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Novel Polymeric Nanoparticles Intended for Ophthalmic Administration of Acetazolamide

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ABSTRACT

Glaucoma is characterized by increased intraocular pressure (IOP) that results in blindness if it remains untreated. Acetazolamide (AZM) is a carbonic anhydrase inhibitor, mainly used to reduce IOP in the treatment of glaucoma. However, the potential of topical treatment is limited, due to its low permeability across the ocular epithelium. An alternative to overcome this limitation is the incorporation of AZM in nanoparticulate systems, such as polymeric nanocapsules (NCs). In this way, the aim of this work was to prepare and characterize NC formulations containing AZM, using ethylcellulose (EC) and Eudragit® RS100 (EUD) as encapsulating polymers. The formulations showed high encapsulation efficiency. Particle size measurements showed that NCs are in the nanometric range. Comparing both groups of formulations, the NCEC proved to be smaller than those prepared with EUD. The formulations prepared with EC showed negative zeta potentials, while NCs of EUD were positively charged. For both groups of formulations, no more than 30% of drug was released in 120 min. *Ex vivo* and *in vivo* studies evidenced that the NCEC formulations were the most efficient, because an increased amount of permeated drug was observed, along with a greater IOP decrease and longer duration of the effect in normotensive rabbits.

Introduction

It is estimated that glaucoma, the leading cause of irreversible blindness in the world, is affecting about 67 million people. Glaucoma is the term used for a group of ophthalmic disorders characterized by an increase in intraocular pressure (IOP), which results in damage of the optic disc and visual field disturbances. IOP increases through an imbalance between the production and drainage of aqueous humor. The main strategy to treat glaucoma is the administration of drugs aiming to decrease IOP. These drugs limit aqueous humor production in the ciliary body and/or enhance aqueous outflow through the trabecular meshwork or the uveoscleral pathway. The drugs used in the long-term management of glaucoma include β-adrenergic blockers, miotics, α-adrenergic agonists, carbonic anhydrase inhibitors (CAIs), prostaglandin analogs, and hyperosmotics. Among them, acetazolamide (AZM), a CAI which has been used in the management of glaucoma for more than 40 years, inhibits the production of aqueous humor without interfering its outflow.[1,2] Although AZM showed to be very effective, systemic side effects, such as diuresis and metabolic acidosis, are the major problems associated with the oral therapy. In this sense, topical administration of AZM could overcome the side effects. However, the potential of topical treatment of glaucoma with AZM is quite limited, mainly due to its poor penetration coefficient (4.11×10^{-6} cm/s) and low aqueous solubility (0.7 mg/mL).[3] An alternative to overcome these limitations is the administration of AZM incorporated in nanostructured systems.

Polymeric nanoparticles are one of the strategies currently used to improve drug absorption across biological membranes. This type of nanocarrier generally presents sizes ranging from 100 to 500 nm and it is subdivided into 2 types of nanostructures, named nanospheres and nanocapsules (NCs).[4,5] Nanospheres are matricial systems, while NCs possess a vesicular organization in which the polymer surrounds a liquid (lipophilic or hydrophilic) core. In such systems, the drug can be entrapped or dissolved inside or adsorbed on to a particle surface.[6] In the case of AZM, its high lipophilicity favors the drug confinement in oily core NCs.

Biocompatible polymers play an important role in safety, stability, and efficiency of nanoparticles for drug delivery. Ethylcellulose (EC) is a hydrophobic cellulose derivative commonly used

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for its mucoadhesiveness and controlled-release properties. Due to the negative surface density of charge, nanoparticles prepared from EC usually present negative zeta potential (ZP).[7] On the other hand, Eudragit® RS100 (EUD), a cationic methacrylate copolymer, produces positively charged nanoparticles that favor mucoadhesion. It is well known that in the case of ophthalmic drug delivery, an appropriate particle size and a narrow size range are highly recommended, in order to ensure low irritation, adequate bioavailability, and compatibility with ocular tissues.[8]

In this work, we report the preparation and some relevant properties of AZM-loaded polymeric nanoparticles made from EC or EUD. The formulations were evaluated in terms of physicochemical characteristics, stability, *in vitro* drug release, transcorneal permeation studies, and *in vivo* IOP reduction, after topical ophthalmic application in nonsedated normotensive rabbits.

Materials and Methods

Materials

AZM was obtained from Parafarm. EC (megawatt 170 KDa) was kindly donated by Colorcon (Cotia, Brazil) and EUD was obtained from Degussa (São Paulo, Brazil). Span 60® (sorbitan monostearate) was purchased from Sigma-Aldrich (São Paulo, Brazil) and Tween 80 (polysorbate 80) was supplied by Henrifarma (São Paulo, Brazil). Medium chain triglycerides (MCT) were purchased from Delaware (Porto Alegre, Brazil). Phosphate buffered saline solution was prepared according to Zimmer et al.[9] All other chemicals and solvents were pharmaceutical grade and used as received.

Preparation of Nanocapsule Suspensions

Nanoparticles were prepared by interfacial deposition of preformed polymers.[10] A solution of polymer (EC or EUD), AZM, lipophilic surfactant (Span 60), and oil (MCT) in acetone was submitted to magnetic stirring for 60 min at 40°C. Then, the organic phase was added into an aqueous dispersion of Tween 80 (hydrophilic surfactant). The mixture was kept under magnetic stirring for 10 min. Then, acetone was removed and the aqueous phase was concentrated by evaporation under reduced pressure. The final volume was adjusted to 10 mL. For comparison, formulations without AZM were prepared (blank formulations). The quantitative composition of formulations is shown in Table 1. Each sample was assayed in triplicate ($n = 3$).

Acetazolamide Content and Encapsulation Efficiency

AZM content was determined after dissolution of an aliquot of NC suspension in methanol, under magnetic stirring. The samples were centrifuged, filtered, and assayed by ultraviolet visible (UV) spectrophotometry at 264 nm.

Encapsulation efficiency (%) was determined by adding an aliquot of the samples in a 10,000 megawatt device (Amicon® Ultra; Millipore). Free drug was separated from the nanostructures by an ultrafiltration and centrifugation technique at 2200 × *g* for 10 min. The difference between the total and the free concentrations of AZM, determined in the nanostructures and in the ultrafiltrate, respectively, was calculated as the encapsulation efficiency (EE%) of the nanoparticles according to the following equation: EE = [(total content − free content)/total content] × 100.

In this experiment, the drug was quantified by high-performance liquid chromatography according to the following conditions: Gemini RP-18 column (150 mm × 4.60 mm, 5 μm; Phenomenex) coupled to a Shimadzu instrument (LC-10AVP Pump, UV-VIS SPD-10AVP Module, Class-VP Software, Shimadzu) at room temperature. The mobile phase was compounded by acetonitrile/sodium acetate 0.1 M (80:20%, vol/vol) adjusted to pH 4.5 ± 0.5 with glacial acetic acid and the flow rate was set at 1.0 mL/min. The volume injected was 20 μL and AZM was detected at 276 nm. Each sample was assayed in triplicate ($n = 3$).

pH Measurements

After preparation, the pH values of nanoparticle suspensions were determined using a potentiometer (Micronal B-474). Each sample was assayed in triplicate ($n = 3$).

Particle Sizes and Polydispersity Index

The particle sizes and polydispersity index of the formulations were determined by photon correlation spectroscopy after dilution of the samples with ultrapure water (1:500) (Zetasizer Nanoseries; Malvern Instruments). Each sample was assayed in triplicate ($n = 3$).

Evaluation of Zeta Potential

The ZP of NC suspensions was measured by the nanoparticle velocity while they were moving due to electrophoresis, after dilution of samples in 10 mM NaCl (1:500) using a Zetasizer Nanoseries Malvern Instrument. Each sample was assayed in triplicate ($n = 3$).

Stability Studies of the Formulations

Drug content, encapsulation efficiency, pH, particle size, polydispersity index, and ZP of all formulations were monitored during 60 days at room temperature and protected from light. Each sample was assayed in triplicate ($n = 3$).

In Vitro *Drug Release From Nanoparticles*

Experiments were performed in a modified Franz diffusion assembly at 35.0 ± 0.5°C. Semipermeable acetate cellulose

Table 1
Composition of Nanocapsule Suspensions

Variable	NCEUDB	NCEUD1	NCEUD1.5	NCEUD2	NCECB	NCEC1	NCEC1.5	NCEC2
Aqueous phase								
Tween 80 (g)	0.077	0.077	0.077	0.077	0.077	0.077	0.077	0.077
Water (mL)	53	53	53	53	53	53	53	53
Organic phase								
EUD (g)	0.100	0.100	0.100	0.100	–	–	–	–
EC (g)	–	–	–	–	0.100	0.100	0.100	0.100
AZM (g)	–	0.010	0.015	0.020	–	0.010	0.015	0.020
MCT (mL)	0.330	0.330	0.330	0.330	0.330	0.330	0.330	0.330
Span 60 (g)	0.077	0.077	0.077	0.077	0.077	0.077	0.077	0.077
Acetone (mL)	27	27	27	27	27	27	27	27

membrane (Sigma® 12000) was placed between the donor and receptor compartments. NC suspensions (1 mL) were placed in the upper compartment while the receptor compartment was filled with 13 mL. The receptor solution was sodium chloride, monobasic sodium phosphate, and dibasic sodium phosphate (pH 7.2), and stirred at 200 rpm with teflon-coated magnetic stirring bar. At selected times, 1 mL aliquots were withdrawn and replaced by the same volume of receptor medium. Data were corrected for dilution. AZM concentration was determined by UV spectroscopy (AZM = 276 nm). Each sample was assayed in triplicate ($n = 3$).

Mathematical modeling of AZM release from nanoparticles was performed through a first-order equation (Eq. 1) as follows:

$$\frac{M_t}{M_\infty} = 1 - \left[e^{-k \cdot t}\right] \quad (1)$$

where M_t is the amount of the drug released at time t, M_∞ is the initial concentration of the drug, and k is the apparent constant of the release kinetic rate.

The release mechanism of AZM from nanoparticles was analyzed by fitting experimental data to Korsmeyer-Peppas model (Eq. 2).

$$ft = \frac{M_t}{M_\infty} = a.t^n \quad (2)$$

where ft is the ratio of absolute cumulative amount of the drug released at time t and at infinite time, a is a constant incorporating structural and geometric characteristic of the carrier, and n is the release exponent indicative of drug release mechanism.

In all cases, the fit of the entire drug release profile was performed using the Scientist 2.0 software (Micromath, St. Louis, MO). The selection of the model was based on the best correlation coefficient and the best model selection criteria, both provided by the software, and the best graphic adjustment.

Transcorneal Permeation Studies

The transcorneal permeation experiments were performed using a modified diffusion chamber. The cell, made of acrylic plastic, consisted in a donor and a receiving receptor compartment (volumes 1.0 and 5.0 mL, respectively).[11] No significant adsorption of the tested formulations to the diffusion chamber surfaces was observed along a 2-h period. The receptor solution is sodium chloride, monobasic sodium phosphate, and dibasic sodium phosphate (pH 7.2). Before use, the receptor solution was aerated with a mixture of 95% O_2 and 5% CO_2 to maintain the oxygenation of the cornea.

Albino rabbits were sacrificed according to the protocols. They were anesthetized with phenobarbital and euthanized with a mixture of 10% O_2 and 90% CO_2 in an acrylic hermetic chamber. Then, the corneas, with a 2-mm ring of sclera, were immediately excised and mounted in a perfusion apparatus diffusion chamber. A 5-mL aliquot of the receptor solution was added to the endothelial face, while 1.0 mL of each formulation was added to the epithelial side face. The temperature in the diffusion chamber was maintained at 35.0 ± 0.5°C by means of a thermostatic water bath. Sample aliquots from the receptor chamber were withdrawn at 15, 30, 45, 60, 75, 90, 105, and 120 min and immediately replaced by previously aerated fresh receptor medium. Samples were filtered through a 0.45-μm microporous membrane, and the filtrate was kept at 4°C until analyzed by UV. The area available for permeation in the cell was 0.785 cm^2.

Linear regression analysis of pseudo steady-state diffusion data allowed calculating the steady-state flux (J, given by $\Delta Q/\Delta t$, where Q is the amount of AZM diffused across the area A at time t) and the apparent permeability coefficient (P_{app}) using the relationship $P_{app} = J/C_i$, where C_i is the initial drug concentration in the donor phase. The lag times for drug absorption (the time required so that the drug can saturate the cornea and reach the receiver compartment) were calculated from the interception of the axis x of regression lines.

Hypotensive Efficacy Studies In Vivo*: IOP Determinations*

In this study, experiments were performed on both eyes of nonsedated normotensive male New Zealand white rabbits (2-2.5 kg). Each formulation was evaluated in 10 animals ($n = 20$ eyes) and each control in 5 ($n = 10$ eyes). The animals were kept in individual cages with free access to food and water and maintained in a controlled 12/12 h light/dark cycle. Animal management procedures conformed to the Association for Research in Vision and Ophthalmology resolution on the use of animals in research, the European Communities Council Directive (86/609/EEC), and the Institutional Care and Use Committee of the Chemistry Faculty of Córdoba University, Córdoba, Argentina, reviewed and approved the protocols. IOP was measured with a TonoVet rebound tonometer (Tiolat, Helsinki, Finland). With this technique, IOP is assessed without the need of topical anesthesia. For each eye, IOP was set at 100% with 2 basal readings taken 30 min before and immediately before the instillation. Then, a single dose of the formulation (50 μL) was applied to both eyes. IOP determinations were performed once every hour over the next 8 h. As a control, rabbits received formulations without the hypotensive agent. The administration protocol included a washout period of at least 48 h between experiments.

Statistical Analysis

Intraocular hypotensive reduction was expressed as means ± standard deviation of the means (SEM). Other parameters as means ± standard deviation were also evaluated. Statistical differences between 2 mean values were evaluated by 2-tailed Student t-test. If necessary, an analysis of variance was employed. Results were taken as significantly different at p values less than 0.05.

Table 2
Physicochemical Characteristics of Nanocapsule Suspensions

Formulation	Theoretical Drug Content (mg/mL)	Drug Content (mg/mL)	Encapsulation Efficiency (%)	pH	Particle Size (nm)	Dispersity Index	Zeta Potential (mV)
NCEUDB	–	–	–	5.03 ± 0.03	223 ± 1.2	0.097 ± 0.011	13.3 ± 0.3
NCEUD1	1.00	1.07 ± 0.04	99.5	4.98 ± 0.06	207 ± 0.9	0.104 ± 0.183	10.7 ± 0.6
NCEUD1.5	1.50	1.55 ± 0.08	99.8	5.17 ± 0.03	229 ± 3.4	0.121 ± 0.043	17.3 ± 0.6
NCEUD2	2.00	1.94 ± 0.05	96.3	5.21 ± 0.07	214 ± 1.5	0.195 ± 0.038	12.9 ± 0.4
NCECB	–	–	–	*5.11 ± 0.06*	*109 ± 0.9*	*0.076 ± 0.002*	*−16.2 ± 0.7*
NCEC1	*1.00*	*1.12 ± 0.03*	*100.1*	*5.18 ± 0.02*	*116 ± 2.5*	*0.176 ± 0.009*	*−14.9 ± 1.4*
NCEC1.5	*1.50*	*1.49 ± 0.02*	*99.7*	*5.23 ± 0.05*	*121 ± 0.6*	*0.094 ± 0.037*	*−13.8 ± 1.3*
NCEC2	*2.00*	*1.99 ± 0.02*	*98.9*	*5.31 ± 0.04*	*106 ± 0.6*	*0.089 ± 0.004*	*−13.3 ± 0.3*

The significance of italics was to differentiate the results of the NCEC from those of NCEUD.

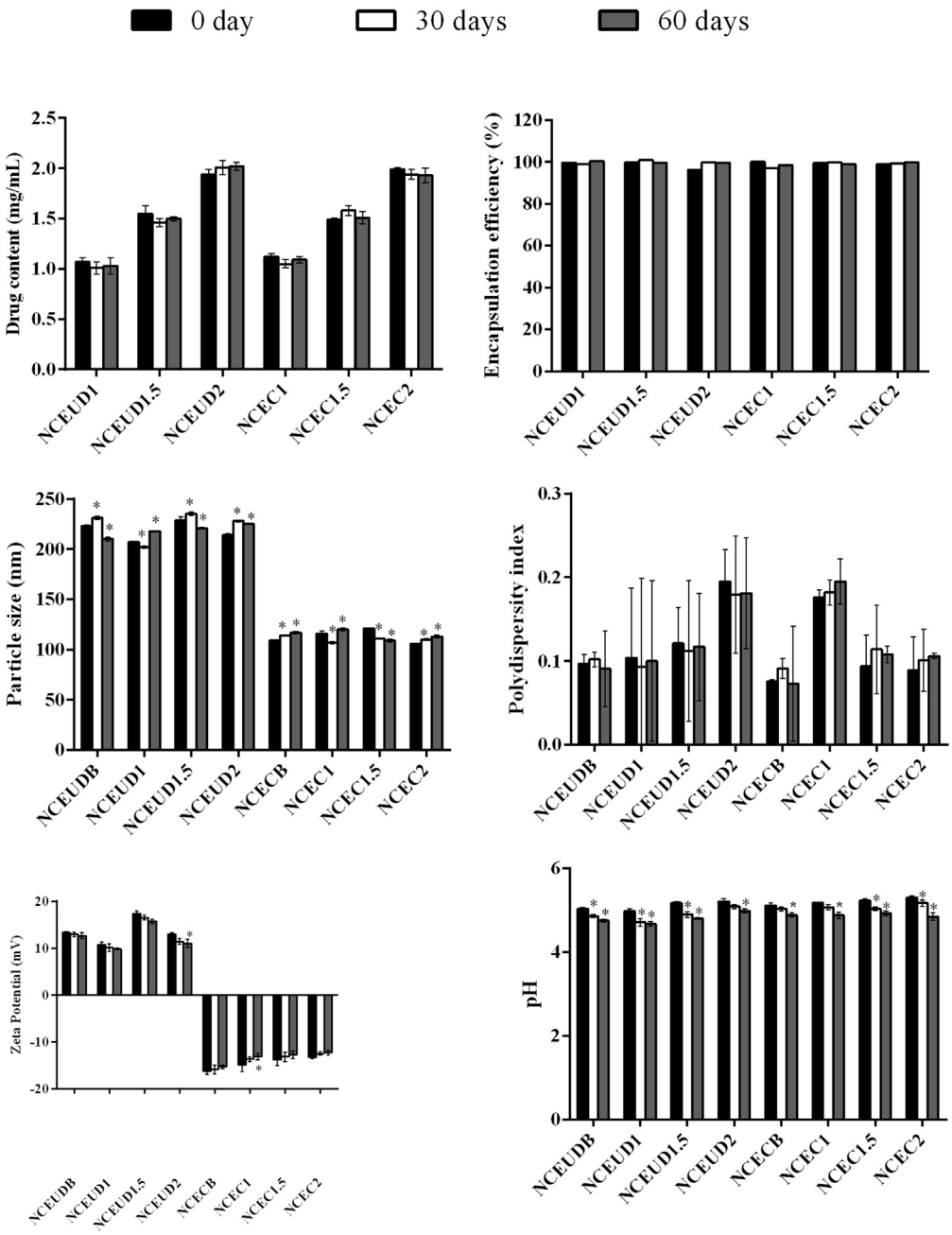

Figure 1. Physical and chemical properties of NC containing AZM (drug content, encapsulation efficiency, particle size, polydispersity index, zeta potential, and pH of the dispersions).

Results and Discussion

After preparation, all formulations presented a macroscopic homogeneous appearance like a milky bluish opalescent liquid (Tyndall effect), regardless of the type of polymer used. Physicochemical characteristics of NC suspensions after their preparation are shown in Table 2. For all formulations, high percentages of encapsulation efficiency were achieved. The pH of formulations was around 5.0, regardless of the type of polymer and AZM concentration. Although the formulations showed a slight acid pH, the values are compatible with ophthalmic administration. In relation to particle size measurements, the nanoparticles are in the nanometric range (106-229 nm) and polydispersity indexes were below 0.2, indicating an adequate homogeneity of these systems. Nanoparticles prepared with EC were smaller than those prepared with EUD.

With regard to ZP, the formulations prepared with EC showed negative values. Similar behaviors were observed by other authors.[12] Suwannateep et al.[13] reported that EC nanospheres of about 280 nm showed a ZP near −30 mV at pH 5.5. These values decreased close to the neutrality at pH 1.2. In our case, we obtained nanoparticles with a quite smaller size, about 120 nm (Table 2), which is in line with a smaller surface and a lower ZP (−15 mV, pH 5.2).

In contrast, nanoparticles prepared with EUD presented positive ZPs due to the cationic nature of the polymer. In both cases, ZPs showed high absolute values suggesting a low probability of particle aggregation.

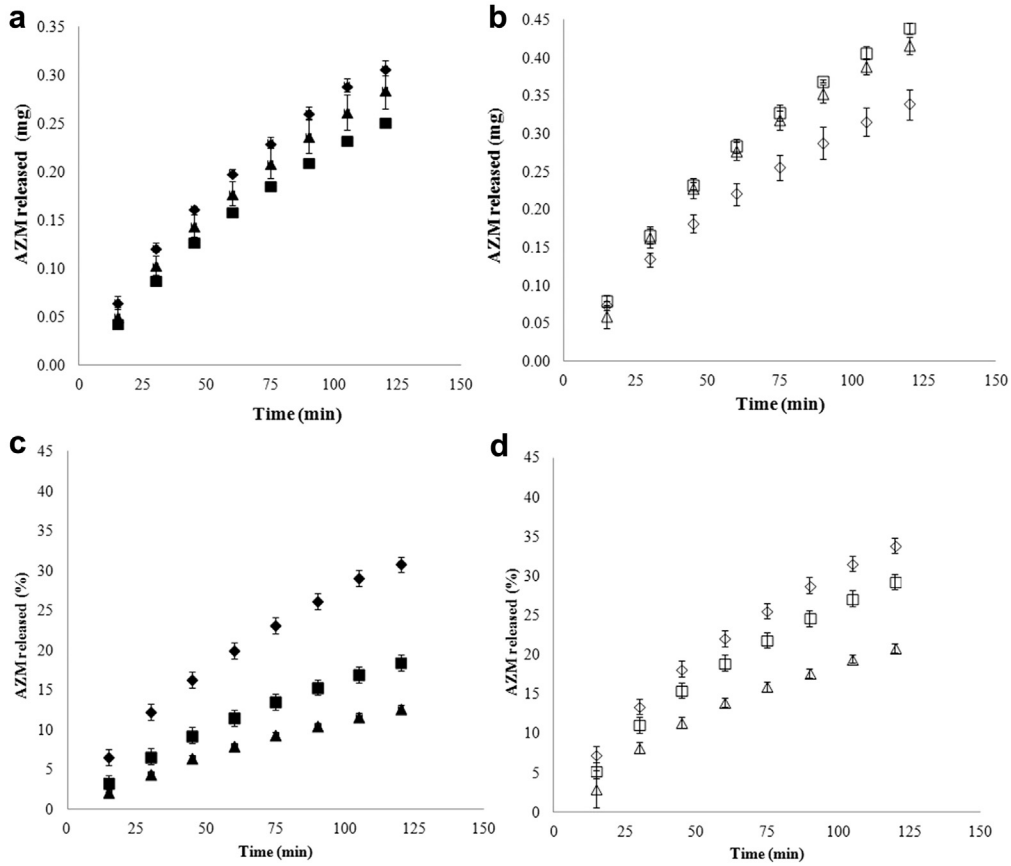

Figure 2. *In vitro* release profile of AZM. Close symbols correspond to NCEUD and open symbols to NCEC. Concentrations: 1 mg/mL (◆, ◊), 1.5 mg/mL (■, □), and 2 mg/mL (▲, Δ). AZM amount (mg) and percentage (%) released are depicted in (a) and (b) and (c) and (d), respectively (mean ± standard deviation, $n = 3$).

Besides, it is well known that the positive charges on nanoparticles surfaces improve the wetability of droplets over the ocular surface, and this strategy becomes a very useful tool for efficacy enhancement of nano-microparticulates such as nanoemulsions.[14]

This antecedent was in line with an unexpected behavior that we observed in NCEUD containing 1.5 mg/mL, because they showed ZP values higher than those from the other 2 concentrations evaluated (1.0 and 2.0 mg/mL). In the framework of this study, we are not able to explain the reason for this observation. However, it is important to highlight it because it seems to influence the permeability and *in vivo* performance (see next paragraphs).

In a similar study reported by Verma et al.,[15] nanospheres from Eudragit RL100 were formulated. In this work, the influence of processing variables over the performance of the system was the main aim. This polymer is relatively more permeable than EUD used in our formulation. In the former, the efficiency entrapment was noticeably lower (about 70%) in comparison to the nanoparticles described in our article (close to 100%), so nanoparticles seem to be more advantageous from this point of view, which could be attributed to the higher solubilization capability of the lipid core.

Regarding physical stability of NC suspensions, no alterations in the initial values of drug content, encapsulation efficiency, ZP, particle sizes, and polydispersity index were observed over storage for 60 days (Fig. 1).

In vitro release profiles of AZM from different formulations of nanoparticles are represented in Figure 2.

Mathematical modeling was used to analyze the release profiles, aiming to elucidate the drug release mechanism. Drug release data (Table 3) were fitted to various kinetic models. First-order kinetic described the drug release kinetics. The data were also analyzed by the Korsmeyer-Peppas kinetic model (Eq. 2). In this case, the value of the release exponent n could infer the mechanism of drug release. For a drug delivery system presenting spherical geometry, n value of 0.43 corresponds to Fickian diffusion of the drug, while n values equal to or higher than 0.85 correspond to a case II transport (relaxation-controlled delivery). Values ranging from 0.43 to 0.85

Table 3
Parameters Calculated by Monoexponential and Korsmeyer-Peppas Models for Acetazolamide *In Vitro* Release

Models or Parameters	Formulations					
	NCEUD1	NCEUD1.5	NCEUD2	NCEC1	NCEC1.5	NCEC2
First order						
k (/min)	0.0114 ± 0.0011	0.0101 ± 0.0009	0.0081 ± 0.0017	0.0110 ± 0.0015	0.0108 ± 0.0014	0.0096 ± 0.0011
R	0.9991	0.9989	0.9993	0.9990	0.9988	0.9990
Korsmeyer-Peppas						
A	0.023 ± 0.002	0.028 ± 0.004	0.021 ± 0.009	0.031 ± 0.010	0.034 ± 0.004	0.039 ± 0.007
N	0.404 ± 0.009	0.427 ± 0.012	0.415 ± 0.008	0.437 ± 0.010	0.432 ± 0.004	0.436 ± 0.003
R	0.9923	0.9983	0.9992	0.9989	0.9991	0.9973

Table 4
Comparison of Various Formulations in Terms of Permeation

Formulations	Microgram Permeated After 2 h	Steady-State Flux (μg/min)	Apparent Permeability Coefficient, P_{app} (cm/min) ($\times 10^{-3}$)
AZM 1 mg/mL	**32.20 ± 2.18**	**0.403 ± 0.05**	**0.51 ± 0.06**
NCEUD1	184.31 ± 19.67	1.50 ± 0.29	1.92 ± 0.37
NCEUD1.5	272.72 ± 11.89	2.50 ± 0.24	2.12 ± 0.21
NCEUD2	232.25 ± 18.09	2.24 ± 0.12	1.42 ± 0.13
NCEC1	*154.97 ± 25.36*	*1.64 ± 0.26*	*1.90 ± 0.01*
NCEC1.5	*220.66 ± 4.24*	*1.78 ± 0.05*	*1.51 ± 0.04*
NCEC2	*320.89 ± 30.16*	*2.37 ± 0.15*	*1.51 ± 0.09*

The significance of italic values was to differentiate the NCEC from NCEUD and the significance of the bold values was to differentiate the solution of AZM from other formulations.

indicate anomalous transport. The AZM release from nanoparticles showed a good fit with the Korsmeyer-Peppas model with "*n*" values around 0.40-0.44 and correlation coefficients higher than 0.99 (Table 3), confirming the Fickian diffusion pattern. This is the classical behavior observed in devices based on a reservoir where the properties of the polymer membrane modulate drug release. As the solvent diffuses through the membrane, the delivery process begins and drug comes out by diffusion.[16]

This observation is in agreement with the profile observed in Figures 2a and 2b, where the kinetic of drug released is represented. Similar patterns for both polymers, practically independent of drug loading, indicate that the polymeric membrane allowed the diffusion of a limited amount of drug, which was practically constant along the experiment.

On the contrary, if the percentage of drug released (%) is considered, as AZM concentration in nanoparticles is increased, the corresponding profile showed lower slopes, indicating that the amount of drug released remained practically constant along the time (Figs. 2c and 2d). On the other hand, it was observed that the release rate from NCEC was higher than those from NCEUD. It is well known that both polymers are water insoluble. However, EUD possesses the lowest permeability of EUD[17] family, which could explain this fact, at least partially. Another fact that is worth taking into account is the size of nanoparticles and its relation with the diffusion process. It is expected that this process be slower in the case of NCEUD compared to NCEC, because the former has a larger size (Table 2).

With respect to permeability of corneal epithelium, it is worth noting that this tissue is the main barrier for drug absorption into the eye. Although the corneal epithelium is more permeable than the stratum corneum, it is relatively impermeable in comparison to other epithelial tissues (intestinal, nasal, bronchial, and tracheal).[18] In addition, the poor aqueous solubility (0.7 mg/mL) and low corneal permeability (4.1×10^{-6} cm/s) of the AZM limit its ocular bioavailability.[3] The results concerning AZM permeation through the cornea are presented in Table 4.

Apparently, the amount of AZM permeated from EC and EUD nanoparticles was quite higher than in the case of AZM solution. This could be explained only if it is hypothesized that nanoparticles may work as carriers (by means of some kind of mechanism) facilitating drug penetration across the cornea.

Besides, NCEC showed to be smaller than NCEUD, which could also facilitate its penetration. Although these topics should be studied in more depth, they allow inferring that these behaviors would be responsible for the higher efficiency observed in the assays related to hypotensive efficacy studies[19] (see next paragraph).

Finally, taking into account that the assay was performed using isolated cornea, it is not possible to argue on some classical eye removing process from cornea surface for AZM solution, which could explain its limited absorption. In fact, because AZM is soluble at this concentration (1 mg/mL, buffer pH 6.8), the drug amount available for absorption is higher in this case, compared to nanoparticles. However, the efficiency of permeation was significantly lower.

In order to evaluate the *in vivo* performance of NC nanoparticles, studies regarding the hypotensive efficacy were carried out by means of IOP determinations in normotensive laboratory animals.

For this study, we selected 3 different concentrations; the lower was similar to that of a marketed solution used for comparison. In this way, formulation of NCs containing 1.0, 1.5, and 2.0 mg/mL of AZM was assayed. The aim of this was to gain information about the potential dose of the NCs because there is not a commercial reference of this kind of nanomedicine.

Along the experiments, we paid special attention on any possible effect on eye tissues and we did not observe any side effect derived from formulation administration. In this way, we concluded that these formulations showed acceptable tolerance in the framework of this study.

The results are depicted in Figures 3a and 3b, and Table 5.

As previously introduced, the efficacy of a drug administered by the ophthalmic route is related to different variables, which may considerably affect its performance. In this case, the diminishing in

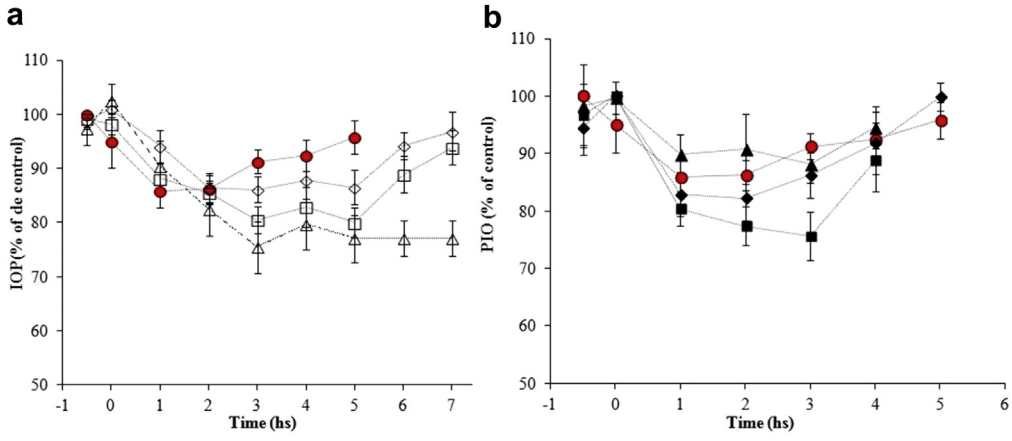

Figure 3. (a) IOP profiles of (●) AZM 1 mg/mL; (◊) NCEC1, (□) NCEC1.5, and (Δ) NCEC2 (mean ± standard deviation, $n = 20$). (b) IOP profiles of (●) AZM 1 mg/mL; (◆) NCEUD1, (▲) NCEUD1.5, and (■) NCEUD2 (mean ± standard deviation, $n = 20$).

Table 5
Maximal IOP Reduction (% SEM), Time Max IOP Reduction (h ± SEM), and Mean Time Effect (h) of AZM

Vehicle	AZM (mg/mL)	Maximal IOP Reduction	Maximal Time Reduction (h)	Mean Time Effect (h)
PBS	1	16.18 ± 2.69	2.00 ± 0.19	5
NCEUD	1	25.38 ± 2.34	2.00 ± 0.33	5
	1.5	27.28 ± 3.37	1.50 ± 0.31	5
	2	10.89 ± 1.70	1.00 ± 0.10	5
NCEC	1	21.47 ± 2.61	3.00 ± 0.24	6
	1.5	26.14 ± 2.49	3.00 ± 0.25	7
	2	27.90 ± 4.22	3.00 ± 0.25	+7

PBS, phosphate buffered saline.

IOP of AZM solution in comparison to nanoparticles was significantly different, although it was dependent on the concentration.

AZM solution showed its highest effect after about 1 h post administration, whereas for NCEUD and NCEC the maximum effect (IOP diminished about 28%, comparatively to AZM solution, $p = 0.05$) was observed after 3 h (1.5 mg/mL) and 7 h (2 mg/mL), respectively.

Unexpectedly for NCEUD, the highest effect was not observed at the highest concentration. The possible explanation for this is the critical influence of the charge on the surface of NCEUD, related to its ZP. For nanoparticles containing 1.5 mg/mL, we measured the highest value of *Z* (Fig. 1) and concordantly such concentration showed the most effective hypotensive effect.[19,20] On the other hand, in the case of NCEC, the intensity of its hypotensive effect was directly proportional to the concentration. The highest effect was observed for NCEC at 2.0 mg/mL, which was able to sustain such effect at least until 7 h. Unfortunately, the assays were not planned to be carried out beyond this time.

Different factors could be involved in the difference in potency observed between NCEC and NCEUD. As discussed above, the *in vitro* drug release showed that the AZM delivery from NCEUD was slower than the one from NCEC and, as it would be expected, this would lead to a lower amount of drug available for absorption.

Other properties of nanoparticles that might influence *in vivo* behavior are potential mucoadhesiveness, particle size, and charge. It was reported that NCEC possesses certain swelling and mucoadhesive properties,[13] whereas EUD has been reported as a material with very low swelling capabilities and without bioadhesiveness possibilities.[21]

With regard to nanoparticles size, it may be directly related to the retention on the mucosa surface. In this case, NCEUD presents a size twice larger than NCEC and, due to the cationic nature of EUD, NCEUD possesses an electropositive charge on the nanoparticle surface. It was argued that positive charges may favor the spreading along ocular surface improving drug absorption.[14] However, for pharmaceutical systems without a minimal retention possibility onto the mucosa, this property becomes disadvantageous because it may accelerate the elimination of the formulation.

Conclusion

AZM, a CAI with ocular hypotensive effect, was vehiculized in polymeric nanoparticles (NC) using EC and EUD (cationic methacrylate copolymer) as constituent of the membrane surrounding the lipid core. These NCs showed very high encapsulation efficiency, they were physically stable, and their size was around 220 and 110 nm for NCEUD and NCEC, respectively. Due to the chemical characteristics of the polymers, NCEUD possesses positive surface charges, whereas for NCEC, the ZP was negative.

In vitro release studies showed a characteristic release pattern corresponding to a classical behavior observed for devices based on a reservoir, where the properties of the polymer membrane modulate drug release. In both cases, the AZM release was practically independent regarding its concentration.

Ex vivo assays, where the permeation through isolated cornea was studied, evidenced that the amount of AZM permeated from EC and EUD nanoparticles was quite higher than in the case of AZM solution. We hypothesized that nanoparticles may work as a carrier (by mean of some kind of mechanism) facilitating drug penetration across the cornea. Besides, the very small size and the mucoadhesive properties of NCs, particularly NCEC, could favor a close contact with the cornea surface facilitating its penetration.

In vivo studies, related to the hypotensive effect of NCs in normotensive rabbits, showed that NCEC formulation was the most efficient, because an increased amount of permeated drug was observed, along with a greater IOP decrease and longer duration of the effect. This novel formulation could be a promising alternative for a more efficient treatment of glaucoma.

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