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Research paper

Cyclodextrin solubilization of carbonic anhydrase inhibitor drugs: Formulation of dorzolamide eye drop microparticle suspension

Phatsawee Jansook [a], Einar Stefánsson [b], Margrét Thorsteinsdóttir [a,c], Baldur B. Sigurdsson [c], Sigrún S. Kristjánsdóttir [a], Jon Fernández Bas [a], Hakon H. Sigurdsson [a], Thorsteinn Loftsson [a,*]

[a] Faculty of Pharmaceutical Sciences, University of Iceland, Reykjavik, Iceland
[b] Department of Ophthalmology, Faculty of Medicine, University of Iceland, Reykjavik, Iceland
[c] ArcticMass Inc., Reykjavik, Iceland

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ABSTRACT

Topically applied carbonic anhydrase inhibitors (CAIs) are commonly used to treat glaucoma. However, their short duration of action requiring multiple daily dosing can hamper patient compliance. The aim of this study was to develop novel aqueous CAI eye drop formulation containing self-assembled drug/cyclodextrin (D/CD) microparticles that enhance and prolong drug delivery to the eye. Phase-solubility of each drug tested (i.e. methazolamide, brinzolamide and dorzolamide HCl) was determined in either pure water or an aqueous eye drop medium. The pH was adjusted to maximize the fraction of unionized drug. Dorzolamide had the highest affinity for γ-cyclodextrin (γCD) and, thus, was selected for further investigation. Hydroxypropyl methylcellulose (HPMC) was the most effective polymer tested for stabilization of the dorzolamide/γCD complexes and gave the highest mucoadhesion at 0.5% w/v concentration. Thus, the dorzolamide eye drop vehicle containing γCD (18% w/v) and HPMC (0.5% w/v) was developed. The physicochemical properties of this formulation complied with the specifications of the eye drop suspension monograph of the European Pharmacopoeia. The in vivo testing of the formulation showed that the drug was delivered to the aqueous humor in rabbits for at least 24 h with the maximum drug concentration at 4 h. Furthermore, this formulation delivered the drug to the posterior segment of the eye after topical administration. These results indicate that this CAI eye drop formulation has the potential of being developed into a once-a-day product.

1. Introduction

Carbonic anhydrase inhibitors (CAIs), such as acetazolamide, methazolamide, brinzolamide and dorzolamide, are commonly used to reduce intraocular pressure (IOP) in glaucoma patients [1]. CAIs are available as aqueous eye drop solutions (dorzolamide) and suspensions (brinzolamide) for topical administration and as tablets for systemic drug delivery (acetazolamide and methazolamide). Systemic CAI administration can lead to various side effects, such as numbness, fatigue and gastrointestinal irritation, and consequent poor patient compliance [2,3]. The first commercially available topical CAI was aqueous eye drop solution containing 2% (w/v) dorzolamide hydrochloride (Trusopt® from Merck) [4]. Several formulations techniques have been investigated in an effort to overcome poor bioavailability of CAIs after topical applications such as gel formulations [2,5,6], water-soluble salts [7], liposomal/niosomal formulations [8–10] and aqueous cyclodextrin (CD) containing eye drop solutions [11–13]. Previously, we have studied aqueous CD containing methazolamide eye drop solutions in rabbits [4]. In humans, these methazolamide eye drop solutions lowered the IOP after topical administration and they were well tolerated and did not cause any adverse effects [14].

CDs are water-soluble complexing agents that are able to solubilize poorly soluble lipophilic drugs, and enhance their permeation through biological membranes, through formation of water-soluble complexes [15,16]. In ophthalmic preparations, drug/CD complexes have been shown to increase drug permeation through cornea [17]. The natural γ-cyclodextrin (γCD) was selected for this study due to its favorable toxicological profile, relatively high solubility and good complexation capabilities. Furthermore, its derivative 2-hydroxypropyl-γ-cyclodextrin (HPγCD) has been shown to cause significantly lower haemolytic effects than, for example, 2-hydroxypropyl-β-cyclodextrin (HPβCD) [18].

The purpose of this study was to investigate several CAI/CD complexes with regard to solubilization and drug delivery and to develop topically effective CAI eye drop CD suspension.

* Corresponding author. Address: Faculty of Pharmaceutical Sciences, University of Iceland, Hofsvallagata 53, IS-107 Reykjavik, Iceland. Tel.: +354 525 4464; fax: +354 525 4071.
E-mail address: thorstlo@hi.is (T. Loftsson).

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2. Materials and methods

2.1. Materials

Dorzolamide HCl was kindly donated by Merck (Rahway, NJ, USA) and brinzolamide by Alcon Laboratories (Fort Worth, TX, USA). Methazolamide was purchased from Fine Chemicals Corporation (Cape Town, South Africa), γ-cyclodextrin (γCD) and 2-hydroxypropyl-γ-cyclodextrin (HPγCD) with molar of substitution (MS) 0.6 (MW 1576 Da) were purchased from Wacker-Chemie (Munich, Germany). Disodium edetate dihydrate (EDTA), sodium chloride, potassium chloride, calcium chloride dihydrate, magnesium chloride hexahydrate and potassium dihydrogen phosphate were purchased from Merck (Darmstadt, Germany), disodium phosphate dodecahydrate, monosodium phosphate dihydrate, glucose anhydrous and sodium bicarbonate were purchased from Norsk Medisinaldepot (Oslo, Norway), magnesium sulfate anhydrous from Fluka (Tokyo, Japan), mucin (porcine stomach, type II), benzalkonium chloride, tyloxapol, hydroxypropyl methylcellulose 4000 (HPMC), polyvinylpyrrolidone MW 38-40,000 (PVP) and carboxymethylcellulose sodium salt medium viscosity (Na CMC) from Sigma (St. Louis, MO, USA), poloxamer 407 (Lutrol® F-127, P407) from BASF (Ludwigshafen, Germany), semipermeable cellophane membranes (SpectaPor®, molecular weight cut off (MWCO) 3500 and 12–14,000 Da) from Spectrum Europe (Breda, Netherlands). All other chemicals used were of analytical reagent grade purity. Milli-Q (Millipore, Billerica, MA, USA) water was used for the preparation of all solutions.

2.2. Methods

2.2.1. pH-solubility profiles

Excess amount of drug to be tested was added to pure aqueous solution. The desired pH (pH from 2 to 9) was obtained by dropwise titration with concentrated aqueous sodium hydroxide or hydrochloric acid solution. The suspension formed was agitated at room temperature (22–23 °C) for 6 days, and if necessary, the pH was readjusted. Excess solid drug was always present during the equilibration. After equilibration, samples were removed, filtered through 0.45 μm cellulose membrane filter, the filtrate diluted with the mobile phase and the amount of dissolved drug determined by reversed-phase high performance liquid chromatography (HPLC). The HPLC Hewlett Packard Series 1100 consisted of a G132A binary pump with a G1379A solvent degasser, a G13658 multiple wavelength detector, a G1313A auto sampler and Phenomenex Luna 5μ C18 reverse-phase column (150 × 4.6 mm). The chromatographic conditions are shown in Table 1.

2.2.2. Solubility determinations

The drug solubility was determined by the previously described heating method after the chemical stability of the drugs during the heating process had been verified [19]. All three drugs tested were shown to be chemically stable in γCD and HPγCD solutions during heating in autoclave. Excess amount of the drug was added to a solution containing 0–20% (w/v) cyclodextrin (pure γCD or HPγCD) in either pure water or eye drop vehicles. The drug suspensions were heated in tightly sealed containers in an autoclave (121 °C, 20 min) and allowed to cool to room temperature. Then a small amount of solid drug was added to the suspensions, and the pH was adjusted to 7.0, 6.5 and 7.5 with concentrated sodium hydroxide solution for brinzolamide, methazolamide and dorzolamide HCl, respectively. The suspensions were allowed to equilibrate at room temperature (22–23 °C) for 7 days under constant agitation. After equilibrium was attained, the suspension was filtered through 0.45 μm membrane filter, and the filtrate was diluted with mobile phase and analyzed by HPLC (Table 1). The apparent stability constant ($K_{1:1}$) and the complexation efficiency (CE) of drug/CD complexes were calculated from the linear phase-solubility profiles [19].

2.2.3. Permeation studies

The permeation of dorzolamide from aqueous γCD and HPγCD solutions (0–20% w/v) through semipermeable cellophane membrane (MWCO 12–14,000 Da) was measured in Franz diffusion cell apparatus consisting of a donor and a receptor compartment (FDC 400 15FF, Vangard International, Neptune, USA). The donor and receptor compartments were separated by the semipermeable cellophane membrane. Prior to the measurement, the membrane was soaked overnight in the receptor phase: phosphate buffer saline, pH 7.4, containing 2.5% (w/v) γCD or HPγCD. CD was added to the receptor phase to allow sink condition. The receptor phase was sonicated under vacuum to remove dissolved air before it was placed in the receptor chamber. The study was conducted at 22–23 °C, and the receptor phase (12 ml) was stirred continuously during the experiment. The donor phase (2 ml) consisted of the sample. A 150-μl aliquot of the receptor medium was withdrawn at 120, 180, 240, 360 and 480 min and replaced immediately with an equal volume of fresh receptor medium. The amount of dorzolamide in the receptor medium was determined by HPLC. The amount of drug release was calculated and corrected for. Each experiment was done in triplicate. The flux (J) was calculated from the linear part of each permeability profile and the apparent permeation coefficient (P_{app}) determined from equation:

$$J = \frac{dq}{A \cdot dt} = P_{app} \cdot C_d \quad (1)$$

where A is the surface area of the mounted membrane (1.77 cm^2) and C_d is the initial concentration of the drug in the donor chamber. The steady state flux was calculated as the slope of linear plots of the amount of drug in the receptor chamber (q) versus time (t).

2.2.4. The effect of polymer on dorzolamide solubility

The effect of polymers on dorzolamide solubility, $K_{1:1}$ and CE in aqueous γCD solutions were determined from phase-solubility profiles. During the phase-solubility study, individual polymer, i.e. HPMC (0.1% w/v), Na CMC (0.25% w/v), PVP (0.25% w/v) and P407 (1% w/v), was added to the eye drop medium containing 0–20% w/v γCD. The composition of eye drop medium was as follows: benzalkonium chloride (0.02% w/v), EDTA (0.1% w/v), sodium chloride (0.05% w/v), dibasic sodium phosphate (0.05% w/v) and monobasic sodium phosphate (0.025% w/v) in purified water. All samples were analyzed by HPLC as previously described.

Table 1
HPLC conditions.

Drugs	Mobile phase[a]	Flow rate (ml/min)	Wavelength (nm)	Retention time (min)
Brinzolamide	ACN:acetic acid:water (24:2:74) with 0.025% w/v SOS	1.4	254	6.8
Methazolamide	ACN:acetic acid:water (12:2:86) with 0.015% w/v SOS	1.3	254	5.3
Dorzolamide HCl	MeOH:THF:acetic acid:water (27:1:2:70)	1.0	254	1.8

[a] Volume ratios. ACN: acetonitrile; SOS: 1-octanesulfonic acid sodium salt; MeOH: methanol; THF: tetrahydrofuran.

2.2.5. Dorzolamide eye suspension formulation

The dorzolamide eye drop suspensions were prepared by suspending 3.3 g of dorzolamide HCl (equivalent to 3 g of dorzolamide) and 18 g of γCD in 100 ml of an aqueous solution containing benzalkonium chloride (20 mg), EDTA (100 mg), monobasic sodium phosphate (25 mg), dibasic sodium phosphate (50 mg), tyloxapol (50 mg) and various amounts of HPMC (0.1, 0.25 or 0.5 g). The suspensions formed were heated in sealed containers in an autoclave (121 °C for 20 min) in reference to the method described in Section 2.2.2. After cooling to room temperature, the pH of the suspensions was adjusted to 7.5 ± 0.05 with concentrated aqueous sodium hydroxide solution, the vials resealed and then agitated by mechanical shaker for 7 days. Initial studies had indicated equilibration was reached within 7 days. After equilibration, the total drug content was determined by dissolving the eye drop suspension in the mobile phase before quantitative determination of dorzolamide by HPLC. The amount of dissolved dorzolamide in the eye drop suspension was done by centrifugation of the sample at 3000 rpm for 10 min at 20 °C (Model Rotina 35R, Hettich, Germany). Then the supernatant was diluted with mobile phase and analyzed by HPLC (Table 1).

2.2.6. In vitro mucoadhesive studies

The mucoadhesive properties of the formulations were evaluated using apparatus described by Bin Choy et al. [20]. This method was modified as follows. Semipermeable membrane with MWCO 3500 was soaked in an aqueous mucin solution (0.1% w/v mucin from porcine stomach, Type II) for 2 h before study. Then, 50 μl of each formulation was pipetted as a single drop at the center of the membrane. The membrane was washed with a continuous flow of Hanḱs solution at the rate of 1 ml/min for 1 min. Then the remaining drug was washed from the membrane by immersing the membrane in volumetric flask containing the HPLC mobile phase carefully avoiding any contamination. After shaking for 5 min, the amount of dorzolamide was finally determined by HPLC. Commercially 2% (w/v) dorzolamide eye drop solution (Trusopt®) was used as a reference.

2.2.7. Physicochemical characterizations

The formulation that had optimum of mucoadhesiveness was selected for further investigation. The pH value of the selected eye drop formulation was determined at room temperature with Thermo Orion Star™ Series pH meter (USA). The osmolality of preparation was measured at room temperature using Knauer K-700 vapor pressure osmometer (Knauer, Germany). The viscosity measurement was performed with a viscometer (Brookfield model DV-I$^+$, USA) equipped with a thermostated water bath at 25 °C and 34 °C (Polystat model, USA). The surface tension of the formulation was measured in quadruplicate at 35 °C by the De Nouy ring method with digital tensiometer K10 (Kruss GmbH, Germany). The particle size was determined by light microscopy (Model BHT, Olympus, Japan). The formulation fulfilled the requirements of the European Pharmacopoeia regarding semi-solid eye preparations [21]. The physical stability of the dorzolamide eye drop suspension was evaluated by determining degree of flocculation (β) and re-dispersion time. The preparation (10 ml) was stored in 10 ml-measuring cylinder for 7 days at room temperature (22-23 °C). Observations were made at every hour for 7 h and then every 24 h for 7 days and β calculated according to Martin [22]. The re-dispersion time measurement was determined as follows. The preparation was filled into a 5-ml colorless glass container. The time required for re-dispersion of the suspension was determined, after the container had been standing in an upright position for 5 days at 25 °C and the suspended particles allowed to be precipitated, by rolling the container in a horizontal position using a mechanical shaker. Each measurement was done in triplicate, and the results are the mean values ± standard deviation (SD).

2.2.8. In vivo studies

The study adhered to the ARVO declaration for the use of laboratory animals in research, and the study was approved by the Icelandic National Animal Research Committee (*Tilraunadýranefnd*). Un-anesthetized pigmented rabbits fed on a regular diet, were placed in restraint boxes. One drop (50 μl) of dorzolamide eye drop microparticle suspension was administered to both eyes. At predetermined time points after administration of the eye drop formulation, the rabbits were anesthetized by fentanyl/fluanisone (Hypnorm®, VetaPharma Limited, UK; 0.1 ml kg^{-1} i.m.) and approximately 0.05 ml of aqueous humor withdrawn by inserting a 30 gauge needle on a syringe into the anterior chamber at the limbus. Blood samples from marginal vein were also collected at each time point (2, 4, 8 and 24 h; n = 6–8). At the end of the experiments (24 h), the rabbits were sacrificed by intravenous injection of T 61 0.3 ml kg^{-1} (1 ml T 61: 0.2 g embutramide, 0.05 g mebezonium iodide, 0.005 g tetracaine hydrochloride; Intervet Deutschland GmbH, Germany) and the eyes were enucleated immediately. Two rabbits, which did not receive the eye drop formulation, were used as a control group.

2.2.8.1. Sample preparation. The cornea was cut from the limbus with a sharp knife and scissors and placed in a sampling tube. Four incisions from anterior to posterior part were performed in the sclera to open the eye, and the iris-ciliary body was removed and placed in another tube. The crystalline lens was removed and placed in a separate sampling tube. The eyeball was then turned inside out, and the vitreous humor emptied into a sampling tube. The retina including choroid was gently scraped away and placed in a sampling tube. The optic nerve was removed from sclera, and then both tissues were placed in separate sampling tubes. Great care was taken to prevent cross-contamination between individual tissue samples and eye fluids. All the samples were kept at −70 °C while dissecting the eyes and following finishing dissection of each rabbit.

Each sample was pipetted or weighed into a glass culture tube. In the case of aqueous humor and serum, proteins were precipitated by mixing the samples with trichloroacetic acid and the mixture allowed to stand on ice for 30 min. For the other tissues (cornea, iris-ciliary body, vitreous humor, sclera, retina and optic nerve), sodium hydroxide solution was added to the sample and it shaken in a 60 °C water bath for 45 min. After cooling to room temperature, the sample was transferred to another glass culture tube. Then, all the samples were buffered to pH 8.0 with 0.2 M tris buffer, which was followed by extraction into ethyl acetate. The samples were mixed by repeated inversion. The obtained mixture was centrifuged. The organic layer was transferred to a glass culture tube and evaporated to dryness under stream of dry nitrogen. The residue was then dissolved in 250 μl of 0.085% aqueous phosphoric acid solution, and 20 μl of the solutions were injected onto the column for quantification.

2.2.8.2. Quantitative determination. A UPLC-MS/MS system, consisting of Waters (USA) Acquity binary solvent manager and sample manager and Waters Quattro Premier XE triple quadrupole mass spectrometer equipped with electrospray ionization (ESI) probe, was used for the quantification of dorzolamide. The chromatographic retention of dorzolamide was achieved using a Waters analytical column, type UPLC BEH C18, 50 mm × 2.1 mm (length × inner diameter), with 1.7 μm particle size, that was maintained at 60 °C in column oven. The gradient system mobile phase consisted of buffer A: 5% acetonitrile/95% water with 10 mM ammonium acetate and buffer B: 95% acetonitrile/5% water with 10 mM ammonium acetate, and the flow rate was 600 μl/min (no splitting).

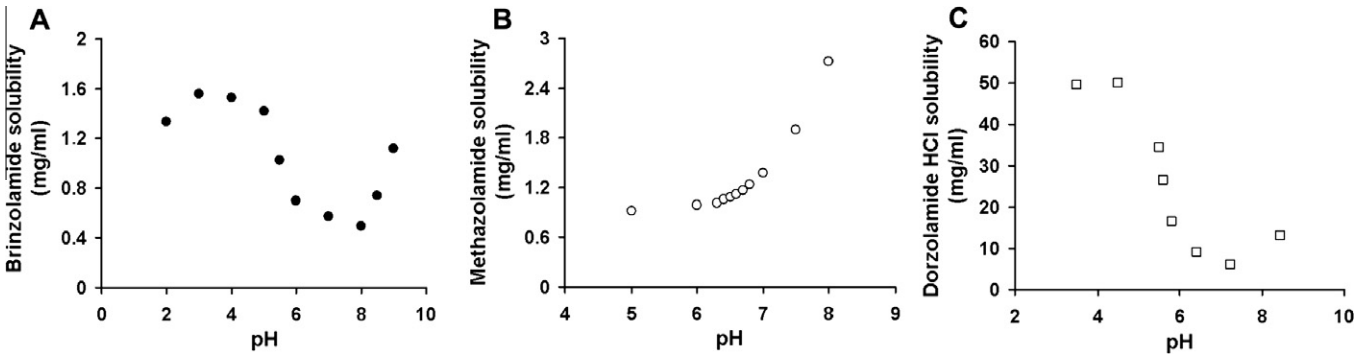
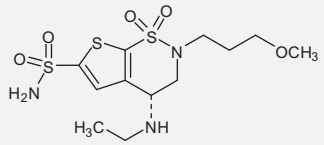
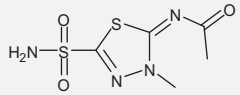
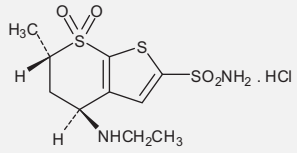


Fig. 1. The pH-solubility profiles of selected CAI drugs in aqueous solution at room temperature (22–23 °C). The pH of the aqueous solution was adjusted with concentrated hydrochloric acid or sodium hydroxide solution. (A) brinzolamide (●); (B) methazolamide (○); (C) dorzolamide HCl (□).

Table 2
Physicochemical properties of the sample compounds [19,29,30].

Physicochemical properties	Chemical structure	Molecular weight	Melting point (°C)[a]	p*Ka*	log $P_{o/w}$[a]	S_0 (mg/ml) in water (at RT[b])
Brinzolamide		383.5	131	5.9; 8.4	6.6 (at pH 7.4)	0.5 (at pH 7.4)
Methazolamide		236.3	213	7.3	0.3	1.9 (at pH 7.5)
Dorzolamide HCl		359.9	264	6.4; 8.5	1.7 (at pH 7.4)	6.7 (at pH 7.4)

[a] The logarithm of the octanol/water partition coefficient.
[b] Room temperature (~25 °C).

The gradient program used was as follows: initial 95% A and 5% B for 0.8 min; linear gradient 5% A and 95% B in 1.2 min; hold for 1 min, return to initial conditions in 1 min. The total chromatographic run time was 4 min. The retention time of dorzolamide was 1.85 min. The sample manager temperature was maintained at 10 °C. The mass spectrometer was optimized for analyzing dorzolamide using multiple reactions monitoring (MRM) to monitor parent → daughter ion (*m/z*) and the transition for dorzolamide 325.2 → 199.0. The ionization source parameters were as follows: capillary voltage 0.4 kV; source temperature 130 °C; desolvation gas temperature 400 °C at a flow rate of 800 L h^{-1} (N_2); cone gas flow rate 50 L h^{-1}. Data acquisition was carried out using MassLynx 4.1 software.

The LC-MS/MS method was validated with respect to sensitivity, linearity, accuracy and precision before the start of study. The lower limit of quantification was set at 5.0 ng/ml (precision 0.84%, accuracy 11.1%). Linearity was confirmed over the concentration range of 5–2500 ng/ml (r^2 0.997). Intra-assay accuracy (−11.9% to −4.5%), intra-assay precision (2.0–3.7%), inter-assay accuracy (0.3–9.2%) and inter-assay precision (2.5–7.9%) were all within the set requirements for the analysts.

3. Results and discussion

3.1. pH-solubility profiles

The pH at which a given drug is formulated is commonly based on its pH-solubility profile [23]. The pH-solubility profiles of brinzolamide, methazolamide and dorzolamide HCl are given in Fig. 1. Table 2 presents some physicochemical properties of the three CAIs tested. Brinzolamide and dorzolamide have similar pH-solubility profiles with a minimum solubility at physiological pH. Both drugs are uncharged at physiological pH, as cations at pH below pK_{a1} (about 6) and as anions at pH above pK_{a2} (about 8.5). Thus, they are in their most lipophilic form at physiological pH and thus have the maximum tendency to partition from the tear fluid into cornea but their solubility in the aqueous tear fluid is at its minimum at physiological pH (Fig. 1 and Table 2). The ranking order of intrinsic solubility of drug substances at physiological pH was as follows: dorzolamide > methazolamide > brinzolamide. Methazolamide possesses higher solubility than brinzolamide, but its lipophilicity is too low for adequate corneal permeability (Table 2). Dorzolamide exhibits more favorable lipophilic/hydrophilic balance than the other two drugs. The phase-solubility of the uncharged drugs was determined at pH 7.0, 6.5 and 7.5 for brinzolamide, methazolamide and dorzolamide, respectively.

3.2. Solubility determinations

Table 3 shows apparent stability constants and the complexation efficiencies of CAI drug/cyclodextrin complexes in pure water and/or the aqueous eye drop medium. The observed $K_{1:1}$ and CE values of all drug tested are relatively low, both for γCD and HPγCD, indicating that relatively large amount of CD is needed to solubilize the drugs. The CE of dorzolamide in eye drop medium

Table 3
Apparent stability constant ($K_{1:1}$), and the complexation efficiency (CE) of CAI drug/cyclodextrin complexes in pure water or the aqueous eye drop medium at room temperature (22–23 °C).

Cyclodextrin	Complexation medium	S_0 (mM)	Slope[a]	Correlation coefficient	$K_{1:1}$ (M^{-1})	CE
Brinzolamide						
γCD	Water	1.29	0.019	0.995	15	0.02
HPγCD	Water	1.29	0.030	0.994	24	0.03
Methazolamide						
γCD	Water	4.09	0.036	0.999	9	0.04
HPγCD	Water	4.09	0.044	0.998	12	0.05
γCD	Eye drop I[b]	4.34	0.038	0.998	10	0.04
HPγCD	Eye drop I[b]	4.34	0.033	0.991	9	0.03
Dorzolamide HCl						
γCD	Eye drop II[c]	8.78	0.126	0.992	16	0.14
HPγCD	Eye drop II[c]	8.78	0.095	0.981	12	0.10

[a] For methazolamide and brinzolamide, these are the initial linear slope of the phase-solubility diagram. Dorzolamide formed B_S-type phase-solubility diagrams with γCD, and thus the slope was calculated from the initial linear section of the profile.
[b] Composition: benzalkonium chloride (0.02% w/v), EDTA (0.1% w/v) and sufficient sodium chloride to obtain isotonicity.
[c] Composition: benzalkonium chloride (0.02% w/v), EDTA (0.1% w/v), sodium chloride (0.05% w/v), dibasic sodium phosphate (0.05% w/v) and monobasic sodium phosphate (0.025% w/v).

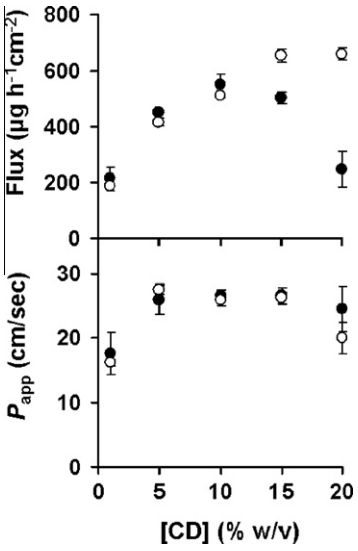


Fig. 2. *In vitro* Flux profiles and apparent permeability coefficient (P_{app}) of dorzolamide from aqueous γCD (●) or HPγCD (○) solutions (1–20% w/v) saturated with the drug through semipermeable membrane with MWCO 12–14,000.

containing cyclodextrins was the highest observed, which can be attributed to its relatively high intrinsic solubility. Thus, dorzolamide was selected to further studies.

3.3. Permeation studies

The dorzolamide permeation fluxes and apparent permeability coefficients from aqueous eye drop medium containing γCD or HPγCD (1–20% w/v), which had been saturated with the drug, are shown in Fig. 2. The flux of dorzolamide from the aqueous HPγCD solutions increased up to HPγCD concentration of 15% (w/v) and then it reached a plateau, while in the case of γCD, it leveled off at 10% (w/v) γCD and then decreased at higher γCD concentrations. The flux profiles showed negative deviation from linearity at the CD concentrations above 5% (w/v). This indicates that the dorzolamide/CD complexes formed nanoaggregates that were too large to pass through semipermeable membrane MWCO 12–14,000 Da [24].

3.4. The effect of polymer on dorzolamide solubility

Dorzolamide in the aqueous γCD containing eye drop medium was selected to study the effect of polymers on the dorzolamide solubility. The values of $K_{1:1}$ and CE, as well as the CE ratios are shown in Table 4. All the polymers tested, except poloxamer 407, increased the γCD solubilization of dorzolamide. Among the polymers tested, HPMC was the most effective enhancer. Based on these results, γCD and HPMC were chosen to develop dorzolamide eye drop suspension as complexing and stabilizing agents, respectively.

3.5. In vitro mucoadhesive studies

Table 5 displays the mucoadhesive characteristics of the dorzolamide eye drop formulation including the commercially available product, 2% w/v dorzolamide eye drop solution (Trusopt®). The mucoadhesiveness was presented in terms of percentage of drug remaining on the mucin layer of the membrane surface. As expected, the eye drop suspension containing 0.5% w/v HPMC appeared to have the greatest mucoadhesion or almost 2-fold higher than those of suspensions containing 0.1% or 0.25% w/v HPMC. Furthermore, the mucoadhesion of all dorzolamide eye drop microparticle suspensions was greater than that of Trusopt®. This indicates that the eye drop suspension that contains solid particles would be washed more slowly from the eye surface than the commercially available product.

3.6. Physicochemical characterizations

The total concentration of dorzolamide in the aqueous dorzolamide/γCD eye drop formulation was determined to be 30.0 mg/ml, and the amount of dissolved dorzolamide was determined to be 9.8 mg/ml, or 33% of the total amount of dorzolamide in the suspension. Table 6 displays the results of the physicochemical characterization of the aqueous eye drop suspension. The pH of the eye drops was determined to be 7.42 ± 0.06. The viscosity decreased with increasing temperature from 38.6 cps at room temperature to 27.5 cps at 34 °C (temperature of the eye surface). The viscosity was lower than that of the commercial product Trusopt® (approximately 100 cps) [25]. The eye drops were slightly hypertonic due to sodium chloride formed during pH adjustments with concentrated aqueous sodium hydroxide solution. The formulation was physically stable and did not form a compact cake during storage. Any sedimentation formed was easily suspended by a moderate amount of agitation. The surface tension was in the range of physiological value of lacrimal fluid's surface tension [26]. The particle size was determined by a light microscopic method. The mean particle size was in micrometer range with the largest particle size observed was 5.4 μm, which was within the limits of the European Pharmacopoeia [21]. The physicochemical

Table 4
Effect of polymers on dorzolamide solubility in aqueous γCD solutions.

Polymer	Concentration (% w/v)	Slope	Correlation coefficient	$K_{1:1}$ (M^{-1})	CE	CE ratio
–	–	0.126	0.992	16	0.14	1.00
P407	1	0.084	0.986	10	0.09	0.64
Na CMC	0.25	0.128	0.999	16	0.15	1.02
PVP	0.25	0.266	0.996	32	0.36	2.52
HPMC	0.1	0.282	0.986	39	0.39	2.73

HPMC: hydroxypropyl methylcellulose; Na CMC: carboxymethylcellulose sodium salt.
PVP: polyvinylpyrrolidone; P407: poloxamer 407.

Table 5
In vitro mucoadhesive properties (mean ± SD; $n = 6$).

Formulation	Total drug (mg/ml)	Initial drug (mg)	Drug remained (mg)	% Drug remained
Dorzolamide eye drop				
HPMC (0.1% w/v)	28.53 ± 0.43	1.43	0.06 ± 0.02	4.2
HPMC (0.25% w/v)	36.44 ± 0.18	1.82	0.08 ± 0.03	4.4
HPMC (0.5% w/v)	29.95 ± 0.84	1.50	0.11 ± 0.05	7.3
Trusopt® (2% w/v dorzolamide)	19.06 ± 0.37	0.95	0.03 ± 0.01	3.2

Table 6
Physicochemical characterization of dorzolamide eye drop formulation.

Physicochemical characterizations	Values (mean ± SD)
pH	7.42 ± 0.06
Viscosity	38.6 ± 2.5 cps (25 °C) 27.5 ± 2.4 cps (34 °C)
Osmolality	371 ± 25 mOsm/kg
Degree of flocculation (β)	2.5 ± 0.3
Re-dispersion time	18 ± 4 s
Surface tension	49.0 ± 2.6 mN/m
Mean particle size	2.2 ± 0.1 μm

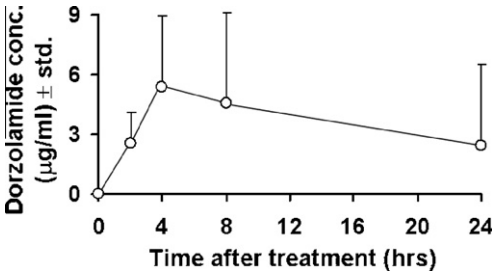


Fig. 3. Dorzolamide concentration (μg/ml) in aqueous humor after topical application (mean ± SD; $n = 6–8$).

properties of the eye drop formulation were acceptable, and thus, it was decided to test the ocular availability of the dorzolamide eye drop suspension in rabbits.

3.7. In vivo studies

The aqueous dorzolamide eye drop suspension was well tolerated after topical application to rabbits, and no macroscopic signs of irritation or redness were observed. Fig. 3 shows the concentration of dorzolamide in aqueous humor after topical application of the 3% (w/v) dorzolamide eye drop suspension. In our previous study, where we tested aqueous dorzolamide eye drop solutions in rabbits using randomly methylated β-cyclodextrin (RMβCD) as solubilizer, peak dorzolamide concentrations (C_{max}) in aqueous humor were determined to be 1.4 and 1.3 μg/ml at 1 h ($t_{max} = 1$ h) after topical administration of 2% (w/v) and 4% (w/v) dorzolamide, respectively, and 2.2 μg/ml at 2 h ($t_{max} = 2$ h) after administration of the commercial product, Trusopt® that contains 2% dorzolamide in aqueous solution [25]. The aqueous 3% (w/v) dorzolamide eye drop suspension containing dorzolamide/γCD complexes both as solid dorzolamide/γCD microparticles and in solution gave peak concentration (C_{max}) of 5.4 μg/ml at 4 h after topical application ($t_{max} = 4$ h). The delay in t_{max} is due to the sustained dorzolamide delivery from the eye drop suspension. The dorzolamide concentration in aqueous humor at 4 and 8 h after single administration of 3% (w/v) dorzolamide was 13–27 and 45-fold higher, respectively, than those obtained after administration of the other three formulations. Furthermore, the eye drop suspension provided sustained high dorzolamide concentrations in aqueous humor for up to 24 h, while the dorzolamide concentration in aqueous humor was essentially zero at 8 h after topical administration of the other three previously tested formulations, including Trusopt® [25]. The new dorzolamide eye drop suspension maintains therapeutic concentrations of the drug in aqueous humor for at least 24 h. Although the studies of the aqueous dorzolamide/RMβCD solutions and Trusopt® were not performed simultaneously with present investigation, the comparison with our previous studies clearly shows the superiority of the aqueous dorzolamide eye drop microparticle suspension as a topical drug delivery system.

Table 7
Concentration of dorzolamide in various parts of the eye 24 h after administration of the 3% (w/v) dorzolamide eye drop suspension (mean ± SD; $n = 6–8$).

Part of rabbit eye	Dorzolamide concentration (μg/g)
Cornea	0.5 ± 0.3
Iris-ciliary body	1.6 ± 1.2
Lens	0.1 ± 0.1
Vitreous humor	0.4 ± 0.3
Sclera	0.6 ± 0.5
Retina	0.8 ± 0.8
Optic nerve	11.3 ± 0.9

The drug distribution in various parts of the eye at 24 h after topical application of the eye drops is shown in Table 7. The results indicate that the eye drop formulation is a very effective drug delivery system for the posterior segment of the eye, i.e. vitreous, retina and the optic nerve. The drug was also found at high concentrations in the anterior segment, in the iris-ciliary body and aqueous humor. These are significantly higher dorzolamide levels than we obtained in our previous study of 2% and 4% dorzolamide eye drop solutions, and Trusopt® [25]. The serum dorzolamide concentrations were relatively low (i.e. 0.3–0.4 μg/ml). Thus, the dorzolamide delivery from the aqueous eye drop suspension did target the eye and minimized systemic drug delivery and consequent systemic side effects [3].

The natural γCD has a very favorable toxicological profile, and it is degraded to linear dextrins and glucose by enzymes in, for

example, saliva. Due to its relatively high molecular weight (1297 Da) and hydrophilic properties, is unable to penetrate into lipophilic membranes. γCD increases the dorzolamide solubility in the aqueous tear fluid and delivers the drug to the surface of the lipophilic cornea, i.e. through the aqueous diffusion barrier, where the drug is released from the γCD complex and the free drug molecules partition into the lipophilic membrane. Furthermore, since 2/3 of the drug is in the form of solid dorzolamide/γCD complex particles, and since these solid particles are retained on the eye surface and slowly dissolved in the tear fluid, sustained high dorzolamide concentration will be maintained for several hours in the aqueous tear fluid. All of these physicochemical characteristics of the formulation lead to sustained high drug concentration within the eye.

The tear fluid is constantly produced and drained from the eye surface at a rate of about 1.5 min^{-1} in humans, and thus, the precorneal drug half-life after application of normal aqueous eye drop solutions is only between 1 and 3 min [27,28]. Consequently, only few percentages of the applied drug dose are delivered into the intraocular tissues. The major part (50–100%) of the administered dose will be absorbed into the systemic drug circulation, which can lead to various side effects. In the aqueous dorzolamide eye drop microparticle suspension, two thirds of the drug is present as solid drug/γCD complex microparticles that are somewhat slowly dissolved in the tear fluid. Consequently, the precorneal drug half-life is increased from couple of minutes to couple of hours. Thus, greater fraction of the drug dose is able to permeate into the eye and smaller fraction is absorbed into the systemic circulation. The aqueous dorzolamide eye drop microparticle suspension looked like normal eye drop suspension and was well tolerated after topical application to rabbits with no observable adverse effects.

4. Conclusion

The development of dorzolamide eye drop microparticle suspension containing γCD and HPMC as complexing and stabilizing agents, respectively, resulted in successful dorzolamide delivery to the aqueous humor and the posterior segment of the eye. The extent of drug permeation was much higher than after administration of the commercial product at 8 h after topical application and gave sustained high dorzolamide concentrations in aqueous humor for at least 24 h. This formulation has the potential of being developed into a once-a-day eye drop formulation leading to improve patient compliance.

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