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Influence of the Interplay between Azone™ as Permeation Enhancer a and Carbopol-974® as a Mucoadhesive upon the *in vitro* Transcorneal Release and the *in vivo* Antiglaucoma Effect of S-Timolol Maleate Ophthalmic Gel Formulations

Mohsen I. Afouna[1]*, Amal K. Hussein[2] and Osama A.Ahmed[3]

[1]Department of Pharmaceutics & Pharmaceutical Technology, College of Pharmacy, Al-Azhar University, Nasr City, Cairo, Egypt.

[2]Department of Pharmaceutics & Pharmaceutical Technology, College of Pharmacy, Minea University, Minea, Egypt.

[3]Department of Pharmaceutics and Industrial Pharmacy, King Abdulaziz University, Jeddah, Saudi Arabia.

***Corres.author: mafouna@pharma.asu.edu.eg**
afounamohsen@yahoo.com

Abstract: The objectives of the current study were to maximize the *in vitro* transcorneal release, the IOP-lowering effect and, the duration of action, of S-Timolol Maleate (STM) ophthalmic formulations. **Method.** The *in vitro* transcorneal release of STM from a 1st set of gel formulations containing different concentrations of Azone™ with a fixed concentration of C-974® were evaluated. Formulation showed highest *in vitro* releasewith lowest concentration of Azone™ was selected for preparation of a 2nd set of ophthalmic formulations using different concentrations of C-974®. Then their*in vitro* permeabilities were assessed. Therefore, the ideal concentrations of both C-974®& Azone™ required for preparation of the best ophthalmic formulation(s) of STM gel(s) have been identified. Thereafter, the *in vivo* IOP-lowering efficacy studies for scaled-up formulations were determined using TONO-PEN™ AVIA tonometer with rabbits for 4-consecutive days. Finally, the most effective formulations were used for a single-dose study to assess the duration of theiractions. **Results.** Majority of tested formulations have showed significant but varied escalations in both, *in vitro* and *in vivo* results. Formulations STMC-2, (STMC-3/STMAZ-4) & STMC-4 showed the higher therapeutic efficacy than that of the reference standard. *Particularly noteworthy with these formulations the IOP base-line didn't reestablished after 24 hours*, and their durations of action in the single-dose study were36±2, 44±3 and 48±4, respectively. **Conclusion.** The *in vitro* release, onset, magnitude & duration of action of STM gels have been potentiated and extended for up to up to 2-days with three test formulations. In other words, the *in vivo* IOP-lowering effects of these formulations were approximately 3-4-time longer and in some cases higher than that of the conventional TM eye drops, and 1.5-2-fold longer and with some formulations (e.g., STMC-3/STMAZ-4) higher than that of the reference standard.

Keywords: Azone; Corneal transport; Ocular delivery; Ocular enhancers; Carbopol-974; Glaucoma; *S*-Timolol; Mucoadhesive.

Introduction:

Timolol maleate (TM) exists as two optically active isomers, S-TM and R-TM. TM has a molecular weight of 432.50. It is a white, odorless, crystalline powder which is soluble in water, methanol, and alcohol. TM is described chemically as (S)-1-[(1,1-dimethylethyl)amino]-3-[[4-(4-morpholinyl)-1,2,5-thiadiazol-3-yl]oxy]-2-propanol,(Z)-2-butenedioate. TM is a potent non-selective β-adrenoreceptor blocking agent has the action of reducing normal, as well as elevated intraocular pressure which is a major risk factor in the pathogenesis of glaucomatous visual field loss and optic nerve damage. It is marketed as the maleate salt of the levo (S-) isomer and is approved for the treatment of hypertension, myocardial infarction, angina, and glaucoma[1,2]. It was reported that R- TM has only 3% of the potency of S- TM in blocking the isoproterenol-induced synthesis of adenosine 3,3,5-monophosphate. The R-enantiomer of TM is 49 times less potent than S-enantiomer, with respect to β_2-adrenoceptor activity in animals, and 13 times less potent in constricting the airways of normal subjects. The R-isomer of TM has been found to be effective in lowering elevated intraocular pressure when applied topically to eye. Yet it is only four-times less potent in reducing intraocular pressure in man[3]. Comparison on the basis of potency, selectivity for β_1-versus β_2- receptor subtypes, partial agonist properties, and non-specific membrane-stabilizing effects shows that TM has the greatest receptor binding affinity than the other available β-blockers including propranolol, metoprolol, nadalol, atenolol, and timolol[4].

It has been reported that TM is about 8 times more potent than propranolol with respect to β-adrenoceptor blockade[5]. When administered orally, TM is well absorbed but it subjects to extensive first-pass metabolism[6]. The half-life in plasma is about 4 hours. Plasma concentrations of TM may become sufficiently high to block pulmonary and cardiac β-adrenergic receptors leading to asthma and congestive heart failure. Hence, for long-term prophylactic use, the maintenance dose should be properly titrated to avoid risks associated with β_2-receptor blockade and recurrence of myocardial infarction. The ability of our biological system to discriminate between two enantiomers of a compound was recognized in 1971[7]. Enantiomers are identical in their physical and chemical properties but behave differently in a chiral environment such as a biological system or a chiral medium. Enantiomers usually differ in the nature and degree of their pharmacological and toxicological properties[8]. For a particular pharmacological action, the more active isomer is called the eutomer and the less active is the distomer. The eudismic ratio (the ratio of activity), is an indication of the degree of stereoselectivity. Chemically and biologically, enantiomers must be considered as different compounds often with greater pharmacological activity than homologous agents[9]. The effect of chirality on the pharmacological behavior of a drug molecule is an interesting and active area in the field of drug design and drug delivery. The discovery that chiral drug molecules differ in their biological activities has been known for over 100 years. Since the initial observation of the existence of asparagine in two enantiomeric forms, numerous studies have been reported about the existence of stereoselectivity with enantiomeric drug substances[10]. Molecular chirality originates because of the existence of configurational isomers, termed enantiomers[11]. The sudden surge in interest in chirality is not only due to advancement in medical sciences per se, but also due to rapid progress in the techniques employed to separate individual enantiomers. It has become feasible to evaluate the biodistribution of enantiomeric drug molecules using chiral reagents, chiral stationary phases, chiral mobile phases, chiral catalysts, and chiral chromatographic separations[12]. For up to two- decades, quite a number of publications have focused on stereochemistry in drug action, metabolism, disposition, and bioequivalence[6,13,14]. Controversies arose as to whether a racemate or a single enantiomer needs to be exploited for therapy. Manipulation of enantiomeric ratio or use of only one enantiomer of a drug may allow minimization of toxicity and efficacy and this may lead to a significant increase in therapeutic ratio and a more rational approach to therapeutics[15]. The development of drugs with chiral centers presents specific challenges that must be addressed at various stages from discovery to clinical evaluation and finally to the market[17].

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[18]. Briefly, glaucoma is a progressive optic neuropathy affecting more than 70 million individuals worldwide and it represents a major cause for irreversible blindness[19]. 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. The main obstacles encountered with ocular drug delivery in a therapeutically effective concentration from ophthalmic deliver systems are, ***i)*** the very short average residence

time of the administered dose, particularly ophthalmic solutions[20,21], ***ii)*** the extensive pre-corneal loss because of the fast tear drainage ***iii)*** solvent evaporation and alteration of the thermodynamics of the drug, ***iv)*** the high possibility of excessive loss of drug through the nasolacrimal drainage that may cause systemic effects &/or side effects particularly, with potent drugs[22], ***v)*** the very limited accommodation capacity of the human eye; range of 10-to-30-▪ l for blinking & non-blinking eye respectively, and ***vi)*** the inherent physiological involuntary defense mechanism of the eye, e.g. blinking & tearing. Subsequently, the ocular bioefficacy of a topically applied ocular drug drops is dramatically very low, only 1-10%[23,24]. 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[25] with restricted/little or no difficulties. Approaches employed to bypass epithelium barrier include; ***a)*** using of a suitable viscosity improving agent (VIA) to prolong the contact-time of drug with the absorbing surfaces, and ***b)*** using of transcorneal penetration enhancer to expedite the 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[26,27]and leads to a measureable decline in the diffusion of drug molecules. Anther formulation-related approaches to maximize the therapeutic efficacy of ocular drugs include using of; ***a)*** extended release dosage form with water-soluble polymer[28,29], and ***b)*** preparation of lipophilic ion-pair from a drug and additives[30,31]. One of the very significant approaches to expedite the absorption of ocular drugs is the incorporation of an ocular penetration enhancer(s)[32-36].

Regardless of the occasional drawbacks of some enhancers such as irritation, morphological, and changes in the corneal membrane[37] an inert, safe, none allergenic non-irritant, expedite 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 (laurocapram) meets the aforementioned requirements of ideal enhancer to varied extents[38,39]. The corneal penetration of hydrophilic compounds (acetazolamide, cimetidine, guanethidine, and sulfacetamide) was enhanced by at least 20-fold at 0.1% Azone[40]. 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[38,41,42].

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[43,44]. Different Carbopol® grades are generally used as thickeners 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[45,46]. However, C-974®certain better mucoadhesiveness of Carbopol C-974® in comparison with the C-971®[47].

Regardless of pre-discussed technical, physiological and physic-chemical difficulties in emerging ophthalmic delivery system, the task of having therapeutically effective, extended, safe and stable ophthalmic formulations of hydrophilic drugs has been fast-growing & very-attractive research area. The present study is devoted to develop and characterize STM that are expedient with extended and possibly controlled drug release and improved therapeutic effects. Therefore, it was necessary to; ***i)*** design, prepare and quantitatively determine the *in vitro* permeability parameters of wide-varieties of STM gel formulations containing combinations between various concentrations Azone (as a transcorneal penetration enhancer), and of C-974® (as a mucoadhesive) and ***ii)*** to evaluate the onset of action of STM ocular formulations, *in vivo* IOP lowering effect and its magnitude.

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 local Institutional Review Board for Preclinical & Clinical Research 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) & stick to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised in 1985). All animals in this study were housed and cared for according to the guidelines from the Association for Research in Vision and Ophthalmology (ARVO).

Drug and Chemicals

S-Timolol maleate was kindly a generous gift from Merck Research Lab., Whitehouse, NJ. Azone (Laurocapram**)**, sodium octane sulfate, acetonitrile, acetonitrile, and benzalkonium chloride sodium hydroxide and phosphoric acid were obtained from Spectrum Chemical Co., Gardena, CA. Ethyl acetate, methanol, isopropyl alcohol, and ethyl alcohol were purchased from were purchased from Sigma Aldrich Chemical Co., St. Louis. NJ. Carpobol-974® (C-974®)was obtained Lubrizol Advanced Materials, Inc. 9911 Brecksville Road, Cleveland, Ohio. Sodium chloride and hydrochloric acid and sorbitol were provided by Fisher Scientific Co., Fair Lawn, NJ. Analytical grades of disodium edetate dihydrate (EDTA) were purchased from Merck (Germany). TIMOPTIC-XE® (timolol maleate ophthalmic gel forming solution), Laboratories Merck Sharp & Dohme-Chibret, Clermont-Ferrand, France was purchased from the market. All other chemicals used in this study were commercially available compounds of special reagent or analytical/HPLC-grade and they were used as received without further modifications.

Equipment

An PermeGear Flow Type modified Franz diffusion system of vertical cells, PermeGear, Inc., Hellertown, PA USA. Hewlett Packard autosampler HPLC system with chime-station, variable wave length UV detector was obtained from Agilent Technology, SL, MI. HPLC chiral column-AGP 100 x 4.0 mm, 5· m, was obtained from ChromTech, ChromTech International AB, Hägersten, Sweden. Thermostatically controlled water bath, vortex/shaker sonicator, hot-plate/magnetic stirrer, pH meter, positive displacement pipettes & tips, were obtained from Fisher Scientific Co., Fair Lawn, NJ. P1803. A digital TONO-PEN™ AVIA tonometer®, Reichert, Inc., NY, Millipore filter paper, (0.45· m, HA), were purchased from Millipore corporation, Bedford, MA.

HPLC Assay of STM

HPLC procedures were employed for analysis of the samples using variable wave length UV at 294 nm wavelength. The column was maintained at 5 °C using a built-in temperature control system. The mobile phase consisted of (ethyl acetate: methanol: isopropyl alcohol: 25% ammonia, in ratios of (80: 20: 2: 1, v/v/v/v). The mobile phase was filtered under vacuum using a 0.45· m filter and degassed using a sonicator. Milli-Q water (ultra-pure de-ionized distilled water) has been used for preparation of mobile phase and throughout the assay procedures. The flow rate of the mobile phase of 1-ml/min throughout the analysis procedures has been maintained. The samples of STM were collected in dark vials and analyzed for their drug contents, and the STM peak has been detected between 10-12-minutes.

Preparation of STM Ophthalmic Gel Formulations

Compositions of two sets of test ophthalmic gel formulations are shown in Tables (1 & 2). Table 1 shows the composition of the 1st set of STM ophthalmic gel formulations that have been prepared with various concentrations of Azone (0.0, 0.0625, 0.125, 0.25, 0.375 and 0.5%), and with a fixed concentration (1.5%) of C-974® as a mucoadhesive to identify the lowest concentration of Azone inducing the highest penetration enhancement upon through analyses of the results of the in *vitro* permeability data. On the other hand, Table 2 shows the composition of the 2nd set of STM ophthalmic gel formulations that have been prepared with a fixed concentration of Azone (0.25%) of Azone; i.e., concentration induced the highest penetration enhancement, steady and prolonged STM release, and with various concentration of C-974® (0.0, 0.5, 0.1, 1.5, 2.0, & 3.0%) to

identify the minimum concentration of C-974® that possess pharmaceutically acceptable STM gel formulation(s). The formulations have been prepared, processed and mixed in accordance to Lubrizol Advanced Materials, Inc.; Technical Data Sheet[48]. The tonicities of the gel formulations were adjusted with sorbitol and the final pH values of all formulations were adjusted to be ~7 (i.e.,the pH value of the commercially available STM; ophthalmic gel forming solution,Laboratories Merck Sharp & Dohme-Chibret, Clermont-Ferrand, France. All formulations were prepared with sterile ingredients and mixed under aseptic conditions. The *in vitro* transcorneal permeability parameters for each formulation were measured and calculated to pinpoint the optimum concentration of both, Azone and C-974®.

In Vitro Corneal Permeability Studies

The factors influencing the penetration of a drug into the skin include: concentration of the dissolved drug, (C_s) in the donor compartment, partition coefficient (K) between the skin and the vehicle, and the diffusion coefficients of the vehicle and the skin(P). The flux (J), i.e., the amount(M), of a drug permeated through a membrane of unit cross-section area (S)in unit time (t) was calculated in accordance to Fick's law:

$$J = \frac{dM}{S} \times dt$$

If a membrane of surface area (*S*) and thickness (h)separates donor and receptor compartments and if the concentrations in the donor and receptor compartments are (C_1) and(C_2), then Fick's law can be rewritten as:

$$J = \frac{dM}{S} \times dt = \frac{D\,(C_1 - C_2)}{h}$$

Under steady-state conditions $(C_1)$is much greater than (C_2) and thus,

$$J = \frac{DC_1}{h}$$

When the cumulative amount of the drug permeated per unit cross-sectional area is plotted with time, the slope of the linear portion of the graph is steady-state flux, from which permeability coefficient can be determined employing the suitable mathematical treatment and Fick's law:

$$J = PSC_1$$

Where (P) is the permeability coefficient, and (C_1) is the donor cell concentration. The enhancement factors (EF) were calculated by dividing of the steady-state flux value (µg.cm^{-2}.hr^{-1}), of the test formulation over the corresponding value for the control. The statistical analyses were done by *t*-test. The animals were sacrificed by administering an overdose of a sodium pentobarbitone solution via the marginal ear vein. Then the corneas were meticulously excised, rinsed with normal saline, and gentlymountedwith its epithelial surface facing the donor chamber using a small pinch clip over a receiver chamber filled with the receiver fluid and stirred gently with appropriate magnetic stirrers (about 600 rpm). The permeation assembly was carried out using an automated modified Franz transcorneal diffusion system employing finite dose (50-µl) 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[36,49,50]. Thereafter, the STM permeability parameters for each test formulation were mathematically calculated employing the equation shown below. All the experiments were conducted in triplicates at 34°C. All samples were analyzed for STM using HPLC methods stated above. 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. During the permeation study, samples have been analyzed at the first and last time points for STM contents and have showed no chiral inversion. All experiments were performed in triplicate. The results of experiments performed 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 (ANOVA) using SPSS software, (SPSS Inc., Chicago, IL, USA) and the criterion for statistical significance was $p< 0.05$.

In vivo IOP Measurement

IOP lowering effects of the Scaled up STM-gel formulations

The *in vivo* studies were performed on normotensive conscious New Zealand albino rabbits weighing 3.0-4.0 kg. The animals were handled and housed, hospitalized and acclimatized at controlled temperature, with a 12/12-hr light/dark to simulate 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 (10-rabbits/group). A dose of 50-µl from each test formulation and the reference standard (Timoptic-XE®; 0.25 TM ophthalmic gel forming solution manufactured by MERCK & CO., Inc. Whitehouse Station, NJ,) was administered onto the conjunctival cavity using a positive displacing pipette once a day to each individual rabbit/group for 4-successive days. Both right and left eye were dosed separately. For IOP measurements, TONO-PEN™ AVIA tonometer (Reichert Inc., Depew, NY, USA), was used to measure the baseline (23±2) as well as the *in vivo* IOP lowering effects after predetermined time intervals after of dosage instillation; i.e., after 1.5, 12,. Thereafter, the IOP measurements were recorded each 4-hour till next dosage (24-hr). Acute eye irritation and ocular irritation potential of the gel formulations were examined in accordance to the Draize Test is an acute toxicity test[51].

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

As it has been indicated in the previous experiments, the IOP-lowering effects of each formula were recorded at the end of each day. Formulations in which the IOP base-line were not reestablished were subjected for further single-dose study to identify their magnitude and extent of actions; i.e., from the time of dosing till reestablishing the IOP base-line. The experimental animals were randomly divided into equal groups (10-rabbits/group). The animals were handled and housed, hospitalized exactly as stated above. Then the animals were treated as described above with a single-dose of each of the test formulation. The IOP lowering effects have been recorded at appropriate time intervals until reestablishing the IOP base-line irrespective of how long it might take.

Results and Discussion

In Vitro Corneal Permeability of STM-Gel Formulations

The first set of STM 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 (Table-1). Figure-1 shows the mean ± SD cumulative amounts of STM (µg/ml) released from the test formulations into the receiver compartment of the diffusion cell as a function of time(n=3). Table 3 shows the calculated permeability parameters for the first set of STM 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. Results in Table 3 revealed that the transport characteristics of STM through excised fresh corneal membrane significantly ($p<0.01$) increased with the increased concentration of Azoneup to concentration 0.5%. Figure 2 reveals a relatively fair-linear (R= 0.7821) and direct relationship between the

apparent permeability coefficient (P_{app}; cm.sec^{-1}) for this set of STM ophthalmic gel formulations and the % of the added permeation enhancer (Azone). In other words, the temporal pattern of STM release from the test formulations appears to be a single-valued function of the concentration of Azone; i.e.,concentration dependent. Moreover, the enhancements factor (EF) of was also found to be a function in the concentration of Azone. 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 diminishes 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]. Accordingly formulations STMAZ-3, STMAZ-4, STMAZ-5 along with the reference standard (Timoptic-XE®; 0.25 TM ophthalmic gel forming solution) were selected for additional *in vivo* IOP lowering effect experiments.

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 STM gel formulations containing a fixed concentration (0.25%) of Azone (lowest concentration that induced the highest permeability parameters in the 1st set of STM ophthalmic gel formulation) as transcorneal penetration enhancer along with different concentrations of C-974® as a mucoadhesive & viscosity improving agent (crosslinker/thickener) were carefully prepared. Formulation STMC-0$_{Control}$ was void of C-974® (a simple eye drops) to serve as a negative control (Table-2). Figure-3 shows the mean ± SD cumulative amounts of STM (μg/ml) of total STM released from the test formulations into the receiver compartment of the diffusion cell as a function of time (n=3). Table 4 shows the calculated permeability parameters for the 2nd set of STM formulations. The data in Table 4 also revealed that the transport characteristics of STM through excised fresh corneal membrane significantly ($p<0.01$) decreased with the increased concentration of C-974®. Figure 4 reveals almost-linear (R= 0.8223) but reversible relationship between the apparent permeability coefficient (P_{app}; cm.sec^{-1}) for this set of STM ophthalmic gel formulations and the % of the added C-974® as a mucoadhesive. In other terms, the viscosity of the formulation adversely affected the drug release, diffusivity and partitioning. 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 a 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]. Formulations STMC-3 (which is identical in composition with formulation STMAZ-4) and STMC-4 have shown significantly ($p>0.01$) higher permeability parameters than formulation STMC-5. Meanwhile, the *in vitro* corneal permeability parameters of formulations STMC-0$_{Control}$ (void of C-974®; eye drops), STMC-1 (containing 0.5% C-974®) and STMC-2 (containing 1.0% C-974®) have shown STM release faster than those of the rest of all test formulations of the 2nd set, particularly during the first 8 hr, depending upon the C-974® concentration. Moreover, formulations designated STMC-1, STMC-2, STMC-3 & STMC-4 have shown measureable 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, elegancy, and recovery time (unpublished data). Therefore, these formulations have been scaled up for further the *in vivo* IOP lowering effects.

Table (1): Composition of the 1st Set of STM Test Ophthalmic Gel Formulations Containing Different Concentrations of Azone as Transcorneal Enhancerand a Fixed Concentration (1.5%) of C-974® as Mucoadhesive & Thickener

Formulation Code	STM (mg/ml) 0.25%	Azone (%)	C-974® (%)	EDTA (%)	BENZ-Cl (%)
*STMAZ-$0_{Control}$	0.0	0.0	1.5	0.1	0.03
*STMAZ-1	2.5	0.0625	1.5	0.1	0.03
*STMAZ-2	2.5	0.125	1.5	0.1	0.03
*STMAZ-3	2.5	0.25	1.5	0.1	0.03
*STMAZ-4	2.5	0.375	1.5	0.1	0.03
*STMAZ-5	2.5	0.5	1.5	0.1	0.03

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

ΔTimolol maleate 3.4 mg/ml ≡ Timolol 2.5 mg/ml.

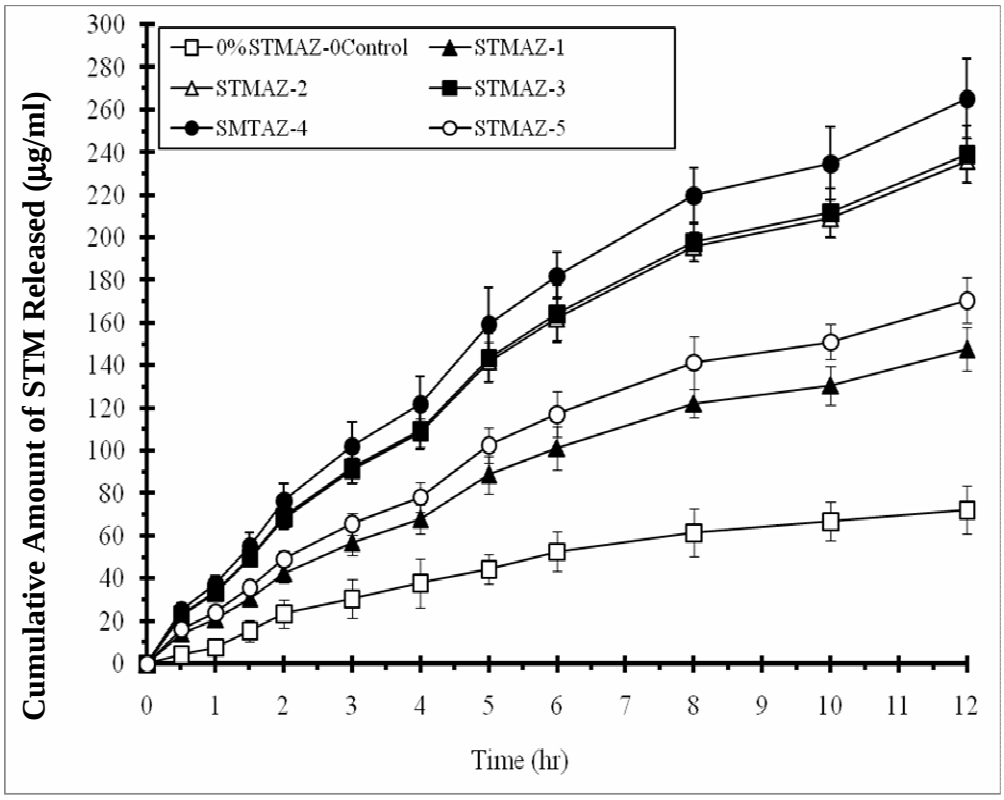

Figure (1): Cumulative amounts of STM(µg/ml) delivered across fresh excised rabbit's cornea into the receiver chamber of a modified Franz diffusion system from STM ophthalmic gel formulations designated STMAZ-$0_{Control}$, STMAZ-1, STMAZ-2,STMAZ-3,STMAZ-4,&STMAZ-5,containing different concentrations; 0.0, 0.0625, 0.125, 0.375, and 0.5% of Azone (penetration enhancer),respectively with a fixed concentration (1.5%) of C-974® as a mucoadhesive & thickener (n=3).

Table (2): Composition of the 2nd set of STM Test Ophthalmic Gel Formulations Containing Different Concentrations of C-974® as Mucoadhesive & Thickener and a Fixed Concentration (0.25%) of Azone as Transcorneal Enhancer

Formulation Code	STM (mg/ml) 0.25%	Azone$ (%)	C-974® (%)	EDTA (%)	BENZ-Cl (%)
°STMC-$0_{Control}$	0.0	0.25	0.0	0.1	0.03
*STMC-1	2.5	0.25	0.5	0.1	0.03
*STMC-2	2.5	0.25	1.0	0.1	0.03
$*STMC-3=(STMAZ-3)	2.5	0.25	1.5	0.1	0.03
*STMC-4	2.5	0.25	2.0	0.1	0.03
*STMC-5	2.5	0.25	3.0	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.

ΔTimolol maleate 3.4 mg/ml ≡ Timolol 2.5 mg/ml.

$It is the lowest concentration of Azone that induced the highest *in vitro* permeability parameters.

$Composition of formulations designated STMAZ-3 & STMC-3 are identical (i.e.; One Formulation).

Table (3): Effects of Different Concentrations of Azone upon the Permeability Parameters of STM Formulations Containing Fixed Concentration 1.5% C-974® as Mucoadhesive & Thickener across Freshly Excised Rabbits Corneal Membrane

Formulation Code	Azone (%)	Mean Steady-State Flux (J_{ss}) ($\mu g.cm^{-2}.sec^{-1}$)	Apparent Permeability Coefficient ($cm.s^{-1}$) x 10^{-6}	Log P_{app}	(EF) $P_{app\ Test}/P_{app\text{-}control}$
*STMAZ-$0_{Control}$	0.000	9.12±1.12	7.31	-4.14	1
*STMAZ-1	0.0625	43.10±3.68	41.45	-3.38	5.67
*STMAZ-2	0.125	118.37±5.37	73.29	-3.20	10.03
*STMAZ-3	0.250	187.80±4.64	89.93	-3.14	12.30
*STMAZ-4	0.375	179.53±4.55	88.95	-3.05	12.17
*STMAZ-5	0.500	172.17±5.07	86.15	-3.07	11.78

Table (4): Effects of Different Concentrations of C-974® upon the Permeability Parameters of STM Formulations Containing Fixed Concentration (0.25%) of Azone as Penetration Enhancer across Freshly Excised Rabbits Corneal Membrane

Formulation Code	C-974® (%)	Mean Steady-State Flux (J_{ss}) ($\mu g.cm^{-2}.sec^{-1}$)	Apparent Permeability Coefficient ($cm.s^{-1}$) x 10^{-6}	Log P_{app}	(EF) $P_{app\ Test}/P_{app\text{-control}}$
°STMC-$0_{Control}$	0.0	179.12±4.49	98.26	-3.01	1.00
*STMC-1	0.5	166.10±7.65	87.32	-3.06	0.89
*STMC-2	1.0	168.37±4.87	88.63	-3.05	0.90
$*STMC-3=(STMAZ-3)	1.5	187.80±4.64	89.93	-3.14	1.00
*STMC-4	2.0	123.10±7.81	73.59	-3.13	0.75
*STMC-5	3.0	105.37±11.09	66.11	-3.18	0.67

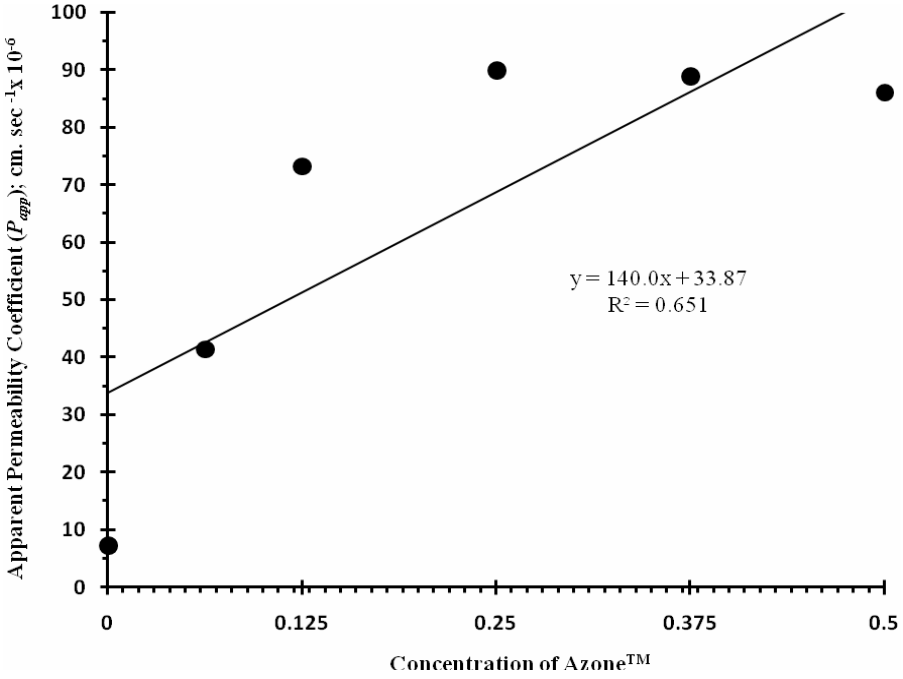

Figure (2): Relationship between the concentration of Azone™ as a transcorneal permeation enhancer and the *in vitro* apparent permeability coefficient of STM ophthalmic gel formulations across freshly excised rabbit's cornea (n=3±SD).

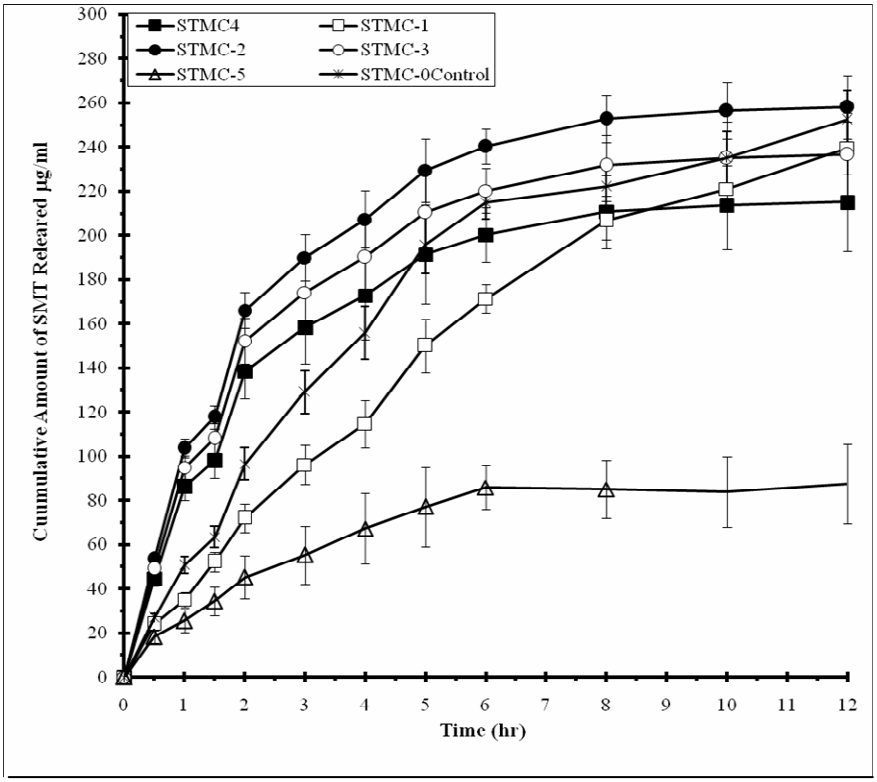

Figure (3): Cumulative amounts of STM (µg/ml) delivered across fresh excised rabbit's cornea into the receiver chamber of a modified Franz diffusion system from STM ophthalmic gel formulations designated STMC-$0_{Control}$, STMC-1, STMC-2,STMC-3,STMC-4,&STMC-5,containing different concentrations; 0.0, 0.5, 1.0, 1.5, 2.0 and 3.0% of C-974®(mucoadhesive), respectively with a fixed concentration (0.25%)of Azone as a penetration enhancer (n=3).

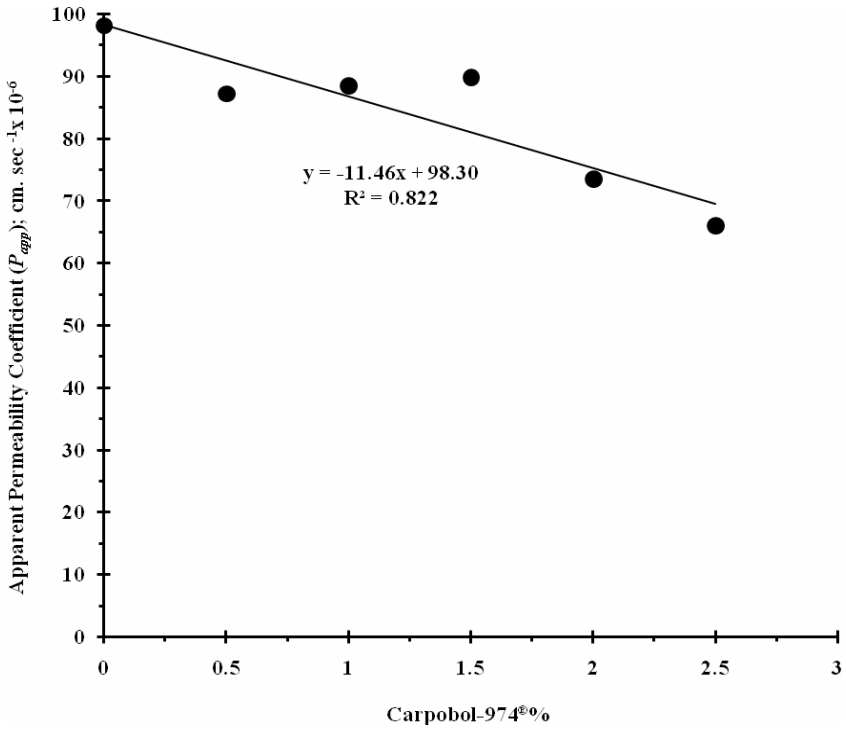

Figure (4): Relationship between the concentration of Carbopol® as a mucoadhesive and the *in vitro* apparent permeability coefficient of STM ophthalmic gel formulations across freshly excised rabbit's cornea (n=3±SD).

In vivo IOP Measurements.

In vivo IOP Lowering Effects of STM Ophthalmic Gels.

As it has been illustrated the current study was conducted to examine the interplay between 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 scaled up ophthalmic gel formulations of the first set; i.e., STMC-3, STMC-4 and STMC-5 along with ΔIOP for the reference standard (Timoptic-XE®; 0.25 TM ophthalmic gel forming solution) applied topically once/day for four successive days. The maximum ΔIOP measurements for the tested formulations; STMC-3, STMC-4, STMC-5 &Timoptic-XE®; 0.25 TM ophthalmic gel forming solution were (8.5±2.5), (6.5±1.5), (5.8±2.0), and (7.0±2.5) mmHg, respectively. 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 STM release from and diffusion though such compacted gel[53]. 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). The higher of Azone concentration the shorter of the onset time up to 0.5%. Nonetheless, formulation STMAZ-3 and STMAZ-4 containing 0.25% and 0.375% of Azone has shown comparable onset, duration of action and ΔIOP with these of formulation STMAZ-5 containing 0.5% and the reference standard.

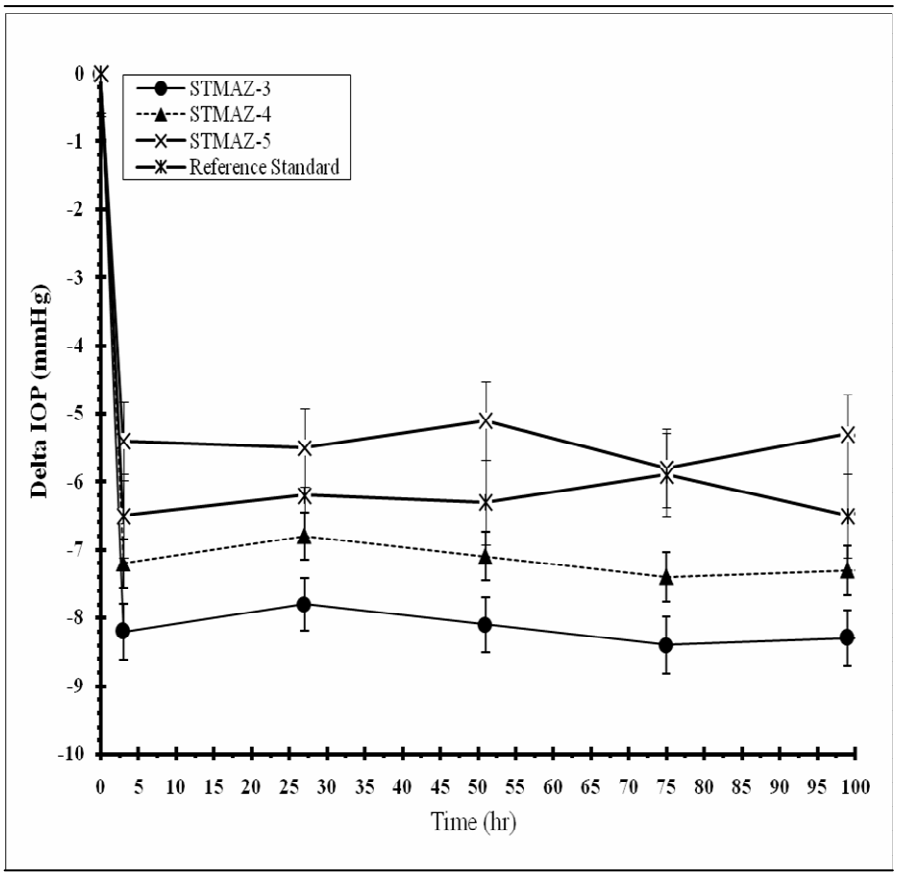

Figure (5): The IOP lowering effects expressed as the difference from the base line (23+0.2 mmHg) for STM ophthalmic gels containing different concentration of Azone™ as a transcorneal permeation enhancer compared to that of the reference standard.

Figure (6) shows the *in vivo*ΔIOP measurements for the scaled up ophthalmic formulations designatedSTMC-$0_{Control}$ (eye drops), STMC-1, STMC-2, (STMC-3/STMAZ-4), STMC-4 of the 2nd set, and the reference standard (Timoptic-XE®; 0.25 TM ophthalmic gel forming solution). The maximum ΔIOP measurements for the formulations of this set were (6.0±2.0), (7.0±2.5), (8.5±2.5), (5.5±1.8), (4.5±1.5) and (7.0±2.5) mmHg, respectively. The onset of action range for these formulations was 0.5-4.5 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 (0-3.0%). 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. Furthermore, the obtained ΔIOP results proves 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 into account, the role of the added penetration enhancer (0.25%) 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 duration of ΔIOP. Evidently, the *in vitro* release and the *in vivo* pharmacodynamics for both sets of STM ophthalmic gel formulations is largely depends upon the combined effect of two of the vital 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 impacts for different extents to the pharmacotherapy as well as to the overall outcomes in developing an ocular drug delivery system.

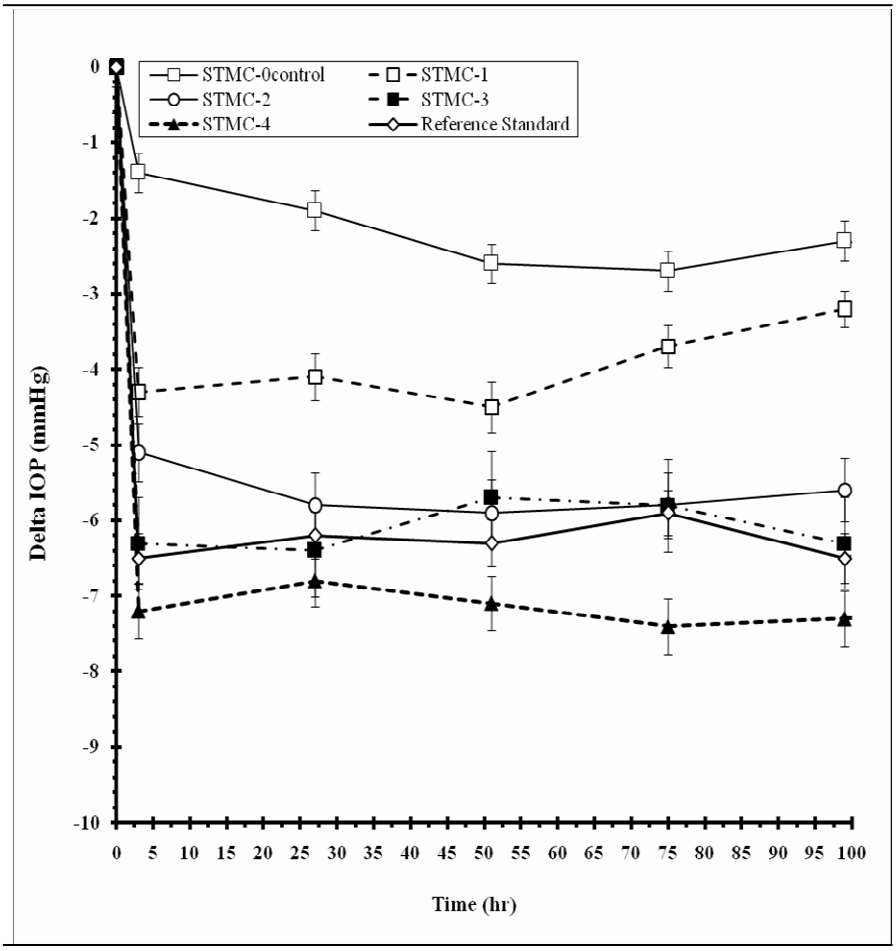

Figure (6): The IOP lowering effects expressed as the difference from the base line (23+0.2 mmHg) for STM ophthalmic gels containing different concentration of C-974® as a mucoadhesive compared to that of the reference standard.

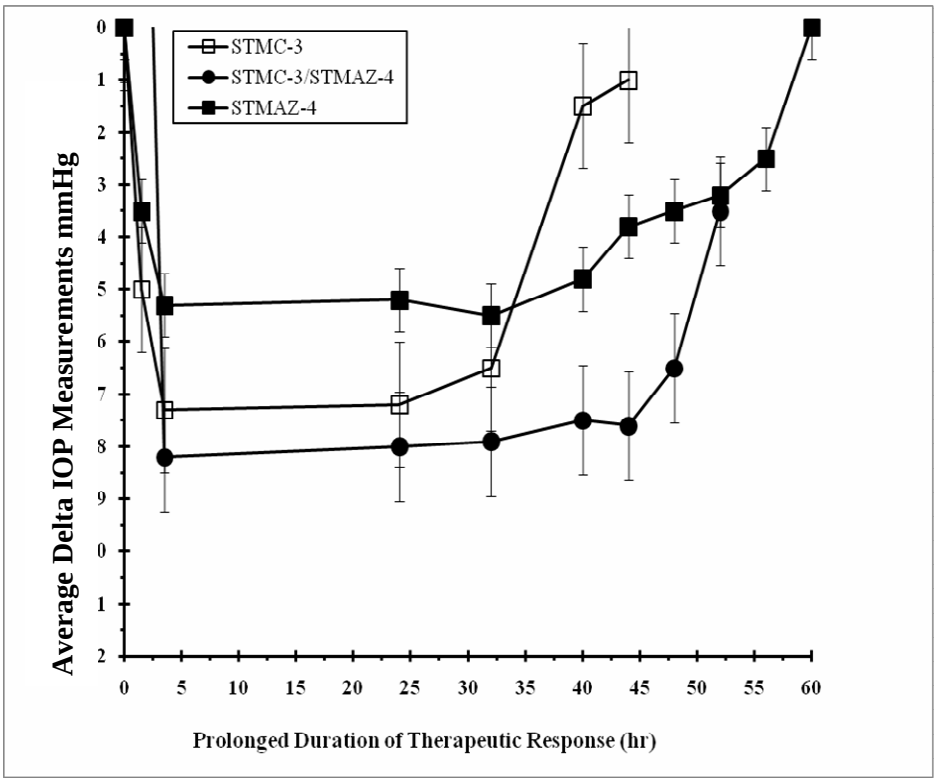

Figure (7): Prolonged duration of action of STMC-2, STMC-3/STMAZ-4, and STMC-4.gel formulations

Extended In vivo IOP lowering effects of scaled up STM-gel formulations

The IOP for each animal has been recorded at the end of each day throughout the previous *in vivo* experiments.Formulations STMC-2, (STMC-3/STMAZ-4), and STMC-4 have showed very promising IOP lowering efficacies, *particularly noteworthy treatment with these formulations, the IOP base-line didn't re-establish after 24 hours*. Therefore, the goal of this study was to examine the hypothesis that the ocular administration of these formulations would be of longer duration of action than those of the rest of tested formulations. Obviously, Figure (7) shows that formulations designated STMC-2, (STMC-3/STMAZ-4), and STMC-4have, have shown significantly comparable potency but significantly longer duration of action therapeutic IOP lowering effects than that of the reference standard (Timoptic-XE®; 0.25 TM ophthalmic gel forming solution. In figure (7) the *in vivo*ΔIOP lowering effects that have been recorded after the first 3 & 24-hr, then each 8-hours till the 40th hr, and finally every 4-hour until the IOP re-establishing the base line (23 ±2) to determine the duration of actions of the each test formulation. The average durations of action for formulations STMC-2, (STMC-3/STMAZ-4), and STMC-4 were 36±2, 44±3 and 48±4, respectively. In other words, the *in vivo* IOP-lowering effects of these formulations were approximately 3-4-time longer and in some cases higher than that of the regular TM eye drops, and 1.5-2-fold longer and with some formulations (STMC-3/STMAZ-4) higher than that of the reference standard Timoptic-XE®; 0.25 TM ophthalmic gel forming solution).The IOP lowering effects for the test 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 STM-C-974® containing gels[29,54-59]. 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 should 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 case in the *in vivo* experiment or in management of patient with glaucoma, where the ocular therapeutic efficacy of an applied dose affected (negatively or positively) by such variables in addition to the physiological, physic-chemical properties of the drug and the formulations vehicles. Regardless of all kinds of co-/lack of--relation and its extent 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[40,60,61]. 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. 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. These results are of great importance and in further substantiate the previous findings of XU et al.,[62] where they have pointed out no clinical and histopathological evidence of ocular toxicity occurred in all Azone-treated and control eyes. The concentration of Azone used in their report was up to 0.9% (i.e., 4-times higher than that at which the maximum *in vitro* transcorneal permeation as well as the highestand the *in vivo* efficacieshave been achievedin the current study). Therefore, Azone can be used as a unique ocular permeation enhancer in ophthalmic delivery systems to increase therapeutic efficacy[62,63].

In conclusion, the *in vitro* corneal drug transport, onset of action, prolonging IOP lowering effect, increasing the magnitude and/or intensity of therapeutic efficacy and the overall success in development of ocular delivery systems essentially depend upon the net outcomes of the interplay between; ***1)***the sustained residence time of STM in conjunctival sac caused by the mucoadhesive/crosslinker (C-974®), ***2)*** the enhanced drug transport induced by the penetration enhancer (Azone), and ***3)*** the bargain diffusivity of the drug throughout the vehicles of gel formulations resulting from the increased viscosity caused by the thickener, ***4)*** The rheological and physicochemical characteristics of the formulation and drug, and/or to ***5)*** the inherent unique physiological and anatomical constraints of the eye. It is clear from the foregoing complex discussions that extra efforts should continue to be devoted toward improving delivery of drugs into the different ocular layers/tissues.

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