulations were tested. The irritation test was carried out in accordance with 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

The IOP for each animal was recorded at the end of each day throughout the experiments. Formulations that exhibited promising IOP lowering effects were subjected to further studies to determine the duration of the action i.e., from the time of dosing until re-establishing the IOP base-line. For this experiment, the experimental animals were randomly divided into equal groups (10-rabbits/group). The animals were handled and housed as stated previously. The animals were then treated as described previously with a single-dose of each formulation. The IOP lowering effects were measured at appropriate time intervals until the IOP baseline was re-established.

RESULTS AND DISCUSSION

In vitro corneal permeability of the LAT-gel formulations

The first set of LAT gel formulations containing different concentrations of Azone as transcorneal penetration enhancer and a fixed concentration (1.5%) of C-974® as a mucoadhesive and viscosity improving agent (crosslinkage thickener) were prepared. Formulation GAZ-$0_{Control}$ did not contain Azone and served as a negative control (Table 1). Figure 1 shows the cumulative amounts of LAT (µ g/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 in hours (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 $^{-1}$, *IOP* P_{app} , and the enhancement factor (EF) calculated as $P_{app\text{-}Tests}/P_{app}$-control. The data in Table 3 also showed that the transport characteristics of LAT through excised fresh corneal

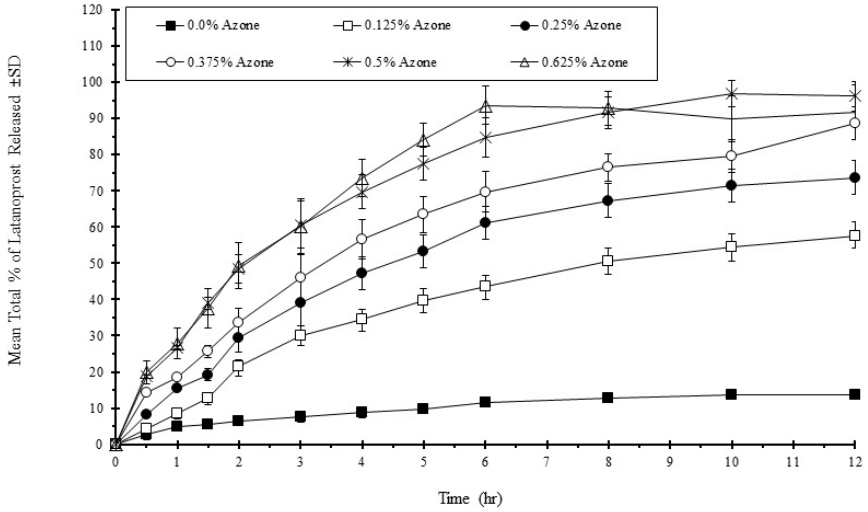

Figure 1 Mean total % of LAT delivered into the receiver chamber of an automated Franz's diffusion system from LAT eye gels containing different concentrations of Azone corneal penetration enhancer and fixed concentration (1.5%) of C-974® as a mucoadhesive across freshly excised rabbit's cornea (n=3±SD).

membrane significantly ($p<0.01$) increased with increasing concentration of Azone up to a concentration of 0.5%.

However, a higher concentration (i.e., 0.625%) did not show any significant increase in the permeability parameters. Figure 2 indicates a 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 percentage of the added Azone as a permeation enhancer. In other words, the temporal pattern of LAT release from the test formulations appears to be

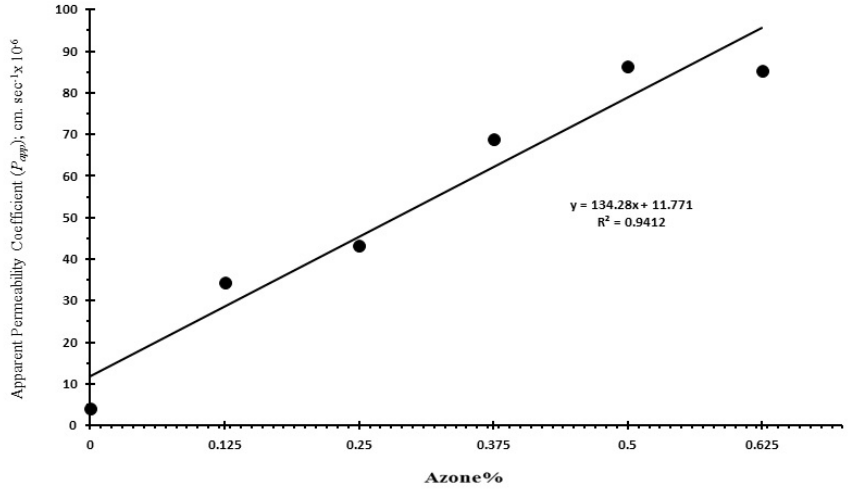

Figure 2 Relationship between the concentration of Azone as a transcorneal permeation enhancer and the *in vitro* apparent permeability coefficient of LAT ophthalmic gel formulations across freshly excised rabbit's cornea (n=3±SD).

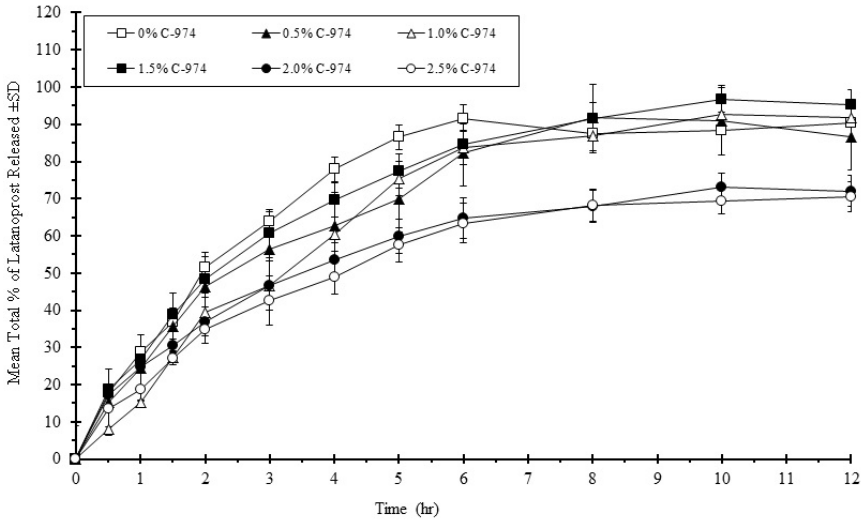

Figure 3 Mean total % of LAT delivered into the receiver chamber of an automated Franz diffusion system from LAT eye gels containing different concentrations of C-974® as a mucoadhesive and fixed concentration (0.5%) of Azone as a corneal penetration enhancer across freshly excised rabbit's cornea (n=3±SD).

a single-valued function of the concentration of Azone, i.e., concentration dependent. This could be related to, but not limited by, the assumption that the enhancement of drug permeation by Azone is due to its ability to reversibly increase 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 drug diffusiion and partitioning.

A different but related hypotehsis is that Azone evokes its effect as permeation enhancer by increasing the drug solubility and/or changing the ratio between ionized and unionized drug molecules in favor of the later (16, 36, 49). The enhancement factor (EF) was also found to be a function in the concentration of Azone. Accordingly, formulations GAZ-3, GAZ-4, GAZ-5 together with the reference standard Xalatan® 0.005% eye drops were selected for additional *in vivo* IOP lowering experiments.

When developing a medicinal drug product it is highly recommended that the lowest possible number of excipients is included at their lowest effective concentration. Therefore, a second set of LAT gel formulations containing a fixed concentration (0.5%) of Azone (lowest concentration that induced the highest permeability parameters in the first set of LAT ophthalmic

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 (n=3)

FORMULATION	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	1.00
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

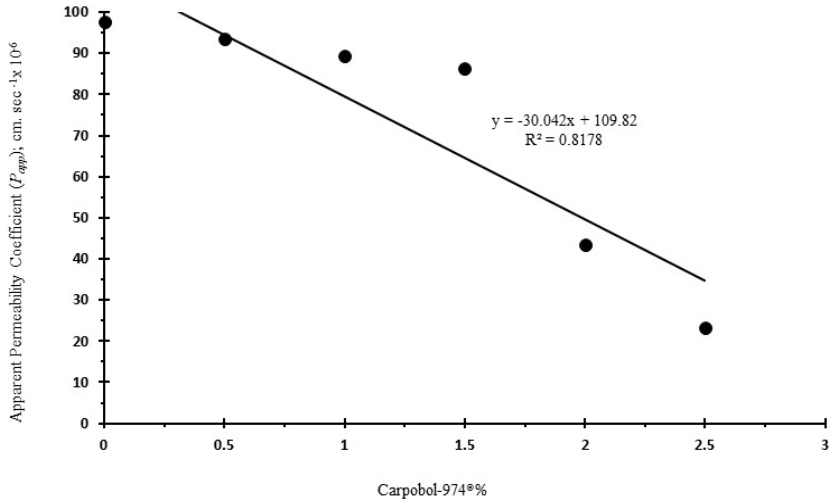

Figure 4 Relationship between the concentration of Carbopol® as a mucoadhesive and the *in vitro* apparent permeability coefficient of LAT ophthalmic gel formulations across freshly excised rabbit's cornea (n=3±SD).

gel formulation) as transcorneal penetration enhancer along with different concentrations of C-974® as a mucoadhesive and viscosity improving agent (crosslinker/thickener) were prepared. Formulation GC-0 $_{Control}$ did not contain C-974® (simple eye drops) and served 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 in hours. Table 4 shows the calculated permeability parameters for the second set of LAT formulations including, mean steady-state flux (J_{ss}), µg.cm^{-2}.sec^{-1}, *IOP* P_{app}, and the enhancement factor (EF) calculated as $P_{app\text{-}Tests}/P_{app}$-$_{control}$. The data in Table 4 also show that the transport characteristics of LAT through excised fresh corneal membrane decreased significantly ($p<0.01$) with increased concentrations of C-974®.

Figure 4 shows that the apparent permeability coefficient remains constant up to a Carbopol concentration of 1.5% and then decreases significantly. 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. This is complicated by the fact that the 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 intermolecular attractive forces (if exists) and to

Table 4 Effects of different concentrations of C-974® on the permeability parameters of LAT formulations containing fixed concentration 0.5% Azone as penetration enhancer through freshly excised rabbits corneal membrane (n=3).

FORMULATION	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

the portioning of the drug from that vehicle to the absorbing surface (50). However, the viscosity of the formulation does affect the drug diffusion. Formulations GC-3 (identical to formulation GAZ-4) and GC-4 showed significantly ($p>0.01$) higher permeability parameters than formulation GC-5. The *in vitro* corneal permeability parameters of formulations GC-$0_{Control}$, GC-1 and GC-2 released LAT at a faster rate than the test formulations of the second set, particularly during the first ~6, 8 and 10 hours, (depending on the C-974® concentration). Formulation GC-$0_{Control}$ showed the fastest onset and the shortest duration of action at ~18-20 hours, which is shorter than, but comparable to, that observed with the reference standard Xalatan® up to ~21-22 hours. Moreover, the formulations GC-1, GC-2, GC-3 and GC-4 showed thixotropic phenomena, i.e., non-Newtonian fluids/semisolid feature in which viscosity decreases with the shearing time, and the subsequently recovers after cessation of shearing (51, 52) (unpublished data). These formulations were scaled up for further *in vivo* IOP lowering studies.

In vivo IOP measurements

In vivo IOP lowering effects of LAT ophthalmic gels

The average IOP base line of the normotensive rabbit (23±2 mmHg) was measured and recorded prior to the administration of each dose. Figure 5 shows the ΔIOP for the scaled up ophthalmic gel formulations of the first set, i.e., GAZ-3, GAZ-4 and GAZ-5 together with ΔIOP for LAT commercial ophthalmic solution (Xalatan®) applied topically once a day for four successive days. The maximum ΔIOP measurements for the tested formulations GAZ-3, GAZ-4, GAZ-5 and 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 action were achieved within the time range of 1.5-3.5 hours, in direct correlation with the concentrations of corneal penetration enhancer (Azone), as well as, the permeability parameters. Formulation GAZ-3

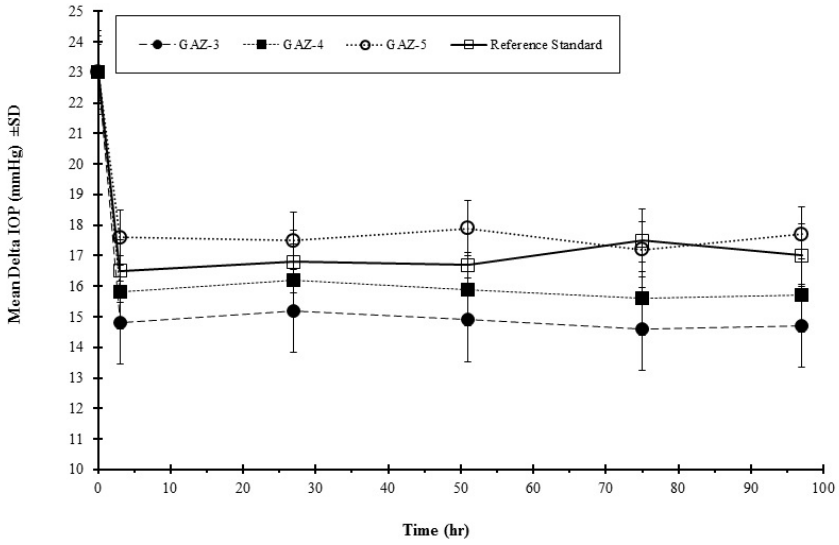

Figure 5 Mean IOP lowering effects expressed as the difference from the base line (23+3.0 mmHg) for scaled up LAT ophthalmic gels containing different concentrations of Azone as a corneal penetration enhancer compared to the reference standard (Xalatan®) in normotensive New Zealand Rabbits (n=3±SD).

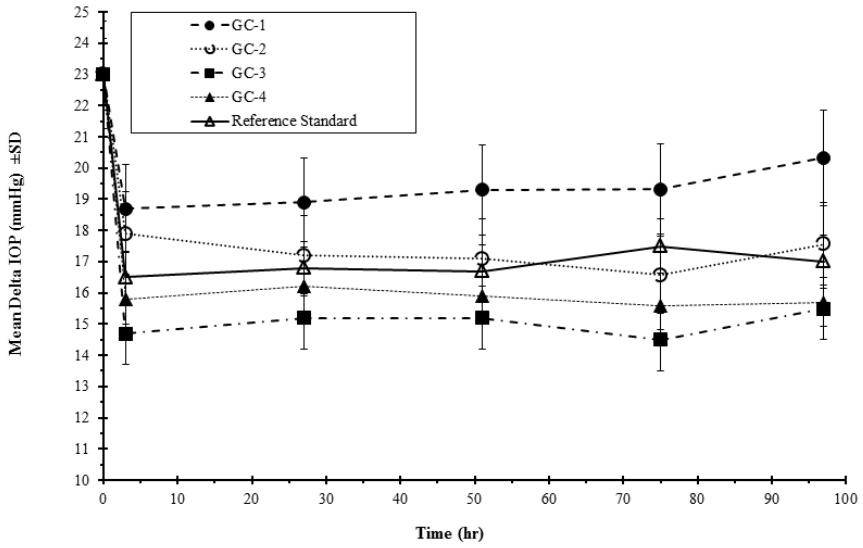

Figure 6 Mean IOP lowering effects expressed as the difference from the base line (23+2.0 mmHg) for rationally scaled-up LAT ophthalmic gels containing different concentrations of C-974® as a mucoadhesive compared to the reference standard (Xalatan®) in normotensive New Zealand Rabbits (n=3±SD).

containing 0.5% of Azone showed comparable onset of action but significantly higher ΔIOP than those with formulation GAZ-5 containing 0.625.0% and with the commercial reference standard Xalatan®. The assumption that a more compact gel network assembly and/or complex could be formed with higher concentrations of the mucoadhesive, crosslinker C-974® could be a reasonable explanation for these results. This might explain 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 the administration of the test gel formulation or one of its ingredients was assessed in New Zealand albino rabbits according to OECD, 2002 protocol (48). Upon inspection, no signs of ophthalmic irritation (i.e., tearing, redness, inflammation, and/or swelling) were recorded with the test gel formulation or any of its ingredients at the used concentrations during the 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 second 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 to 4 hours. Such a wide range of the onset of action is likely because this set of formulations encompasses formulations that contain different concentrations of the C-974® mucoadhesive (0-2.5%). The duration of action was a function of the mucoadhesive concentration. Concentrations of C-974® greater than 1.5% significantly ($p>0.05$) extended the duration of action, but reduced the efficacy due to delaying the onset of action. The presence of C-974® as mucoadhesive functions to increase the contact period with the ocular absorbing surfaces, i.e., providing more time for the drug to be delivered, while the addition of Azone to the formulation increases the permeability with a resultant increase in the

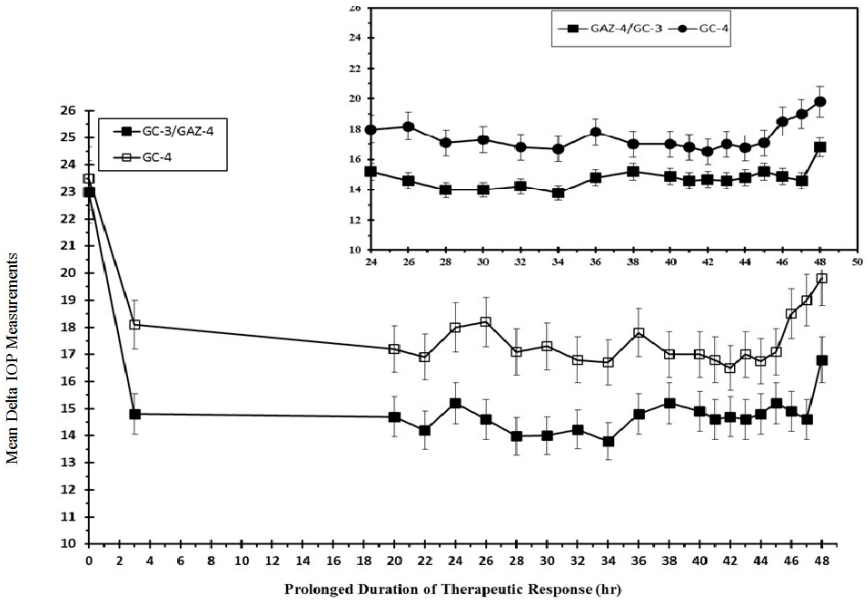

Figure 7 Mean extended duration of action of the two scale-up LAT ophthalmic gel formulations (i.e., GC-3/GAZ-4 and GC-4) in normotensive New Zealand Rabbits (n=3±SD).

ΔIOP. Evidently, the *in vitro* release and the *in vivo* pharmacodynamics for both sets of LAT ophthalmic gel formulations is largely dependent on the combined effect of these two formulation ingredients.

Extended *in vivo* IOP lowering effects of selected LAT-gel formulations

The IOP for each animal was recorded at the end of each day throughout the previously described *in vivo* experiments. Formulations GAZ-4/GC-3 and GC-4 showed significant IOP lowering effects, a particularly noteworthy point being that both formulations sustained the IOP lowering effect past 24 hours. Figure 7 shows that formulations designated GAZ-4/GC-3 and GC-4, induced significantly higher therapeutic IOP lowering effects than that of the reference standard Xalatan®, i.e., 7.8±1.8 mmHg and 6.5±2.1 mmHg respectively. In Figure 7 the *in vivo* ΔIOP lowering effects that were recorded after the first 3 and 24 hours, then each two-hours until the 40th hour, and finally each subsequent hour until the IOP reestablished the base line (23±2 mmHg) 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 lasted for 47±2.25 hours, and 48±1.5 hours, respectively. The IOP lowering effects for the two ophthalmic gel formulations remained essentially unchanged during the duration of the action. This relatively high steady magnitude of the IOP lowering effect 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 molecular-dimensions micro-viscosities. The presence of these channels could help increase the initial release rate, as well as, the *in vivo* IOP lowering effects of LAT-C-974® containing gels (29, 52, 53).

In vitro release experiments are usually designed to maintain the drug formulation in constant, direct contact with the corneal epithelium layer throughout the entire experiment. However, this is not true for *in vivo* experiments or the

management of patients with glaucoma, where the ocular therapeutic efficacy of an applied dose is affected (negatively or positively) by the unique physiological and anatomical constraints of the eye in addition to the physicochemical properties of the drug formulation. The results of this study suggest that, for an ocular drug delivery system, the correlation between *in vitro* release data and the *in vivo* efficacy is complex and controlled by a number of inter-related and in some cases contradictory factors that should be taken into consideration prior to developing ocular drug delivery systems, as well as, extrapolating or generalizing the *in vitro* studies outcomes to the *in vivo* clinical situation.

In conclusion, the *in vitro* corneal drug transport, onset of action, augmenting IOP lowering effect and increasing the extent of LAT therapeutic efficacy for the test formulations largely depend upon the net outcomes of the interaction between (a) the prolonged residence time of LAT in conjunctival cavity caused by the mucoadhesive and crosslinker C-974®, (b) the accelerated drug transport by the penetration enhancer (Azone), (c) the reduced diffusivity of the drug throughout the vehicles of gel formulations resulting from the increased viscosity by the thickener, (d) the rheological and physicochemical characteristics of the formulation and drug, in addition to (e) the inherent unique physiological and anatomical constraints of the eye represent further vital element that constrains successful development of ocular delivery systems. As this study shows, improving the delivery of drugs to ocular tissues is a non-trivial exercise that involves measuring, understanding and universalizing the mechanisms of pharmacodynamics and drug targeting. Although significant challenges remain regarding the deconvolution of a plethora of effects in the delivery of drugs to the eye, the problem is not unsurmountable.

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