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RESEARCH ARTICLE

Influence of acetazolamide loading on the (*in vitro*) performances of non-phospholipid-based cationic nanosized emulsion in comparison with phospholipid-based anionic and neutral-charged nanosized emulsions

Shunmugaperumal Tamilvanan[1,2] and Balakrishnan Ajith Kumar[2]

[1]*International Medical University (IMU) SDN BHD, Jalan Jalil Perkasa, Bukit Jalil, Kuala Lumpur, Malaysia, and*
[2]*Arulmigu Kalasalingam College of Pharmacy, Krishnankoil, Tamil Nadu State, India*

Abstract

Context: Acetazolamide (ACZM)-loaded anionic, cationic, and neutral-charged oil-in-water nanosized emulsions were prepared and compared with their mean droplet diameter, surface charge, entrapment efficiency, freeze–thaw cycling stability, *in vitro* drug release, and transcorneal permeation.

Objective: The present study aims to determine the influence of ACZM loading on the performances of non-phospholipid-based cationic nanosized emulsion in comparison with phospholipid-based anionic and neutral-charged nanosized emulsions.

Results and discussion: Regardless of charges, all of these emulsions exhibited a nanometer range mean particle diameter (240–443 nm) following autoclave sterilization. While the anionic and cationic emulsions did show high negative (−36.9 mV) and positive zeta potential (+41.4 mV) values, the neutral-charged emulsion did not. Presence of cryoprotectants (5% w/w sucrose + 5% w/w sorbitol) improved the stability of cationic emulsion to droplet aggregation during freeze–thaw cycling. The *in vitro* release kinetic behavior of drug exchange with physiological anions present in the simulated tear solution appears to be complex and difficult to characterize using mathematical fitting model equations. Augmentation in drug permeation through goat cornea, *in vitro*, was noticed for cationic emulsion.

Conclusion: ACZM-loaded cationic nanosized emulsion could be suitable for topical application into eye to elicit better therapeutic effect in comparison with its anionic and neutral-charged emulsions.

Keywords: Arachis oil, *in vitro* release, zeta potential, cryoprotectant, cornea, permeation

Introduction

Acetazolamide (ACZM), the most effective carbonic anhydrase inhibitor (CAI), is used conventionally via oral tablets in large doses for the reduction of intraocular pressure (IOP) in patients suffering from glaucoma. This treatment leads to unpleasant systemic side effects such as central nervous system (CNS) depression, renal failure, diuresis, vomiting, anorexia, and metabolic acidosis. So, its oral use has become unpopular and several scientists have sought to replace oral CAIs with topical CAIs to abolish systemic side effects.[1] Furthermore, in spite of the advent of newer CAIs, like dorzolamide and brinzolamide, interest in ACZM is not lost and it is still in clinical use, mainly because it is more effective than these new topical CAIs.[2] However, two major problems that hinder the topical effectiveness of ACZM are its poor aqueous solubility (0.7 mg/mL) and low permeability coefficient of 4.1×10^{-6} cm/sec.[3] Topical formulations of ACZM solution (in the form of sodium salt) were initially unsuccessful because of its limited ocular penetration, which

Address for Correspondence: Shunmugaperumal Tamilvanan, International Medical University (IMU) SDN BHD, No. 126, Jalan Jalil Perkasa (Jalan 19/155B), Bukit Jalil, 57000 Kuala Lumpur, Malaysia. Tel: +60-3-2731 7484. Fax. No: +60-3-8656 7229.
E-mail: tamilvanan2k@yahoo.com

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caused an insufficient amount of the drug to reach the ciliary body of the eye.[1] Other significant attempts have been made to formulate effective ACZM topical preparations that include contact lenses containing ACZM,[4] topically active surfactant gel preparation of ACZM,[5] aqueous ACZM solution using 2-hydroxypropyl-β-cyclodextrin,[6] aqueous ACZM solution using cyclodextrins in combination with bioadhesive polymers, penetration enhancers, and co-solvents[7] and polymeric suspensions of ACZM-containing viscolyzers and penetration enhancers.[8] Although all these topical ocular drug-delivery systems offer numerous advantages over conventional oral tablet, they are not devoid of pitfalls including poor patient compliance and difficulty of insertion, as in contact lenses, and tissue irritation, as well as damage and toxicological complications caused by penetration enhancers.[9] In order to overcome these problems, the researchers envisaged the concept of vesicular drug-delivery systems for ocular topical therapy. Kaur et al.[9] recommended the incorporation of CAIs in vesicular delivery systems to enhance the bioavailability of these agents by improving their corneal penetration. Niosomes have been reported as a possible approach to improve the low corneal penetration and bioavailability characteristics of ACZM.[10] Also, topical ocular formulation of ACZM using large unilamellar[11] and multilamellar[12] liposomes as a vehicle have also been reported in the literature.

In past decades, several studies have indicated the use of oil-in-water (o/w) nanosized (submicron) emulsions not only for parenteral drug delivery but also for ocular topical application.[13,14] The main advantage of such nanosized emulsion formulation is the potential to increase the solubility and bioavailability of drugs as well as to reduce the local irritation. When compared with the other already reported different ACZM-containing solutions (prepared based on sodium salt, surfactant gel, co-solvent, and cyclodextrin), polymeric suspensions, liposomes, and niosomes, the nanosized emulsion formulation itself would offer better formulation stability as the ACZM is expected to entrap into either oil phase of the emulsion or oil-in-water interface of the emulsion. Furthermore, the emulsion formulation would even produce lesser local ocular tissue irritation effect after topical application into human eye.[13,15] It should be added that the o/w nanosized emulsions having either anionic or cationic charges are now being exploited commercially as delivery system to improve the ocular bioavailability of the drug like cyclosporin A. For instance, the first anionic lipid emulsion containing cyclosporine A 0.05% (Restasis®, Allergan, Irvine, CA) for the treatment of chronic dry eye disease was approved for clinical use by the Food and Drug Administration (FDA) in December 2002, and is now available in the United States. Furthermore, an over-the-counter product, Refresh Endura®, a non-medicated anionic emulsion has also been exploited in the United States for eye-lubricating purposes in patients suffering from moderate-to-severe dry eye syndrome. In addition, according to the manufacturer's press release,[16] Cyclokat® a cationic nanosized emulsion formulation containing cyclosporin A is currently under advanced Phase II clinical trials for improvement with signs and symptoms in patients suffering from moderate-to-severe dry eye syndrome. Furthermore, since the extraocular surface tissues (cornea and conjunctiva) of the eye are negatively charged at physiological pH,[17] the cationic emulsion (Novasorb®, Novagali Pharma, Evry, France) have the electrostatic attraction with and even better spreadability onto these ocular tissues to elicit better therapeutic effect in comparison with its anionic counterpart.[16]

The so far commercially exploited o/w nanosized (anionic and cationic) emulsions was stabilized through the electrostatic repulsive and steric hindrance forces generated by the mixed emulsifier film, which comprises of a phospholipid component, a nonionic surfactant molecule, and a interfacial charge (anionic or cationic)-producing molecule. Antioxidants such as α-tocopherol is also included in most of the developed nanosized emulsions in order to prevent the oxidative degradation of phospholipid or other emulsion components and thus the minimization of free fatty acid formation in phospholipid-based emulsions following autoclave sterilization and subsequent storage at stipulated conditions. However, even with the addition of antioxidants, the phospholipid-containing emulsion was prone to the liberation of free fatty acids (from phospholipid components) leading to the generation microclimate acidic pH in the vicinity of oil phase, oil–water interface, and water phase of the emulsion.[18,19] The microclimate pH thus generated might be of harmful to the emulsion-incorporated drug molecules. In this context, it becomes necessary to develop an emulsion without the addition of phospholipid emulgator but still shows the required kinetic stability for at least over months. In addition, in order to overcome the instability problems of liposomes in the aqueous state and to facilitate the development of liposome lyophilization, non-phospholipid-based liposomal adjuvants were reported.[20] Recently, a non-phospholipid-based injectable nanosized emulsion containing cationic droplets stabilized by poloxamer–chitosan emulsifier film was prepared.[21]

The objectives of the present study are, therefore, to formulate ACZM-loaded anionic, cationic, and neutral-charged o/w nanosized emulsions based on lecithin–oleic acid–poloxamer, poloxamer–chitosan, and lecithin–poloxamer emulsifier combinations, respectively, and to investigate the influence of ACZM loading on the (*in vitro*) performances (mean droplet diameter, surface charge, entrapment efficiency (EE), freeze–thaw cycling stability, *in vitro* drug release, and transcorneal permeation) of non-phospholipid-based cationic nanosized emulsion in comparison with the phospholipid-based anionic and neutral-charged nanosized emulsions. It should be emphasized that one part of this work was presented in abstract form at Tamil Nadu Pharmaceutical Sciences Welfare Trust, Chennai, India in 2007 and received a research start-up grant from

it (available in http://www.pictrust.com/links/scholorships.phtml, accessed on December 30, 2010). Another part was presented in a poster form at Indian Association of Pharmaceutical Scientists & Technologists (IAPST), Madras Medical College, Chennai, India in 2008.[22]

Materials and methods

Materials

ACZM was obtained from FDC Pharmaceuticals Ltd., Goa, India as a gift sample. Arachis oil (refined and GLC tested, POSTLINE™) was purchased from S.K. Oil Industries, Jalgaon, Maharastra, India. Arachis oil consisted of high mono unsaturated fatty acids (MUFA), comprising 8–10 carbon atoms according to manufacturer's specifications. Chitosan (medium molecular weight; deacetylation, 81%; viscosity of 1 wt% solution in 1 wt% acetic acid, 286 Cps; moisture, 4.6 wt%; ash, 0.5 wt%) was obtained from Aldrich Chemical Co., St. Louis, MO. Polyoxyethylene–polyoxypropylene emulsifier, poloxamer 188 (Pluronic F68), was furnished by BASF (Parsippany, NJ). Lecithin (Epikuron®200, a soy phospholipids complex with a minimum of 92% phosphatidylcholine) was purchased from Degussa Bioactives GmbH (Freising, Germany). Soybean oil, oleic acid, and α-tocopherol were purchased from Fluka (St. Louis, MO). MCT (Estasan®3580) was obtained from Uniquema Asia Pacific (Kuala Lumpur, Malaysia). All other ingredients were obtained commercially and used as received.

Methods

Solubility determination

The solubility of ACZM in water, soybean oil, MCT, castor oil, arachis oil, sesame oil, oleic acid, and mixtures thereof in various ratios were determined. An excess amount of ACZM was added to each solvent in bottle. The bottles were then tightly closed under N_2 and shaken in a thermostated shaking water bath at 25°C until equilibrium was reached (24–48 h). Each experiment was performed in triplicate. The samples were then filtered through 0.45 μm membrane filters in order to remove undissolved drug particles in the saturated solution. The first few milliliters of the filtrate were rejected to avoid problems arising from adsorption on the filter. Some samples were also subjected to centrifugation. One hundred microliters of the filtrate was dissolved in acetonitrile and aliquot volumes after suitable dilution was assayed for ACZM content by HPLC. No significant difference was observed between the solubility in the filtered and centrifuged samples.

Formulation development

ACZM-loaded anionic, cationic, and neutral-charged emulsions were prepared according to the method described elsewhere.[13,14] Following procedure is meant for preparing the phospholipidless and arachis oil-based cationic nanosized emulsion. In brief, arachis oil, ACZM, and α-tocopherol were taken in a beaker and heated up to 70°C. Chitosan was dissolved in 0.05 M acetic acid and heated up to 70°C. Poloxamer 188 was dissolved in double-distilled water (DDW) containing glycerin in a separate beaker and heated up to 70°C. Arachis oil–drug–tocopherol solution was mixed to chitosan solution and then, poloxmer–glycerin solution was added and stirred well by means of a magnetic stirrer. The resulting mixture was further heated to a temperature of 85°C. At this temperature, the obtained crude emulsion was further mixed by a high shear mixer Polytron (Kinematica, Luzerne, Switzerland) for 5 min and rapidly cooled to below 20°C. After cooling, the emulsion was homogenized using a two-stage homogenizer valve assembly (Gaulin Homogenizer, APV Gaulin, Hilversum, the Netherlands) at 9000 £/in.^{2} for 5 min. The emulsion was then filtered through a TE membrane filter (Schuell and Schleicher, Dassel, Germany) with a pore size of 0.45 μm. The emulsion was packed under nitrogen atmosphere in siliconized glass bottles and then sterilized by steam autoclave at 121°C for 15 min.

The typical cationic emulsion consisted of arachis oil (5 mL), ACZM (50 mg), chitosan (250 mg), α-tocopherol (0.02 mL), poloxamer 188 (500 mg), glycerol (1.125 g), and DDW (up to 50 mL). The anionic emulsion consisted of same ingredients but instead of 250 mg chitosan, 1.2 g lecithin, and 0.125 g oleic acid were added to dissolve ACZM along with α-tocopherol. The neutral-charged emulsion was prepared based on the same ingredients as that of the anionic emulsion but omitting the oleic acid. Since the anionic and neutral-charged emulsions consisted of a phospholipid component (lecithin), microclimate acidic pH was generated due to the liberation of the free fatty acids following autoclave sterilization. Therefore, a pH adjustment step was included for anionic and neutral-charged emulsions, where the pH was adjusted to 7.0 from the acidic side using sodium hydroxide (0.1 N). Three replicate emulsion samples were prepared for each one of the charge types. In addition, ACZM solution (1% w/v) was prepared using polyethylene glycol 400 (7%, v/v) and propylene glycol (53%, v/v).[23]

Zeta potential, pH, and viscosity measurements

The zeta potential measurements were carried out using the Malvern Zetasizer 3000 (Malvern Instruments, Ltd., Malvern, UK). The samples were diluted in DDW and the measurements were carried out in 10 mM NaCl solution. Each sample was analyzed twice, and each analysis consists of three replicates. The pH was recorded at given time intervals using a pH meter under different storage temperatures (MP220 pH meter, Mettler Toledo, UK). An Ubbelohde capillary viscometer (Schott, Hofheim, Germany) was used to measure the viscosity of the emulsion samples.

Particle size analysis

Mean droplet diameter was determined utilizing an ALV NonInvasive Back Scattering High Performance Particle Sizer (ALV-NIBS HPPS; Langen, Germany) at 25°C and

using water (refractive index: 1.332; viscosity: 0.894543) as the dilution medium. A laser beam at 632 nm wavelength was used. The sensitivity range was 0.5 nm–5 mm. Values reported were the mean droplet diameter of triplicate emulsion samples.

Entrapment efficiency

Ultracentrifugation method The EE of the emulsions were determined by measuring the concentration of ACZM in the aqueous layer obtained by ultracentrifugation (UC).[24] Centrifugation was carried out using a HITACHI UC apparatus, operated at 50,000 rpm (~162,000 *g*) at 4°C for 2 h. Polyallomer tubes were used and their bottoms were pricked after centrifugation with a syringe needle to collect the aqueous phase. Concentrations of ACZM in both the aqueous layer and the whole emulsion were determined by high-performance liquid chromatography (HPLC).

The EE was calculated according to the following equation[25,26]:

$$\text{EE}\,(\%) = \left[\frac{(C_{\text{total}} \times V_{\text{total}} - C_{\text{water}} \times V_{\text{water}})}{C_{\text{total}} \times V_{\text{total}}}\right] \times 100 \qquad (1)$$

where C_{total} is total drug concentration (50 mg), V_{total} is total volume of emulsion (50 mL), and C_{water} is drug concentration in water phase.

Determination of drug amount at oil–water interface

Ultrafiltration method Ultrafiltration (UF)[27] was performed using VIVASPIN 4 filters (molecular weight cutoff of ~10,000 Da; VIVASCIENCE Ltd. Co., Germany) at 810 *g* for 30 min with all types of the developed emulsions. The amount of ACZM in the separated aqueous phase was measured by HPLC. The drug amount (%) at o/w interface of the emulsion was determined using the following formula:

$$\begin{matrix}\text{Drug amount (\%)}\\ \text{at o/w interface}\\ \text{of the emulsion}\end{matrix} = \left\{\frac{\begin{matrix}\text{(drug amount in whole emulsion -}\\ \text{drug amount in water phase)}\end{matrix}}{\begin{matrix}\text{(initial drug amount added during}\\ \text{emulsion preparation)}\end{matrix}}\right\} \times 100 \qquad (2)$$

Transmission electron microscopy In order to evaluate the emulsification efficiency of the different size reduction equipments used during the preparation process, the ACZM-loaded cationic emulsion containing 5 mL of arachis oil was followed up at each of the stirring stages by means of a transmission electron microscope (CM 12; Philips, Endhoven, The Netherlands) using a negative staining technique (1% solution of phosphotungstic acid (PTA) sodium salt at pH 7.5).

Quantitative drug analysis

ACZM content was analyzed using a HPLC system. In the present study, the already reported[28] method has been fully validated and was adapted to meet the requirements for the ACZM stability-indicating test. The Kontron HPLC system consisted of Kontron 420 pump, a UV detector 332, and autosampler 360 (Kontron Instruments, Zuerich, Switzerland) equipped with a Rheodyne sample injector with a 50 µL sample loop. A reversed-phase 250 × 4.6 mm Luna™ C_{18} (5 µm) column furnished by Phenomenex™ (Torrance, CA, USA), and a 2 × 8 mm precolumn of the same material were used. The mobile phase, at 1 mL/min flow rate, consisted of 20% acetonitrile and 0.1 M sodium acetate adjusted to pH 4.5 with glacial acetic acid, which was filtered and degassed before use. The analytical column and the guard column were kept inside a column heater held at 25°C. ACZM was monitored with a UV–visible absorbance detector at 245 nm, and under these experimental conditions, the run time was 5.2 min. Six standard solutions of drug in acetonitrile were first prepared and appropriately diluted with acetonitrile to final ACZM concentrations ranging from 12.5 (minimum detectable concentration) to 200 µg/mL. Calibration curves of peak area versus drug concentrations were constructed and yielded a linear relationship ($R^2 = 0.9999$). The ACZM-loaded emulsion was directly dissolved in acetonitrile to appropriate dilutions (1:10–1:100). Twenty microliters of these solutions were injected into the HPLC. All the solvents used were of HPLC grade. All the experiments were duplicated and the deviation ranged from 0% to 3%, indicating that the various experimental conditions were well-controlled.

Stability assessment

The drug content, zeta potential, and droplet mean diameter were monitored over up to 6 months time in all of the developed emulsions stored at 4°C, 25°C, and 37°C. Possible phase separations were assessed visually at given time intervals. All other visible changes were recorded.

Freeze–thaw cycling stability

ACZM-loaded cationic nanosized emulsion was diluted with high concentration of cryoprotectant solutions. Different cryoprotectants were added in increasing amounts to assess the optimum concentration (Table 4). Combinations of cryoprotectant were also tried at different concentration levels. The freeze–thaw cycling stability of the emulsion in presence of cryoprotectants or in absence of cryoprotectants was tested by transferring the emulsion sample (5 mL) into plastic test tubes and subsequent placing of it in a –10°C freezer for 16 h and then thawed in a water bath at 30°C for 2 h. This freeze–thaw cycle was repeated five times, and its influence on emulsion property (particle mean diameter) was measured after each cycle.

Accelerated stability test

Since sterilization at 121°C for 15 min is a drastic operation, it constitutes an accelerated stability test by itself. Creaming and phase separation were then assessed

visually after sterilization. To evaluate the mechanical and physical resistance, the emulsion should be subjected to an accelerated mechanical stress. For this purpose, those emulsion bottles that were stored at the already stated temperature conditions for up to 6 months time were shaken at 100 strokes/minute by an oscillatory agitation motion over 24 h at 25°C. The droplet size was evaluated before and after this accelerated test.

In vitro *release of ACZM*

The *in vitro* release kinetics of ACZM from the nanosized emulsion was carried in DDW or simulated tear solution (STS). The composition of STS consisted of 192.4 mg $NaHCO_3$, 111.0 mg KCl, 2.29 mg $CaCl_2$, 672.8 mg NaCl, 669.0 mg albumin, 2.5 mg glucose, and DDW up to 100 mL.[29] The 1600, 800, and 200 µL of ACZM-loaded nanosized emulsions were diluted in 8 mL of DDW or STS to elicit 1:5, 1:10, and 1:40 dilution ratios, respectively. The samples were placed in a glass vial and incubated at 37°C on a water bath throughout the experiment. At every time point (1, 16, 36, 56, and 86 min corresponding to the equivalent time intervals of 5, 20, 40, 60, and 90 min including centrifugation time), 400 µL samples were withdrawn from the vial and filtered through a 300 K Nanosep filter over 4 min at 4000 rpm. Aliquots of 10 µL from the filtrate were dissolved in ethanol/acetonitrile and assayed for ACZM content by the HPLC method as described previously. Ten microliters aliquots from the originally diluted drug-loaded nanosized were also assayed for ACZM content and were used as the total reference content. All kinetic experiments were done in triplicate.

In vitro *transcorneal experiment*

The animal experiments are conducted in full compliance with the Institutional Review Board resolutions on the use of animals in research, Arulmigu Kalasalingam College of Pharmacy, Krishnankoil, Tamil Nadu State, India (Ethical committee research number: MD-58.14-8).

The transcorneal permeation experiments were performed with a modified diffusion chamber. The cell, made of acrylic plastic, consisted of a donor and a receiving compartment (volumes 2.0 and 5.0 mL, respectively).[30] No significant adsorption of the tested formulations to the diffusion chamber was observed over the 2 h period of the permeability experiments. The receptor solution consisted of STS. Before use, the receptor solution was aerated with the mixture of 95% O_2 and 5% CO_2 to maintain oxygenation of cornea. Following humanely killing of the goats at the local slaughter house, three to four eyes were enucleated and placed over a separate plastic bag containing the aerated fresh receptor medium to preserve the eyes during transportation/before dissection. Sterile surgical procedures were used and for avoiding cross-contamination, excision of cornea along with 2–4 mm ring of surrounding scleral tissue was done from a single eye at a time. The excised corneas were then preserved separately in the medium. One cornea with 2–4 mm ring of sclera was mounted by sandwiching surrounding scleral tissue between clamped donor and receptor compartments of a perfusion apparatus in such a way that its epithelial surface faced the donor compartment. The corneal area available for diffusion was 1.0 cm^2. A 5 mL aliquot of the receptor solution was added to the endothelial side, whereas 1.0 mL (1000 µg) of either the anionic, cationic, and neutral-charged nanosized emulsions or the ACZM solution (1% w/v or 10,000 µg) was added to the epithelial side. The temperature in the diffusion chamber was maintained at 37°C ± 0.5°C by a thermostatic water bath. Sample aliquots from the receptor chamber were withdrawn at 15, 30, 60, 75, 90, 100, 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 HPLC. According to previous studies, it could corroborate that at the end of the study, the final concentration of ACZM was at least 20-fold below the maximal solubility in the acceptor medium.

The apparent corneal permeability coefficient (P_{app}) of these formulations was determined according to the following equation[31]:

$$P_{app} = \Delta Q/(\Delta t \times C_o \times A \times 60)\ (cm/sec) \quad (3)$$

where C_o is the initial concentration of ACZM in the donor compartment, 60 represents the conversion of minutes to seconds, and A is the area of the cornea. For the calculation of the apparent permeation coefficient in the present study, A is the surface area of goat cornea, 1.0 cm^2. $\Delta Q/\Delta t$ is the steady-state rate of drug permeation across the intact cornea, as obtained from the slope of the straight line relating corneal permeability (drug amount, Q) to time (t) plot (Q vs. t). The lag time was also determined from this graph by extrapolating the linear portion of the x-axis.

The flow rate of the steady state (J_{ss}) can be calculated by the formula:

$$J_{ss} = C_o \times P_{app} \quad (4)$$

Statistical analysis

The mean and standard deviation of measurements were computed. All the data were subjected to Student's t-test to find out whether or not for the statistical difference. The alpha (α) value for statistical significance is 0.05 (i.e. $P < 0.05$).

Results

ACZM solubility

Preliminary ACZM solubility study was carried out for the identification of the best lipophilic vehicle to formulate nanosized emulsions. Table 1 lists the solubility of ACZM in the selected single oils or oil mixtures.

Since the arachis oil dissolved the highest drug amount per unit volume (10 mg/mL), it was selected as oil phase to design o/w emulsions with low oil content while still dissolving the required therapeutic dose of ACZM. Thus, in the present formulation development stage, the arachis oil was used at a concentration levels ranging from 1.25% to 10% and the characteristics like viscosity, fluidity, ease of manufacturing of the formed emulsion were followed up with time. Accordingly, it was decided to work with 5 mL of arachis oil for dissolving 50 mg of drug resulting in a concentration of 0.1% w/v of ACZM along with other oil soluble components to form the inner oil core phase of the final emulsion.

Transmission electron microscopy

The transmission electron microscopic images, following negative staining with sodium phosphotungstate, showing the population droplet size of a ACZM-loaded cationic nanosized emulsion (5% arachis oil) immediately after mixing of oil and water phases with mild constant stirring (A), after polytron (B), and after final droplet size homogenization (C) are presented in Figure 1. There was a significant reduction in the mean droplet diameter of the emulsion at each step of the emulsion preparation.

Table 1. Acetazolamide (ACZM) solubility in different solvents at 25°C.

Solvents	ACZM solubility (mg/mL)
Water	0.7
Castor oil	2
MCT	2
Arachis oil	10
Sesame oil	1.5
Soybean oil	2
Oleic acid	1
Sesame oil + MCT (1.5:1)	3
Sesame oil + castor oil (1.5:1)	3
Castor oil + MCT (1:1)	4
Sesame oil + MCT (1:1)	3
Soybean oil + MCT (1:1)	4

MCT = medium-chain triglycerides.

Physicochemical characteristics of emulsions

Table 2 shows the physicochemical characteristics (mean droplet diameter, zeta potential, drug EE, and drug amount (%) at o/w interface) of ACZM-loaded anionic, cationic, and neutral-charged nanosized emulsions. Regardless of the modifications made in the compositions of the developed emulsions, which render them anionic, cationic, or neutral, the mean droplet diameters of these emulsions were in the range of 240 ± 19 nm to 443 ± 23 nm and presented a monodispersed droplet size distribution. The data presented in Table 2 show that the zeta potential of the developed formulations was of the same order of magnitude, either positive charge (41.4 ± 1.85) for cationic emulsion or negative charge (–36.9 ± 2.25) for anionic emulsion. The drug EE of cationic emulsion was higher followed by neutral and anionic emulsions. Similarly the order for drug amount (%) at o/w interface of the emulsion was cationic > neutral > anionic.

Long-term storage stability of emulsions

Table 3 presents the effect of storage temperature and time on the mean droplet diameter and zeta potential values of ACZM-loaded cationic nanosized emulsion. Storing the cationic emulsion for up to 6 months at 4°C, 25°C, and 37°C caused a statistically insignificant change (slight reduction from 240 to 218 nm) in the mean droplet diameters of the emulsion. However, the cationic emulsion's zeta potential was found to reduce significantly after 6 months at all studied storage temperatures. It is needed to point out that following 1 to 2-month storage at the studied storage conditions, the ACZM-loaded anionic and neutral-charged emulsions were shown for the separation of oil and water phases. This indicates that these two emulsions were not stable any more.

Freeze–thaw cycling stability and cryoprotectants

Table 4 shows the cryoprotectants added to study the freeze–thaw cycling stability of ACZM-loaded cationic nanosized emulsion. In the pretest, three methods (A, B, and C) were compared when adding the cryoprotectant to the AZCM-loaded emulsions. (A) Adding the cryoprotectant to the water phase of the emulsion in the preparation process. This will increase the viscosity of the

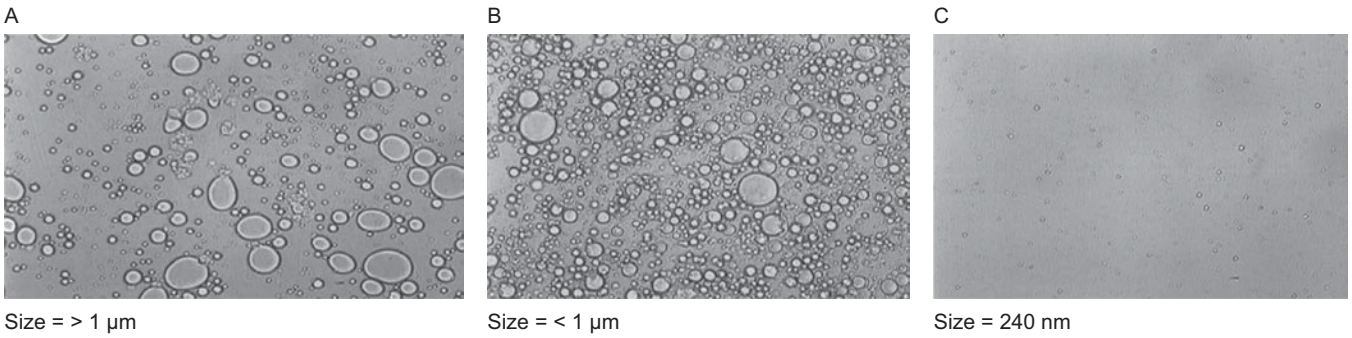

Figure 1. Transmission electron microscopic images, following negative staining with sodium phosphotungstate, showing the population droplet size of a ACZM-loaded cationic nanosized emulsion (5% arachis oil) immediately after mixing of oil and water phases with mild constant stirring (A), after polytron (B) and after final droplet size homogenization (C).

system with the increasing mean particle diameter being observed. The conformation of the particles may be changed in the system. (B) Dissolving equal solid sugars directly into the final AZCM-loaded emulsions. However, some poorly water-soluble sugars, such as mannitol and lactose, needed ultrasound, which would increase the mean particle diameter and induce leakage of the drug. (C) The drug-loaded emulsions were diluted with a high concentration of cryoprotectant solution. When using this method, cryoprotectants showed better protective effect, and it was much easier to perform this in the laboratory. So, method (C) was chosen.

Among the tested cryoprotectants in single or in combination at different concentration levels, sucrose and sorbitol combination (at 5% w/w each) was found to produce a satisfactory result in terms of mean particle diameter of the emulsion after five freeze–thaw cycles (Figure 2). The pH of the emulsion after cryoprotectants addition was 6.8–7.0. No variation in the mean particle diameter of the drug-loaded cationic nanosized emulsion was observed when sucrose and sorbitol cryoprotectants were used. It should be added that ACZM-loaded anionic and neutral-charged nanosized emulsions with any of these added cryoprotectants were fully aggregated during freeze–thaw cycling study. Similarly ACZM-loaded cationic nanosized emulsion without cryoprotectant was also undergone aggregation of dispersed oil droplets as reflected in the progressive increase in the mean particle diameter over five freeze–thaw cycles shown in Figure 2.

In vitro release of ACZM

Figure 3 shows ACZM *in vitro* release profiles from a typical cationic nanosized emulsion formulation as a function of increasing dilution ratios (1:5–1:40) of STS over 90-min time. At 1:5 dilution, about 72% drug release was achieved for neutral-charged emulsion followed by 62% for anionic emulsion and only 30% for cationic emulsion. About 65% of the drug was released from neutral-charged emulsion at 1:10 dilution, whereas 55% drug was liberated from anionic emulsion. At 1:40 dilution, 58% and 48% drug release were seen, respectively, from neutral and anionic emulsions. However, only 19–20% of the drug was released at 1:10–1:40 dilution from the cationic emulsion. Therefore, it appears that drug release from cationic emulsion was delayed when compared with anionic and neutral-charged emulsions under the studied experimental conditions.

In vitro transcorneal permeability studies

Figure 4 shows the *in vitro* transcorneal permeation profiles of the ACZM solution, ACZM-loaded anionic, cationic, and neutral-charged emulsions in goat cornea. All the formulations were shown linear relationships in the time versus drug amount permeated plot as the correlation (R^2) values were in the range from 0.9912 to 0.9969. Permeation data of ACZM solution and ACZM-loaded emulsion formulations are shown in Table 5. All of the tested formulations had similar lag time of 60 min to exert their permeation effect through goat cornea epithelial cells. Cationic emulsion possessed the % permeation value of 45.00 ± 4.3 followed by anionic and neutral-charged emulsions (14.80–17.50%) and ACZM solution (7.80 ± 0.86%). The steady-state flux (J_{ss}) value was also higher for cationic emulsion (2.22 ± 0.1 μg/min). Again the ACZM solution exhibited the lowest J_{ss} value (0.25 ± 0.3 μg/min), whereas

Table 2. Physicochemical characteristics of acetazolamide (ACZM)-loaded anionic, cationic, and neutral-charged nanosized emulsions.

Formulations	Mean droplet diameter (nm ± SD, n = 3)	Zeta potential (mV ± SD, n = 3)	Drug entrapment efficiency (%)	Drug amount (%) at oil–water interface of the emulsion
ACZM-loaded anionic emulsion	290 (±25)	−36.9 (±2.25)	69 (±2)	25
ACZM-loaded cationic emulsion	240 (±19)	+41.4 (±1.85)	99 (±1)	48
ACZM-loaded neutral-charged emulsion	443 (±23)	Nil	85* (±2)	39*

Figures in parentheses indicate standard deviation ($n = 3$).
*Statistically significant when compared with cationic emulsion ($P < 0.05$).

Table 3. Effect of storage temperature and time on the mean droplet diameter and zeta potential values of acetazolamide (ACZM)-loaded cationic nanosized emulsion.

	Physicochemical properties					
	Mean droplet diameter (nm) at different storage temperature (°C)			Zeta potential (mV) at different storage temperature (°C)		
Storage time (month)	4	25	37	4	25	37
0	240 (±19)	240 (±19)	240 (±19)	41.4 (±1.85)	41.4 (±1.85)	41.4 (±1.85)
1	250 (±56)	238 (±50)	240 (±35)	—	—	—
3	235 (±45)	230 (±46)	228 (±46)	—	—	—
6	221 (±52)	221 (±40)	218 (±50)	37.8* (±0.89)	37.2* (±2.35)	30.6* (±1.37)

—: Not determined.
Figures in parentheses indicate standard deviation ($n = 3$).
*$P < 0.05$.

the neutral and anionic emulsions possessed 1.10 ± 0.2 and 0.96 ± 0.1 μg/min, respectively. As expected, the cationic emulsion showed a higher apparent permeability coefficient (P_{app}) value of 13.32 ± 0.92 × 10^{-6} cm/sec, whereas the anionic and neutral-charged emulsions demonstrated, respectively, the P_{app} values of 5.64 ± 0.14 × 10^{-6} cm/sec and 4.87 ± 0.52 × 10^{-6} cm/ sec. The ACZM solution was illustrated the lowest P_{app} values of 2.52 ± 0.42 × 10^{-6} cm/ sec.

Discussion

Emulsion stabilization

The o/w nanosized emulsions are heterogeneous dispersions of two immiscible liquids. Unlike microemulsions, which are transparent and thermodynamically stable,[32] macro (coarse)- and nanosized emulsions are only kinetically stable and/or metastable dispersions. Nevertheless, the stability of the o/w macro (coarse)- and nanosized emulsions can substantially be improved with help of suitable emulsifiers or emulgators that are capable of forming a mono- or multilayer-coating film around the dispersed oil droplets in order to reduce interfacial tension and to increase droplet–droplet repulsion.[33] Depending on the concentrations of these three components (oil–emulgators–water) and the efficiency of the emulsification equipments used to reduce droplet size into a nanosized and/or monodispersed size range, the o/w nanosized emulsion with improved stability over desired time span (in comparison with coarse emulsion) can be obtained.

Table 4. List of cryoprotectants and the concentrations used to study the freeze–thaw cycling stability of acetazolamide (ACZM)-loaded cationic nanosized emulsion.

Cryoprotectant	Concentration tested (% w/w)
Maltose	5, 10, 20
Glucose	5, 10, 20
Sucrose	5, 10, 20
Sorbitol	5, 10, 20
Trehalose	5, 10, 20
Mannitol	5, 10
Lactose	4, 8
Fructose	5, 10, 20

The o/w nanosized emulsions were prepared following a well-established combined emulsification technique. The developed emulsions consist of lecithin–oleic acid–poloxamer, poloxamer–chitosan and lecithin–poloxamer emulsifier combinations to confer anionic, cationic, and neutral charges over dispersed oil droplets, respectively. However, irrespective of the different charges, arachis oil was used as the oil phase to prepare all of these emulsions. In addition, the viscosity and refractive index of these emulsions were measured and found to be 1.5 cps and 1.345, respectively, close to the viscosity and refractive index values of water indicating that the emulsion can be suitable for ocular use.

Further, it is needed to point out that manufacturing an emulsion with nanometer size range has to process through various traditional droplet size reducing steps as already described in "Materials and methods" section. Most of the droplets exhibit an average size diameter ranging from 1 to 4 μm (Figure 1A) following mixing of oil and water phases with constant mild stirring and subsequent raising of temperature to 85°C. To improve emulsification efficiency, rapid Polytron mixing at a high speed is necessary to break the droplet sizes in between 250 nm and 1 μm levels. It should be emphasized that the number of large droplets diminished greatly as compared with the previous step (Figure 1B). Rapid cooling after Polytron and homogenization steps lead to a final

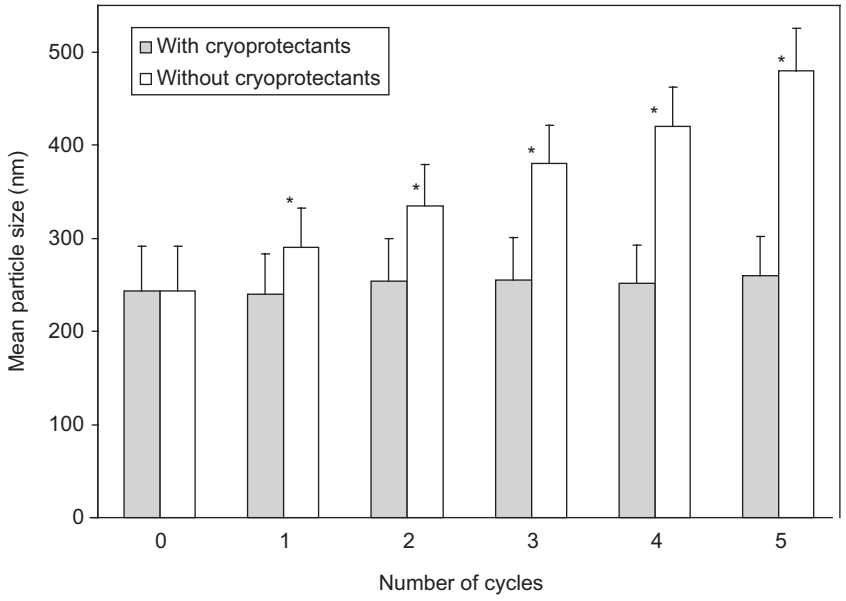

Figure 2. Dependence of mean particle diameter of the acetazolamide (ACZM)-loaded cationic nanosized emulsion on number of freeze–thaw cycles (−10°C for 22 h/30°C for 2 h) in the absence and presence of cryoprotectants *($P < 0.05$).

droplet size of <250 nm (Figure 1C). The ACZM content after emulsion preparation and at each step during the preparation of emulsion through various processing steps such as oil phase, polytron, and homogenization, before and after sterilization was determined. No significant variation was observed between the drug content determined at each of the processing steps and the initial drug content.

Long-term and freeze–thaw stabilities

An important parameter that evaluates the stability of the formed emulsion upon over the storage conditions used is ultimately the measured mean droplet size. Since anionic and neutral-charged emulsions did not produce stable formulations after 1 or 2 months storage at three different temperature conditions, the data of long-term and freeze–thaw stability studies observed with cationic

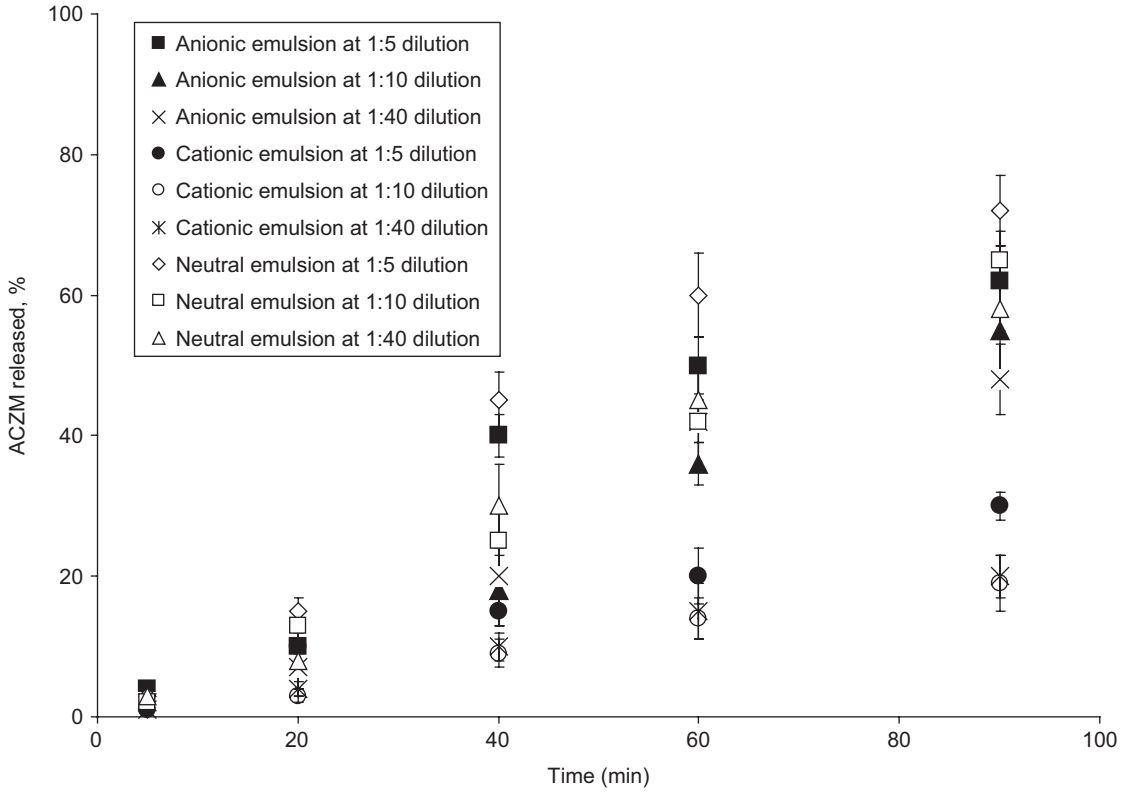

Figure 3. Acetazolamide (ACZM) *in vitro* release profiles from a typical cationic nanosized emulsion formulation as a function of increasing dilution ratios (1:5–1:40) of simulated tear solution (STS).

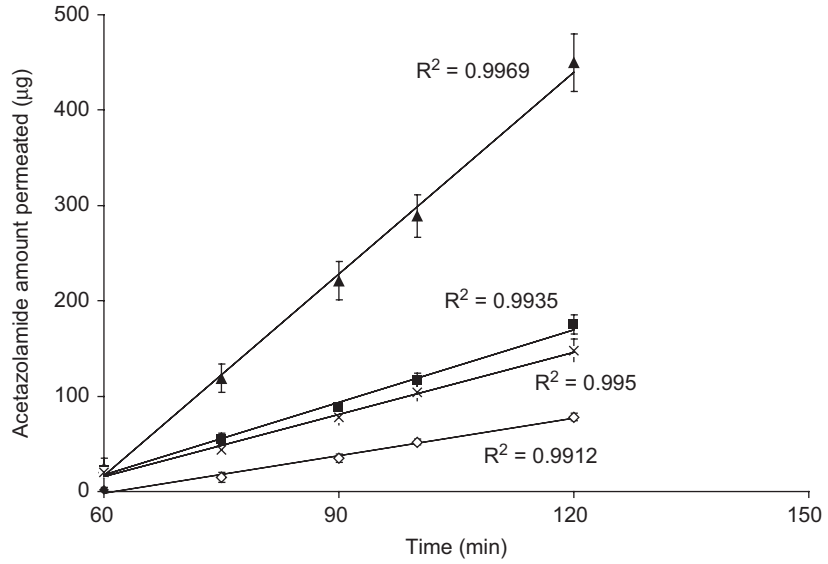

Figure 4. Acetazolamide (ACZM) amount (µg) permeated through goat cornea versus time (min). Key: ◊ ACZM solution; ■ ACZM-loaded anionic emulsion; ▲ ACZM-loaded cationic emulsion; × ACZM-loaded neutral-charged emulsion.

Table 5. Comparison of acetazolamide (ACZM) formulations in terms of % permeation, steady-state flux, and apparent permeability coefficient P_{app} from *in vitro* permeation studies using goat cornea.

Formulations	% Permeation	Lag time (min)	Steady-state flux (μg/min)	Apparent permeability coefficient, P_{app} (cm/sec) ($\times 10^{-6}$)
ACZM solution	7.80 ± 0.86	60	0.25 ± 0.3	2.52 ± 0.42
ACZM-loaded anionic emulsion	17.50 ± 1.4	60	1.10 ± 0.2	5.64 ± 0.14
ACZM-loaded cationic emulsion	45.00 ± 4.3	60	2.22 ± 0.1	13.32 ± 0.92
ACZM-loaded neutral-charged emulsion	14.80 ± 1.3	60	0.96 ± 0.1	4.87 ± 0.52

All values are expressed as mean ± SD ($n = 3$).

emulsion were presented (Table 3 and Figure 2). In spite of an insignificant statistical difference among the mean droplet diameters of the ACZM-loaded cationic emulsion, there was a moderate tendency of the mean diameter values to decrease slightly from 250 to 218 nm with increasing storage time irrespective of the storage temperature (Table 3). Therefore, the identified experimental conditions that yielded the smallest emulsion droplet size are considered the optimal conditions for the manufacturing and processing of the specific emulsion formulation.

Dependence of mean particle diameter of the non-phospholipid-based cationic emulsion on number of freeze–thaw cycles in the absence and presence of cryoprotectants is depicted in Figure 2. In the absence of cryoprotectants, the mean particle size of the emulsion was found to increase progressively from 243 to 480 nm after five cycles. A number of physicochemical mechanisms may be responsible for the extensive droplet aggregation observed in the absence of cryoprotectants. First, when the emulsion was placed in the freezer, some of its water crystallized. This caused the dispersed oil droplets to come into closer proximity because the oil droplets were confined to the nonfrozen regions remaining in the aqueous phase.[34] When there was no presence of sufficient free water to fully hydrate the oil droplet surfaces,[35–37] the droplet–droplet interactions was forced closer together to effect coalescence to occur. Second, ice crystallization led to an increase in the ionic strength of the freeze-concentrated nonfrozen aqueous phase surrounding the emulsion droplets.[36] Third, it is possible that ice crystals formed during freezing may have penetrated into the oil droplets and disrupted their interfacial membranes. This allowed the oil droplets more prone to coalescence between them. Fourth, cooling may have caused some of the fat in the emulsion droplets to crystallize promoting partial coalescence due to penetration of a fat crystal from one droplet through the membrane of another droplet.[38,39]

In contrast, the presence of cryoprotectants improved the stability of emulsions to droplet aggregation during freeze–thaw cycling. For example, after one cycle there was no significant change in the mean droplet diameter in the arachis oil-based nanosized emulsion containing 5% sucrose and 5% sorbitol (remained the same around from 240 nm), and even after five cycles, the mean droplet diameter increased to 260 nm only.

A number of mechanisms have been proposed to account for the ability of cryoprotectants to improve the stability of emulsions to aggregation during freeze–thaw cycling. First, cryoprotectants increased the osmolyte concentration in the aqueous phase, thereby reducing its crystallization temperature. Subsequently, the total amount of ice crystals formation was limited and therefore the volume of nonfrozen aqueous phase available to the oil droplets was increased.[36] Second, cryoprotectants formed hydrogen bonds with emulsifiers adsorbed to droplet surfaces, thereby reducing the tendency for interactions to occur between droplet surfaces when the free water content was reduced by ice crystallization.[37]

In vitro drug release

To define optimal experimental conditions for the preparation of drug-laden nanosized emulsions that can retain its adsorbed/incorporated ACZM in the presence of electrolytes under the physiological conditions existing in the lachrymal sac in the presence of tear, ACZM release profiles from emulsions diluted in different ratios of DDW and STS ranging from 1:5 up to 1:40 were determined. Preliminary experiments show that irrespective of the charges, <10% of ACZM was released from the nanosized emulsions following various DDW dilutions up to 1:40 (data not shown). However, when DDW was changed by a STS, a rapid release up to 72% of ACZM from the neutral-charged nanosized emulsions over 90-min time was observed (Figure 3). This indicates that the presence of electrolytes in the STSs induced rapid release. It should be emphasized that proteins and specifically albumin can displace anionic drug like ACZM from the emulsions. However, the concentration of albumin in STS is only 0.1 mM whereas the concentrations of chlorides and carbonates are 130 and 7.6 mM, respectively. Thus, the displacement effect should be mainly mediated by the anions in the STS. Cl^{-1} and CO_3^{-2} probably enabled the rapid release of the ACZM from the neutral-charged emulsion over a 90-min period, indicating that an anion-exchange process was probably involved. Although this type of anion-exchange process is not expected to occur in anionic nanosized emulsion, the drug release was found to be higher than that of the cationic nanosized emulsion. The observed higher drug release from anionic emulsion could be corroborated with the inherent property of the emulsion system. It is well-known that emulsions

especially the anionic ones possessed a poor drug-retarding effect when sink condition prevails. Meanwhile, electrostatic repulsion may occur between the drug and negatively charged emulsion droplets resulting in a higher percentage of drug release. On the other hand, it is noteworthy that increasing the dilution ratio from 1:5 to 1:10 and more particularly to 1:40 did not show any sudden increment in initial fraction released from any of the emulsions at 5-min period. After the 5-min period, a progressive increment in the drug release was noted for all the three emulsions studied. But cationic emulsion showed somewhat delayed drug release behavior in comparison with the other two emulsions. Being a holder of an anionic drug, ACZM, the cationic emulsion might have liberated its cargo rapidly on contact with anions-possessing medium like STS through the above-said anion-exchange process. In spite of that, the cationic emulsion delayed the drug release, which could possibly be related to the emulsion's ability to hold the drug even in the presence of competing anions or to the delay in drug partitioning out of the charged emulsifier (mono- or multilayer) film. It is to be noted that the *in vitro* release results are consistent with those of the EE, as the cationic emulsion with the highest drug EE (i.e. low leakage ability) showed the lowest drug release percentage.

To substantiate our explanation of delay in drug partitioning out of the charged emulsifier even in the presence of competing anions, Hagigit et al.[40] previously suggested a mathematical model equation developed by Bhaskar et al.[41] to test the particle diffusion controlled release of drug from the cationic nanosized emulsions (phospholipid-based) instead of the Fick's diffusion law-based traditional equations. In the present study, the same model equation was examined for the non-phospholipid-based cationic emulsion and the equation is given below.

$$-\mathrm{Ln}(1-F) = 1.59\,(3/r)^{1.3} \times D_{\mathrm{i}}^{0.65} \times t^{0.65} \quad (5)$$

This equation suggests that particle diffusion control can be tested by simply testing for linearity between Ln(1–F) and $t^{0.65}$. In the majority of cases studied so far, the rate-determining step of the ion-exchange process was established to be diffusion of the counter ions rather than an actual chemical exchange reaction at the fixed ionic groups. Thus, in the present study, the ACZM release experiments were tested for counter ion diffusion as suggested by Equation (5). Plots of Ln(1–F) versus $t^{0.65}$ for ACZM exchange is shown in Figure 5. It can be seen from Figure 5 that the kinetic data do not conform with counter ion diffusion since the R^2 value is not close to 1. The exchange with anions from STS should not be a simple exchange process leading to significant deviations from the expected mathematical model. The *in vitro* release kinetic behavior of ACZM exchange with physiological anions appears to be complex and difficult to characterize using mathematical fitting equations. The non-conformity does not exclude potential therapeutic efficacy since it is expected that despite infinite dilutions and the presence of anions a majority of the associated ACZM molecules that remain within the cationic nanodroplets would elicit the desired pharmacological action once the drug-loaded cationic nanosized emulsion droplets reach the target cells following topical application. It can be deduced that non-phospholipid-based cationic nanosized emulsion can maintain the initial physicochemical properties, associate ACZM molecules, and release them under appropriate physiological conditions. Furthermore, the ACZM release

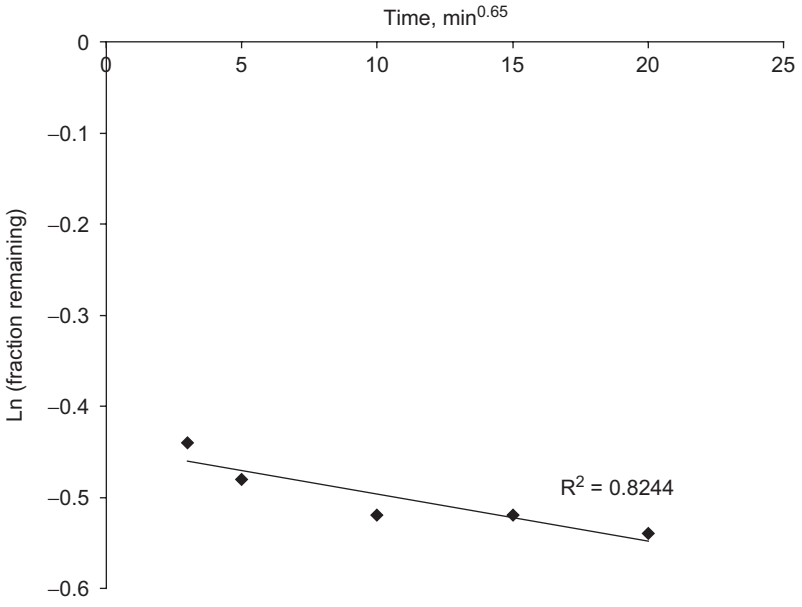

Figure 5. Ion-exchange equation release plot of acetazolamide (ACZM) from typical non-phospholipid-based cationic nanosized emulsion in 1:40 dilution with simulated tear solution (STS).

kinetic process will not be governed by a film diffusion process but rather by low oil degradation at the tissue of target. Further pharmacokinetic and pharmacological animal studies are needed to validate our assumptions and verify the *in vitro* ACZM release kinetic hypothesis.

In vitro transcorneal permeation

ACZ is a poorly water- and lipid-soluble drug. Its poor lipid solubility limits its transit through the corneal epithelium and endothelium, whereas poor aqueous solubility prevents the transit through the hydrophilic stroma.[42] The analysis of data from Table 5 indicates that the ACZM-loaded cationic emulsion was the most efficient in drug permeation according to % of permeation, steady-state flux, and P_{app} values. The anionic and neutral-charged emulsions were showed lower permeation than the cationic emulsion. On the other hand, the ACZM from its solution was permeated in a much lesser extent compared with the emulsions. It is important to highlight that the ACZM concentration in its solution is 10-fold higher than in the case of the currently developed ACZM-loaded anionic, cationic, and neutral-charged nanosized emulsions. The observed noticeable differences in permeation efficiency between solution and emulsion formulations can be attributed to the fact that the ACZM was entrapped at oil–water interface of the emulsion or simply dissolved in a vesicular type oil droplet protected by a mono- or multilayer emulsifier films. Moreover, a hydrophilic solution form of the drug is not at all favorable for the permeation phenomenon to occur through goat cornea probably due to the lesser contact time between the drug and cornea.

A remarkable fact from the results of the present study is that the cationic emulsion provided higher ACZM permeation coefficient value than anionic and neutral-charged emulsions. There are evidences that colloidal delivery systems can facilitate the penetration of drugs into ocular surface tissues through an endocytic mechanism.[43] As previously suggested, the endocytic effect is probably more pronounced with the phospholipid-based cationic emulsion.[44] In experiments of keratocyte cell-culture exposed to either anionic or cationic blank emulsion (phospholipid-based), only the positively charged oil droplets were internalized in the keratocytic cells (unpublished data). In spite of differences in blink frequency, ocular surface permeability, and aqueous humor dynamics between goat and man, a mean ACZM % permeation of 45.00 ± 4.3 as observed with non-phospholipid-based cationic emulsion, in this present study, was of further clinical interest. A high and fast penetration achievement after *in vitro* transcorneal permeability through goat cornea might be an indication that the topical administration of ACZM-loaded cationic emulsion to patients with glaucomatous condition should provide adequate levels of ACZM in aqueous humor resulting in a decreased production of aqueous humor and hence a lowering of IOP.

Conclusion

The influence of ACZM (0.1% w/v) loading on the *in vitro* performances of non-phospholipid-based cationic nanosized emulsion in comparison with phospholipid-based anionic and neutral-charged nanosized emulsions were investigated. Whereas anionic emulsion was prepared based on lecithin–oleic acid–poloxamer emulsifier combination, cationic emulsion was developed using poloxamer–chitosan emulsifier mixture. Neutral-charged emulsion was manufactured by utilizing a lecithin–poloxamer emulsifier blend.

All the comprehensive ACZM-associated experimental results suggest that an electrostatic attraction occurred between the cationic dispersed oil droplets of the emulsion and the anionic ACZM molecules at the o/w interface of the cationic nanosized emulsion. It was noted in the kinetic experiments that the presence of electrolytes in the STSs induced a rapid drug release from the neutral-charged emulsion mediated by an anion-exchange process. ACZM-loaded cationic emulsion possesses a better permeation through corneal tissues than the anionic and neutral-charged emulsions.

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Declaration of interest

The authors report no declarations of interest.

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