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Original Article

NANOEMULSION AS A NOVEL OPHTHALMIC DELIVERY SYSTEM FOR ACETAZOLAMIDE

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ABSTRACT

Objective: The aim of this work was to formulate the antiglaucoma drug acetazolamide as ocular nanoemulsion of high therapeutic efficacy and prolonged effect.

Methods: Eighteen nanoemulsion formulaions consisting of different oils, surfactants and cosurfactants at various ratios and constant water content (39%) were prepared based on their constructed pseudoternary-phase diagrams. According to the *In vitro* release studies, three nanoemulsions which exhibited fast drug release were used to prepare acetazolamide nanoemulsions with higher water content (59%). The six nanoemulsions at either water content (39 or 59%) were evaluated for their physicochemical properties and ex- vivo corneal permeability. In addition, Draize rabbit eye irritation test was performed. Moreover, biological evaluation of acetazolamide nanoemulsions for their intraocular pressure lowering activity on glaucomatous albino rabbits was carried out.

Results: Isopropyl myristate nanoemulsion prepared with cremophor EL and transcutol P exhibited the fastest drug release among all isopropyl myristate nanoemulsions. Oleic acid nanoemulsion prepared with mixture of tween 80 and cremophor EL as surfactants together with transcutol P showed the fastest drug release among other oleic acid nanoemulsion formulae. Similar results were also observed for peanut oil nanoemulsions. The above mentioned formulations either at 39% or 59% water content showed acceptable physicochemical properties and higher acetazolamide permeability coefficient through goat corneas than that reported for the free drug. They also were non irritant to rabbit eye. Therapeutic efficacy testing revealed that peanut oil nanoemulsion at 39% water content showed better and prolonged intraocular pressure lowering effect relative to either commercial brinzolamide eye drops (Azopt®) or the commercial oral acetazolamide tablet (Cidamex®).

Conclusion: Acetazolamide was successfully formulated in nanoemulsion form which revealed high therapeutic efficacy in treatment of glaucoma together with prolonged effect.

Keywords: Acetazolamide, Glaucoma, Nanoemulsion, Topical ophthalmic formulation, Ocular irritation.

INTRODUCTION

Glaucoma, one of the leading causes of irreversible ablepsia in the world, is estimated to be suffered by 70 million people. It generally occurs in people aged above 40, but it may also influence the visual acuity of much younger people, even children [1]. Glaucoma is an ophthalmic disorder characterized by an increase in intraocular pressure (IOP), which results in a damage to the optic disc and visual field disturbances. Increase in IOP (above 21 mmHg or 2.8 kPa) is a consequence of an imbalance between the production and drainage of aqueous humor.

Carbonic anhydrase inhibitors (CAIs), as a mainstay in the medical treatment of glaucoma, have been used since 1954 [2], owing to their ability to lower IOP by reducing aqueous humor formation in the ciliary body.

Amongst the available CAIs, acetazolamide (ACZ) is still the most effective drug for the treatment of glaucoma [3]. Yet, it is not available in market as topical ophthalmic formulation due to two major problems that hinder the topical effectiveness of ACZ which are its poor aqueous solubility (0.7 mg/ ml) and low permeability coefficient of (4.1×10^{-6} cm/ sec). To obtain the desired lowering in IOP, large oral doses of ACZ are used which lead to unpleasant systemic side effects such as central nervous system depression, renal failure, diuresis, vomiting, anorexia, and metabolic acidosis [4, 5]. Thus, to abolish systemic side effects many attempts were performed to formulate ACZ in topical dosage forms with higher effectiveness as ophthalmic application of drugs remains the primary and patient well accepted administration route for the treatment of various eye diseases.

These include topical formulations of ACZ solution in the form of sodium salt [3], contact lenses containing ACZ [6], aqueous ACZ solution using 2-hydroxypropyl-β-cyclodextrin [7], aqueous ACZ solution using cyclodextrins in combination with bio adhesive polymers, penetration enhancers, and co-solvents [8] and polymeric suspensions of ACZ containing viscolyzers and penetration enhancers [9]. However, all these attempts 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 [10]. More recent attempts investigated incorporation of ACZ in vesicular drug delivery systems for ocular topical therapy. Niosomes have been reported as a possible approach to improve the low corneal penetration and bioavailability characteristics of ACZ [11], topical ocular formulation of ACZ using large unilamellar [12] and multilamellar [13] liposomes as a vehicle and a trial to formulate ACZ in nanosized emulsion [14] have also been reported in literature. For several years, nanoemulsions (NEs) have been investigated as new drug delivery systems, and their potential uses in ophthalmology have been studied by several research teams [15]. Formulations based on NEs have several interesting characteristics such as enhanced drug solubilization, good thermodynamic stability and ease of preparation [16, 17]. However, the most critical problem regarding the NE based drug carriers is the toxicity of the components. Recent efforts have been focused on how to decrease or eliminate the toxicity or irritation of the NE formulations [15].

The objective of the present study was to formulate ACZ in the form of NE for topical ocular administration of the drug using ocular safe components. The NE form is thought to offer a sustained release effect and high penetration of the drug through the cornea. This would therefore provide the advantages of conventional eye drops (ease of application and high patient compliance) and eliminate their disadvantages (low bioavailability and frequent administration).

MATERIALS AND METHODS

Materials

ACZ (99.9% purity) was obtained from Chemical Industries Development Pharmaceutical Company (CID), Egypt. Tween 80,

tween 20, propylene glycol (PG), oleic acid, castor oil, potassium dihydrogen phosphate, disodium hydrogen phosphate and sodium chloride were obtained from Gomhorya Company, Egypt. Peanut oil (arachis oil) was obtained from Nefertari Body Care, Egypt. Transcutol P (diethylene glycol monoethyl ether) was kindly donated by Gattefosse, France. Isopropyl myristate (IPM) was supplied by Sigma Chemical Co., USA. Cremophor EL (polyoxyl 35 castor oil) was kindly donated by BASF, Germany. All other chemicals were of analytical grade.

Methods

Determination of ACZ saturated solubility in different NE components

To find out appropriate surfactants, cosurfactants and oils that have good solubilizing capacity of ACZ, the solubility of ACZ in various components was measured. Surfactants employed were tween 80, tween 20, labrasol, brij 30 and cremophor EL. Cosurfactants used were PG and transcutol P. Oils employed were IPM, oleic acid, peanut oil and castor oil.

In screw capped vials, an excess amount of ACZ was added to 2 ml of each selected NE component, and shaken in a thermostated shaking water bath (GFL 1083, Germany) at room temperature for 24 h. The suspensions were centrifuged at 3000 rpm for 30 min. Then, 1 ml of the supernatant was dissolved in 50 ml dimethyl sulfoxide (DMSO) and diluted to 100 ml using phosphate buffer saline (PBS) pH 7.4. Concentration of ACZ was determined spectrophotometrically at λ_{max} 263 nm (Shimadzu UV-1601 PC, Kyoto, Japan). Each determination was performed in triplicate. The blanks for the experiments were prepared using 1 ml of pure substance dissolved in DMSO and diluted to 100 ml using PBS.

Construction of pseudo-ternary phase diagrams

Pseudoternary phase diagrams were constructed to obtain the components and their concentration ranges that can result in large existence area of plain NE.

The pseudoternary phase diagrams of oil (IPM, oleic acid and peanut oil), surfactant (tween 80 and cremophor EL), and cosurfactant (PG and transcutol P) were developed using water titration method at 25°C [18] at various surfactant/cosurfactant (S/COS) ratios (km ratios).

In case monophasic, clear, and transparent mixtures were visualized after stirring, the samples were marked as points in the phase diagram. The area covered by these points represents the region of NE and was measured using AutoCAD® software (Autodesk, Inc., San Rafael, CA, USA).

Preparation of ACZ loaded NEs

From the constructed pseudoternary phase diagrams, eighteen NEs were selected at the predetermined optimum km ratio in the area of dilutable NEs in order to prepare stable NE formulae that withstand physiological dilution process occurring after ocular administration. To prepare drug loaded NEs, 1% w/w ACZ was sonicated with surfactant / cosurfactant / oil blends until complete dissolution of the drug then the aqueous phase containing 3% w/w DMSO was added dropwise to prepare NEs containing 39% w/w aqueous phase, while to prepare NEs at 59% water content, aqueous phase containing 20% DMSO was used. DMSO was added in order to prevent any precipitation of the drug after the addition of the aqueous phase. Drug loaded formulae were preserved with benzalkonium chloride (0.01%) [19].

Examination under cross polarized microscope

In order to exclude liquid crystalline systems and to verify the isotropic behavior of NEs, all formulae were examined under cross polarized microscope (Olympus, Japan) to insure the absence of birefringence [20, 21].

***In vitro* release studies of ACZ from different formulae**

In vitro drug release studies of the developed drug loaded NEs (0.5 g each) were performed in triplicate in a USP dissolution tester apparatus type II (Hanson SR8 - Plus, USA) at 34 ± 0.5 ºC to simulate the ocular surface temperature [22]. The receptor phase was composed of 100 ml PBS pH 7.4 and it was constantly stirred at 50 rpm throughout the experiment. Drug release was performed through a semipermeable membrane (Spectra Por©). At definite time intervals, 1 ml of the receptor phase was withdrawn and replaced by fresh buffer to maintain a constant volume. Drug concentration was determined spectrophotometrically at 263 nm. Percent of released drug was plotted versus time. The mean dissolution rate (MDR) was calculated for each NE formulation according to the following equation:

$$MDR = \left\{\sum_{j=1}^{n} \Delta M_j \left| \Delta t \right.\right\}/n$$

Where n is the number of dissolution sample times, Δt is the time at midpoint between t and t_{-1} (easily calculated with $(t + t_{-1})/2$) and ΔMj is the additional amount of drug dissolved between tj and t−1 [23].

The drug release profiles were fitted to zero order, first order and Higuchi diffusion models to estimate the best fitting kinetic model having the highest correlation coefficient.

Physicochemical characterization of NEs

Evaluation of rheological properties

Viscosity of the prepared formulae was determined using cone and plate viscometer (Brookfield, USA), spindle 40. Shear rate in the range of (37.5 - 375 S^{-1}) was applied and the resulting shear stress was measured.

Droplet size determination

The mean droplet size (MDS) was determined by photon correlation spectroscopy (PCS) using a Zetasizer Nano ZS (Ver.6.20, Malvern Instrument Ltd., Worcestershire, England). All measurements were performed in triplicate at room temperature (25 ºC) at 90° to the incident beam.

pH measurements

pH of the NE formulations was measured by pH meter (JENWAY model 350, JENWAY Ltd., UK).

Refractive index measurements

Refractive index was determined at 25 ºC using refractometer (M 46.17 / 63707 Higler and Walts Ltd., England).

Surface tension measurements

Surface tension measurements were carried out room temperature using a thermostatically controlled processor tensiometer K100 (Kruss GmbH, Germany) provided with a Du Nouy ring (ring radius 9.545 mm, wire diameter 0.37 mm).

Osmolality determination

Osmolality was measured using Micro Osmometer (model 3300, Advanced Instruments Inc., USA).

Thermodynamic stability studies

The thermodynamic stability of the formulae was evaluated on three phases according to the protocol designed by Shafiq *et al*. [24]. Initially, the formulae were subjected to six heat (45 ºC) – cool (4 ºC) cycles with storage for 48 h at each temperature. Then, they were centrifuged at 3500 rpm for 30 min and finally exposed to three freeze (−21 ºC) – thaw (25 ºC) cycles with storage for 48 h at each temperature.

Ex vivo corneal permeation studies using goat corneas

Goat corneas were used to study the permeation across the corneal membrane. Whole eyeballs of goats were procured from a slaughter house and transported to laboratory in normal saline maintained at 4 ºC. The corneas were carefully removed along with a 5 – 6 mm of surrounding scleral tissue and washed with cold saline. The washed corneas were kept in cold freshly prepared solution of simulated tear fluid (STF) of pH 7.4 [25]. The study was carried out by using Franz-diffusion cells in such way that epidermal side was in an

intimate contact with formulation in the donor compartment. The corneal area available for diffusion was 0.785 cm^2. The receptor compartment was filled with STF pH 7.4 at 34°C ± 0.5 ºC. The receptor medium was stirred at 50 rpm. The samples were withdrawn at different time intervals and replenished with an equal volume of STF pH 7.4 at each time interval.

The permeation study was carried out for 4 h, and samples were analyzed for ACZ spectrophotometrically at 263 nm. Results were expressed as the mean of three experiments ± S. .D.

The amount of drug permeated per unit area through excised cornea (µg / cm^2) versus time (h) was plotted and the permeation parameters of ACZ in the different formulae were calculated [26-28].

The steady state flux (J) values across excised cornea were evaluated from the linear ascents of the permeation graphs by means of the relationship

$$J = dQ / dt\ A\ (\mu g / cm^2\ hr)$$

The permeation coefficient P was calculated as:

$$P = J / C_0 \times 60 \times 60\ (cm / s)$$

Where C_0 represents the initial drug concentration in donor compartment (µg / cm^3)

The drug absorption lag times (indicating the time taken by the drug to saturate the cornea and to reach the receiving compartment) were calculated from x-axis intercept values of regression lines.

Corneal hydration

At the end of the experiment, each cornea (freed from adhering sclera) was weighed, soaked in 1 ml methanol, dried overnight at 80 °C, and reweighed. From the difference in weights, corneal hydration was calculated [29-31].

Ocular irritation studies

Animal housing and handling

Qualitative as well as quantitative estimation of ocular irritancy of ACZ loaded NE formulations was studied on adult male New Zealand albino rabbits weighing about 2 – 3 kg. The protocol of the study was approved by the Research Ethics Committee in the Faculty of Pharmacy, Cairo University, Egypt. The rabbits were housed singly. The standard environmental conditions, i. .e. dark: light cycle 12 h each, 20 – 25 °C temperature and 40 – 70% relative humidity were maintained. Rabbits were fed with standard pellet diet and water was provided ad libitum. All the animals used in the experiment were healthy and free from any clinically observable ocular abnormalities.

Ocular irritancy test

The test was conducted as per the Modified Draize Test [32, 33] on a group of 12 New Zealand albino rabbits. All the glass wares used in the experiments were sterilized by autoclaving (Remi Equipments Pvt. Ltd., Mumbai, India). All applied formulations were sterilized by filtration technique using 0.22 µm membrane filters (Millipore, USA). 25 µl of each formulation was instilled into the lower cul-de-sac of the eye of each rabbit with the help of needleless syringe. The untreated contralateral eye was used as a control. A separate control group of two rabbits received normal saline. The eyelids were gently held together for about 10 S for avoiding the loss of instilled solution. Each animal was observed for ocular reactions (redness, discharge, conjunctival chemosis, iris and corneal lesions) at 5, 10, 15, 30 min and 1, 2, 3, 6, 9, 12, 24 h post instillation. The ocular irritancy test was performed by providing 0 (absence) to 4 (highest) grades on clinical evaluation scale as described in table 1 [32, 33]. Overall ocular irritation index (Iirr) was calculated by summing up the total clinical evaluation scores over the observation time points. A score of 2 or 3 in any category or Iirr more than 4 was considered as an indicator of clinically significant irritation.

Table 1: Modified Draize grading scale for clinical evaluation of ocular irritation.

Parameter	Observation	Score
Conjunctival discharge	Normal	0
	Slight discharge	1
	Severe discharge covering a small area around the cornea	2
	Severe discharge covering a large area around the cornea	3
Conjunctival chemosis	Normal	0
	Slight chemosis including nictating membrane	1
	Severe chemosis with eye partially closed	2
	Severe chemosis with eye closed	3
Conjunctival redness	Blood vessels normal	0
	Some blood vessels definitely hyperaemic	1
	Diffuse color, individual vessels not easily discernible	2
	Diffuse beefy red	3

Therapeutic efficacy studies

Twelve New Zealand albino rabbits weighing 2.0 – 3 kg were randomized in groups of four having three in each. Experimental glaucoma was induced in the rabbits by an intraocular injection of 0.25 ml of 2% w/w sodium carboxy methylcellulose [34].

The IOP of the rabbits were measured using an indention tonometer (Schoetz tonometer, Teufel Germany). Before taking the measurement, eyes were anesthetized with 1 – 2 drops of benoxinate hydrochloride (Benox®) eye drops.

The resting glaucomatous IOP was measured twice a day in the 2 days before drug application. In this way, the normal baseline of each animal was established before treatment. All experiments were carried out at the same time of the day.

A single 50 µl dose of 1% ACZ loaded peanut oil NE with 39% and 59% aqueous content (NE 18a and NE 18b) was applied topically to the cornea of the first two groups. In addition, 50 µl aliquots of Azopt® (brinzolamide, 1% w/v) were administered to the rabbits of the third group for comparison. The fourth group received orally, a fraction of ACZ tablet (Cidamex®) equivalent to 9 mg ACZ. All the measurements were performed 2 times, at each interval, and the mean was calculated. The IOP was measured at 30, 60, 90, 120, 150, 180, 240, 300 and 360 min.The reduction of IOP versus time plots; provide the therapeutic profiles of ACZ NEs versus ACZ tablet and 1% brinzolamide. These profiles were used to calculate the pharmacodynamic parameters: area under the curve (AUC_{0-6}), time required to achieve peak IOP reduction (t_{max}), maximum percentage decrease in IOP and the mean residence time (MRT) using WinNonlin® software (Ver. 1.5, Scientific consulting Inc., Cary, NC, USA).

Statistical analysis of the results was performed using one way analysis of variance (ANOVA) which was computed with GraphPad Instat software (GraphPad software, USA).

RESULTS AND DISCUSSION

Determination of ACZ saturated solubility in different NE components

Preliminary ACZ solubility study was carried out for the identification of NE components that show highest solubility and

thus would be used for preparation of ACZ loaded NEs. table 2 lists the solubility of ACZ in different surfactants, cosurfactants and oils.

Table 2: ACZ solubility in different NE components at room temperature

	NE component	Solubility (mg/ml)
Surfactants	Tween 20	12.9 ± 0.41
	Tween 80	17.2 ± 0.89
	Cremophor EL	15 ± 0.75
	Labrasol	8.2 ± 0.22
	Brij 30	3.4 ± 0.14
Cosurfactants	Transcutol P	22.4 ± 0.9
	PG	3.7 ± 0.21
Oils	IPM	1.2 ± 0.11
	Peanut oil	9.3 ± 0.24
	Oleic acid	1.6 ± 0.2

Selection of NE components

For the present study, tween 80 and cremophor EL were used as non ionic surfactants, as they showed the highest solubility of ACZ among other surfactants. In addition, tween 80 is widely used in ophthalmic preparations due to its safety. It is listed for ophthalmic preparations in US Pharmacopeia-National Formulary, European Pharmacopeia, and the Japanese Pharmacopeia [35]. Moreover, an ocular irritation evaluation test was made by Alany *et al*. [36] and classified tween 80 as practically nonirritant. On the other hand, according to informations provided by the manufacturer, the instillation of 0.05 ml of cremophor EL in the rabbit's conjunctival sac caused only slight reddening of the conjunctiva which disappeared within a few hours. It is also reported that the application of a 50% aqueous solution of cremophor EL caused slight irritation with lacrimation, which disappeared rapidly whereas 30% aqueous solution had no irritant effect [37].

An additional important criterion for selection of the surfactants is their HLB values. The HLB value required to form o/w NEs should be greater than 10 [38]. Tween 80 and cremophor EL have HLB values of 15 and 12–14, respectively.

Transcutol P and PG were used as cosurfactants. Transcutol P showed the highest ACZ solubility among all tested components. It is known as common emulsion excipient suitable for dissolving or dispersing lipophilic drugs in ocular preparations [39]. Also, an ocular irritation study was performed by Ammar *et al* [19] on ocular NEs in which transcutol P was one of its component and they found that they were non irritant to rabbit eyes. Furthermore, it is reported that solutions of up to 50% PG caused no irritations to rabbit eyes, whereas the undiluted application was associated with a weak conjunctival redness [40].

Although peanut oil showed highest ACZ solubility, IPM and oleic acid were also used as oil phase in NE in order to study the effect of different oil structures on area of NE formation.

Peanut oil, IPM and oleic acid are structurally different oils. IPM is an ester of isopropanol and myristic acid which is a fatty acid of 14 carbon atoms. Oleic acid is a monounsaturated fatty acid of chain length of 18 carbons. Peanut oil is a vegetable oil that consists mainly of triglycerides of oleic acid, linoleic acid and linolenic acid, which are C_{18} fatty acids containing 1, 2 and 3 double bonds, respectively.

Construction of pseudoternary phase diagrams

Eighteen systems were prepared each at S/COS ratio of 1:1, 2:1, 3:1, 4:1, 5:1, 7:1 and 9:1; the pseudoternary-phase diagrams were mapped with the water titration method at 25 °C to identify the area of NE regions.

As expected from the previously reported data [41], the phase behavior of different systems was strongly influenced by the molecular volume of the oil incorporated within the NE. It was found that, NE regions obtained upon using IPM as an oily phase, were larger than in case of oleic acid which in turn were larger than those obtained using peanut oil. The latter failed to form NEs with S/COS ratios 1:1 and 2:1 (Fig. 1). Such findings coincide with that obtained by Aveyard *et al* who found that if the hydrocarbon chain length of the oil being dispersed is considerably less than the length of the hydrophobic part of the surfactant used, the oil will significantly penetrate the interfacial surfactant layer resulting in more efficient spontaneous NE formation and higher oil solubilization [42]. In addition, the presence of polar ester groups in oil structure decreases the oil hydrophobicity and results in stronger interactions between surfactant and oil molecules which lower interfacial tension [43, 44] and favor the formation of NE. In contrast, the presence of double bonds increases oil hydrophobicity [43].

Consequently, oleic acid showed smaller NE regions than that showed by IPM due to longer carbon chain, the absence of polar ester groups and the presence of one double bond.

Peanut oil showed the smallest NE regions. This finding could be on one hand due to its high triglyceride content which decreases oil surfactant interactions to a greater extent and on the other hand due to the presence of double bonds in fatty acid chains which lead to an increase in oil hydrophobicity and a more complicated spatial arrangement of the triglyceride structure [43]. The use of cremophor EL as surfactant resulted in smaller NE existence areas compared to tween 80 (Fig. 1). In order to increase the oil solubilizing capacity of cremophor EL and due to the fact that the flexibility of surfactant layer and its ability to partition at higher levels into the oil–water interface might be enhanced by combined surfactants [45-49], NEs with a mixture cremophor EL and tween 80 (1:1 weight ratio) were also prepared during the formulation of ACZ loaded NEs.

With respect to the cosurfactants used, the use of transcutol P resulted, generally, in higher NE existence areas than those obtained using PG. This may be attributed to the longer carbon chain length in transcutol P (6 carbons versus 3 carbons in PG) and also due to the increase in the number of hydroxyl groups in PG (2 versus 1 in transcutol P) which was found to reduce NE existence area. This was in agreement with El Maghraby [50] and Alany *et al* [51].

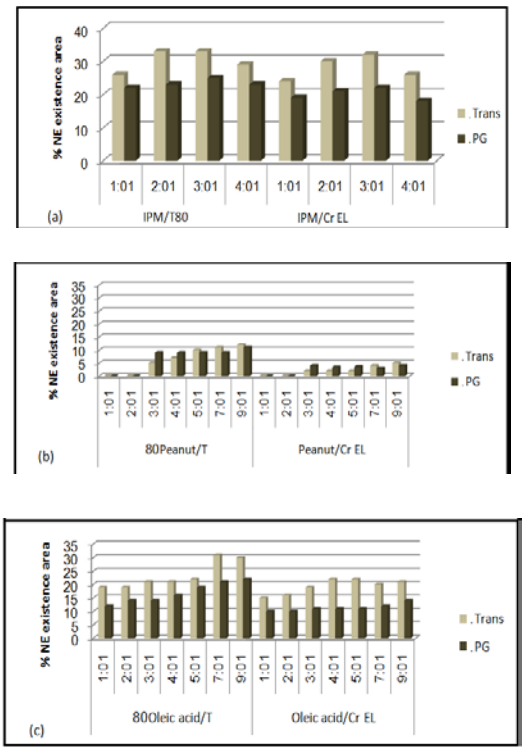

Fig. 1: Dependency of NE existence area on types of oil, surfactant, cosurfactant and surfactant to cosurfactant weight ratio: (a) IPM, (b) peanut oil and (c) oleic acid. (T80: tween 80, Cr EL: cremophor EL, Trans: transcutol P and PG: propylene glycol)

It is evident from Fig. 1 that for systems containing IPM the optimum S/COS weight ratio was found to be 3:1 while for systems containing peanut oil and oleic acid the optimum S/COS ratio was found to be 9:1.

Preparation of ACZ NEs

Eighteen NEs were formulated at the predetermined optimum S/COS weight ratio and at 39% (w/w) water content.

The prepared NEs were loaded with 1% (w/w) ACZ. The NE compositions are shown in table 3.

Examination under cross polarized microscope

In all previously prepared NEs, no liquid crystalline domain was observed. All the formulations appeared dark when viewed between crossed polarizers indicating isotropic nature of NEs.

Table 3: Composition of ACZ loaded NEs.**

Component (% w/w)	IPM	Oleic acid	Peanut oil	T 80	Cr EL	PG	Trans
NE 1	6	....	....	40.5	....	13.5	....
NE 2	6	....	....	40.5	....	....	13.5
NE 3	6	....	....	....	40.5	13.5	....
NE 4	6	....	....	....	40.5	....	13.5
NE 5	6	....	....	20.25	20.25	13.5	....
NE 6	6	....	....	20.25	20.25	....	13.5
NE 7	....	3	....	51.3	....	5.7	....
NE 8	....	3	....	51.3	....	....	5.7
NE 9	....	3	....	....	51.3	5.7	....
NE 10	....	3	....	....	51.3	....	5.7
NE 11	....	3	....	25.65	25.65	5.7	....
NE 12	....	3	....	25.65	25.65	....	5.7
NE 13	....	....	3	51.3	....	5.7	....
NE14	....	....	3	51.3	....	....	5.7
NE 15	....	....	3	....	51.3	5.7	....
NE 16	....	....	3	....	51.3	....	5.7
NE 17	....	....	3	25.65	25.65	5.7	....
NE 18	....	....	3	25.65	25.65	....	5.7

** All NE formulae contain 1% ACZ and 39% water

***In vitro* release studies of ACZ from different formulae**

The *In vitro* drug release profiles of the drug loaded IPM, oleic acid and peanut oil based NEs are graphically illustrated in Fig. 2. To facilitate comparison between release behaviors of different NE formulae, MDR was calculated and the results are represented in table 4.

The release results of IPM NEs revealed that formulae containing cremophor EL surfactant (NE3, NE 4) showed higher drug release than those prepared using tween 80 (NE1, NE2). This finding can be attributed to the higher thermodynamic activity of the drug in NE 3 and NE 4 as ACZ is more soluble in tween 80 than in cremophor EL. This was in agreement with Rhee *et al* [52].

For oleic acid and peanut oil NEs, the results revealed that formulae containing mixture of tween 80 and cremophor EL surfactant showed higher drug release than those prepared using either tween 80 or cremophor EL alone. This can be correlated to the fact that combined use of surfactants is significantly more effective in generating NEs, as it results in smaller droplet size.

The decrease in droplet size resulted in an increase of total number of oil globules and subsequent increase in their surface area which led to an increase in drug release.

This finding was observed in oleic acid and peanut oil NEs but not in NEs prepared using IPM which probably could be referred to the larger molecular volume of oleic and peanut oil which makes their nano emulsification more difficult.

For all prepared NEs, drug release from PG NEs was significantly lower than those containing transcutol P as cosurfactant. This is probably due to the higher viscosities of PG NEs than transcutol P NEs (Table 4) This was in agreement with Idrees *et al* [53]. Mathematical modeling of the data revealed that the dissolution profiles of all formulae followed Higuchi diffusion model.

Preparation of ACZ NEs with different aqueous phase content

NEs 4, 12 and 18 that exhibited highest drug release among NEs prepared using different oils were chosen to prepare NEs with 59% aqueous phase content in an attempt to prepare stable NEs with minimum surfactant concentration and to study the effect of increasing water concentration on the physico-chemical properties and permeation efficiency through goat corneas.

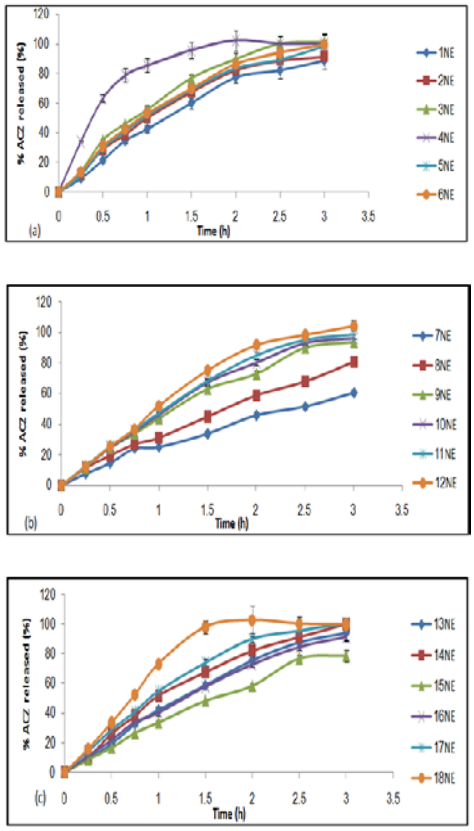

Fig. 2: Release profiles of ACZ from different NE formulations: (a) IPM NEs, (b) oleic acid NEs and (c) peanut oil NEs

Table 4: The effect of NE composition and viscosity on release efficiency represented by MDR

Formulation	Viscosity (cp)	MDR
NE1	486.6	0.18
NE2	157	0.75
NE3	1497	0.88
NE4	334.8	1.8
NE5	465.6	0.73
NE6	282.3	0.81
NE7	518	0.42
NE8	371.5	0.58
NE9	1048	0.68
NE10	805.7	0.72
NE11	685.4	0.73
NE12	402.9	0.74
NE13	1355	0.56
NE14	831.9	0.66
NE15	2736	0.49
NE16	1778	0.62
NE17	1758	0.83
NE18	1135	1.01

The same S/COS/oil ratios in these formulae were used to prepare NEs of higher water content (59% water content) (Table 5). The formulae with lower (39%) and higher (59%) were subjected to various physicochemical characterization.

Physicochemical characterization of NEs

Evaluation of rheological properties

It has been assumed that ophthalmic instillation of a formulation should influence the normal behavior of tears. Systems with low viscosity allow good tolerance with little blinking pain. In contrast, systems with enhanced viscosity, although less tolerant, induce an increase in ocular contact time by reducing the drainage rate and, as a consequence, improve bioavailability [54].

ACZ NEs exhibited a Newtonian behavior. The viscosity values of studied ACZ NEs at minimum shear rate were shown in (Table 6).

From the viscosity results, it could be observed that NE formulae prepared with IPM showed the lowest viscosities while that prepared with peanut oil showed the highest viscosity values. The viscosity of all NE formulae decreased upon dilution with aqueous phase.

Table 5: Composition of NEs exhibited highest drug release (NE 4, 12 and 18) at lower 39% and higher 59% water content. **

Component (% w/w)	NE 4 a	NE 4 b	NE 12 a	NE 12 b	NE 18 a	NE 18 b
IPM	6	4	….	….	….	….
Oleic acid	….	….	3	2	….	….
Peanut oil	….	….	….	….	3	2
T 80	….	….	25.65	17.1	25.65	17.1
Cr EL	40.5	27	25.65	17.1	25.65	17.1
Trans	13.5	9	5.7	3.8	5.7	3.8
Water	39	59	39	59	39	59

** All NEs contain 1% (w/w) ACZ.

Droplet size determination

The selected NEs showed MDS of 23.81 - 90.26 nm (Table 6). This small MDS could be related to the penetration of the cosurfactant molecules into the surfactant film. This would decrease the fluidity and surface viscosity of the interfacial film, lower the radius of curvature of the droplets and thus form transparent systems [55].

Three oils (IPM, oleic acid and peanut oil) were investigated at two different ratios (6, 4 in NEs prepared with IPM and 3, 2 % w/w in case of oleic and peanut oil). With respect to the effect of oil on MDS, it was found that IPM results in the smallest MDS followed by oleic acid and peanut oil which shows the largest MDS. This was consistent with the molecular volume of the different oils. The greater the molecular volume of oil, the lower its penetration in the S/COS interfacial film. Therefore, peanut oil which is a triglyceride with high molecular volume revealed highest MDS [56, 57].

For NEs containing the same oil, it was found that the larger the oil percentage, the larger the MDS. This finding was consistent with a previous report showing that the MDS increases significantly when more oil is incorporated owing to the expansion of oil droplet of the NE by further addition of the oil [58].

pH measurements

The ideal pH for maximum comfort when an ophthalmic preparation is instilled in the eye should be in the range of 7.2 ± 0.2 [19]. In some cases, the instillation of a solution with a pH different from tears causes irritation and painful sensation. This depends on the instilled volume, the buffering capacity, the composition and the contact time of the solution with the eye surface [19]. Different pH values can be tolerated if the preparation is not or is only very slightly buffered because in this case the limited buffering capacity of the tears is able to adjust the pH to physiologic levels on administration [59]. The pH of therapeutic substances applied as eye drops can vary from 3.5 to 8.5 [19]. The pH values of the developed NEs were in the range of 4.9 to 5.5 (Table 6) being therefore adequate to their application to the eye. The obtained values were also able to preserve drug stability as ACZ is highly unstable at an alkaline pH and has maximum stability at pH 4 to 5 [60, 61].

Refractive index measurements

Refractive index measurements detect possible impairment of vision or discomfort to the patient after administration of eye drops [59]. Refractive index of tear fluid is 1.340 to 1.360 [59]. It is recommended that eye drops should have refractive index values not higher than 1.476 [62].

Table 6 shows that ACZ NEs had refractive index values ranging from 1.347 to 1.366 which are within the recommended values.

Surface tension measurements

The surface tension values obtained for the NEs were in the range of 32.5 - 38.7 mN/m (Table 6) which is lower than the physiological value of the lachrymal fluid surface tension which ranges from 40 to 50 mN/m [59]. This result was expected because of the large amounts of surfactants used in the preparation of NE

Administration of eye drops with lower surface tension than that of the lachrymal fluid resulted in destabilization of the tear film [63, 64]. The low surface tension obtained for the developed NEs, thus, destabilized the tear film which can guarantee a good spreading effect on the cornea and mixing with the precorneal film constituents, thus possibly improving the contact between the drug and the corneal epithelium [65].

Osmolality Determination

The osmolality of the prepared ACZ NEs ranged from 860 to 1750 mOsm/kg (Table 6). A study on human tear osmotic pressure stated that the osmolality of lachrymal fluid is between 280 and 293 mOsm/kg on waking. As a result of evaporation when the eyes are open, osmolality may vary between 231 and 446 mOsm/kg [66]. Depending on the drop size, solutions with an osmolality lower than

100 mOsm/kg or higher than 640 mOsm/kg are irritant. However, Haße and Keipert [65] formulated ocular microemulsions with osmolality ranged from 1,200 to 2,400 mOsm/kg and found that they were nonirritant using hen's egg test on the chorioallantoic membrane and Draize test on rabbits' eyes. In addition, the original osmolality was restored 1 or 2 min after instillation of the nonisotonic solution [67].

Thermodynamic stability studies

The stability of the developed systems was evaluated via heat–cool cycles, centrifugation, and finally freeze–thaw cycle stress tests. The systems passed the alternate heat–cool cycles and remained clear. The second phase exploring the effect of centrifugation revealed promising physical stability; no signs of creaming, phase separation or cracking. Cloudiness was observed when the systems were frozen at −21 °C.

However, their clarity was recovered upon thawing. Similar findings were reported by Ammar *et al.* [19] and Tayel *et al.* [68] who related this transient instability to the coagulation of the internal phase at such low temperature and to the pressures exerted by the ice crystals on the dispersed globules and the adsorbed layers of S/COS.

Table 6: Physicochemical properties of ACZ NEs.

Formulation	Viscosity (cp)	Droplet diameter (nm)	Refractive index	pH	Surface tension (mN/m)	Osmolality (mOsm/Kg)
NE 4 a	334.8	40.86	1.362	5.2	32.5	1195
NE 4 b	15.7	27.75	1.347	5.3	35.8	860
NE 12 a	402.9	63.78	1.358	4.9	34.7	1750
NE 12 b	193.6	27.76	1.348	5.1	38.7	1246
NE 18 a	1135	90.26	1.366	5.2	33.2	1586
NE 18 b	83.7	23.81	1.358	5.5	36.9	1070

Ex vivo corneal permeation studies using goat corneas

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 [69]. From the results of the present study, it is obvious that water content and oil type in NEs affect ACZ permeation through goat corneas.

It was found that the increase in water content results in a decrease in permeation (Fig. 3). This may be attributed to the decrease in concentration of surfactants and cosurfactant in NE which act as permeation enhancers by increasing the membrane permeability and consequently the drug uptake. This result is consistent with Baroli *et al.* [70], Peltola *et al.* [71], Yuan *et al.* [58] and Zhu *et al* [72]. In addition, the increase in water content (39 to 59 %) resulted consequently in a decrease in the oil percentage in the formula thus the contact area between oil and lipophilic surface of corneal epithelium decrease. The increase in oil content in NEs resulted in more oil which was available to adhere to lipophilic surface of corneal epithelium and therefore could promote ACZ permeation.

By comparing the effect of oil type, it was evident that peanut oil showed the highest permeation followed by oleic acid then IPM. This observation could be attributed to ACZ solubility in these oils (Table 2) in addition to the differences in oils' lipophilicity. Peanut oil which is a triglyceride is the most lipophilic among the studied oils, so its nanodroplets are more likely to adhere to the surface of corneal epithelium and consequently resulted in an increased ACZ permeability through cornea either by lipophilic association through the corneal membrane or via endocytosis [73, 74]. The analysis of data from table 7 indicates that NE prepared with peanut oil at 39% aqueous phase content (NE 18a) was the most efficient in drug permeation according to the cumulative amount permeated per unit area, steady state flux, and permeability coefficient values.

A six fold increase in ACZ permeability coefficient was obtained in case of NE 18a as if compared with permeability coefficient of ACZ stated in literature.

Corneal hydration

Corneal hydration was calculated to evaluate the damage to corneal tissue. Normal cornea has a hydration level of 75 to 80% [75]. An alteration in this level indicates damage to the endothelium or epithelium. Corneal hydration of all of the formulations was between 75 to 80%, which indicated that the formulations did not cause any damage to the corneal tissue.

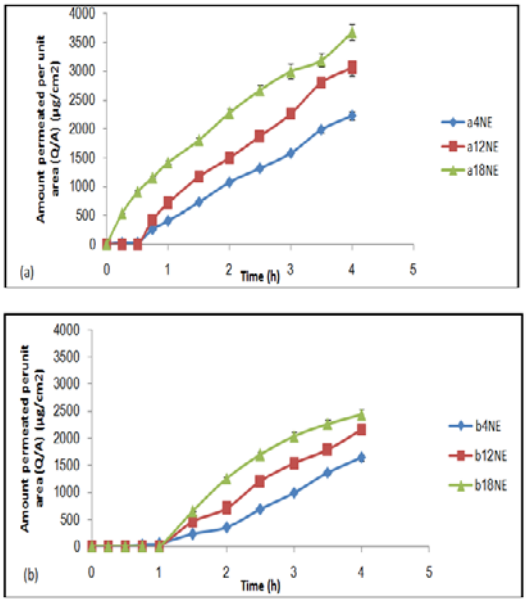

Fig. 3: ACZ permeation through goat corneas from different NEs: (a) NEs prepared at 39% water content and (b) NEs prepared at 59% water content

Table 7: Comparison of ACZ NEs in terms of permeation parameters obtained from ex vivo permeation studies using goat cornea.

Formula	Amount permeated /unit area (Q/A) after 4 hrs (µg/cm²)	Lag time (min)	Steady state permeation (J, µg/cm². .hr)	Permeability coefficient (cm/s)
NE 4a	2232.58 ± 76.09	30	591.1	1.64×10^{-5}
NE 12a	3057.32 ± 126.21	30	822.1	2.28×10^{-5}
NE 18a	3681.51 ± 144.7	0	852.5	2.36×10^{-5}
NE 4b	1644.54 ± 59.5	60	422.6	1.17×10^{-5}
NE 12b	2167.8 ± 74.19	60	588.9	1.64×10^{-5}
NE 18b	2448.11 ± 100.28	60	727.5	2.02×10^{-5}

Ocular irritation test

According to the scores of the Modified Draize Test (Table 1), the application of NEs revealed a very slight redness of the conjunctiva and a slight reflex lachrymation but no chemosis during the first 0.5 h after application of NE formulations with 39% aqueous content. In none of the six rabbits did the Iirr score exceed 4 at any time (Table 8). It has to be noted that lachrymation decreased until complete disappearance 1 h post administration while redness completely disappeared after 2 h. In case of NE formulae prepared with 59% aqueous content, rabbits showed very slight lachrymation after instillation which disappeared completely within 15 min and no signs of chemosis or redness were observed (Fig.4).

From the above results, it is evident that the NE formulations were non irritant and could be tolerated by the rabbit eye. Taking into consideration that the rabbit eye is more susceptible to irritant substances than the human eye [76], this result could be considered very promising.

Table 8: Observed irritation scores for different formulations

Formulation	Time for observing total Iirr (min)					
	5	10	15	30	60	120
NE 4 a	1	1.5	2	1.5	0.5	0
NE 12 a	1	1	2	1.5	0.5	0
NE 18 a	0.5	1	1.5	2	0.5	0
NE 4 b	0.5	0.5	0	0	0	0
NE 12 b	1	0.5	0	0	0	0
NE 18 b	1	0.5	0	0	0	0
0.9% NaCl	0	0	0	0	0	0

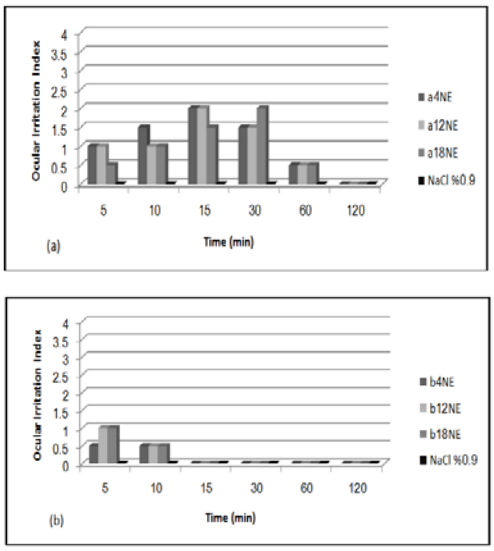

Fig. 4: Ocular irritation index of different formulations: (a) NEs prepared at 39% water content (b) NEs prepared at 59% water content

Therapeutic efficacy studies

Fig. 5 represents the percent decrease of IOP with respect to the baseline in glaucomatous rabbits versus time. It is evident from Fig. 5 that NE 18a shows optimum pharmacodynamic parameters. The calculated pharmacodynamic parameters namely; AUC_{0-6} (area under the percentage decrease in IOP – time curve), t_{max} (time required to achieve maximum decrease in IOP), maximum % decrease in IOP and MRT (mean residence time) are listed in table 9.

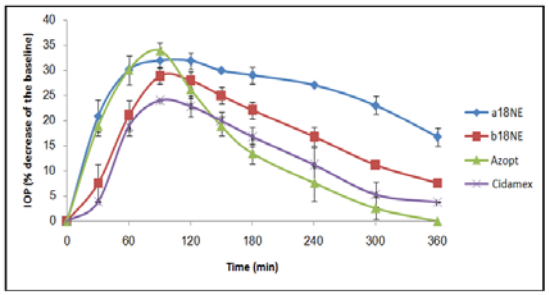

Fig. 5: Percentage decrease in IOP after administration of NE 18a, NE 18b, Azopt® eye drops and Cidamex® tablet

Table 9: The calculated pharmacodynamic parameters after administration of NE 18a, NE 18b, Azopt eye drops and Cidamex tablet.

Formulation	Max. % decrease in IOP	t_{max} (h)	AUC_{0-6}	MRT
NE 18 a	31.96±1.52	1.2±0.29	152±1.87	7.5±1.02
NE 18 b	29.9±0.01	1.7±0.29	104.4±5.5	4.07±0.184
Azopt	33.93±1.57	1.5±0	83.96±7.86	2.2±0.25
Cidamex	24.01±0.02	1.5±0	75.5±7.55	3.13±0.18

Considering the parameter AUC_{0-6} as a measure of the drug ocular bioavailability, it is evident that the value of this parameter was ranked in the following order: NE 18a, NE 18b, Azopt® and finally Cidamex®. By applying ANOVA statistical test, the differences were statistically significant ($p < 0.05$) with the exception of Azopt® and Cidamex® where variation among them was considered non significant ($p > 0.05$).

In the view of the current result, it could be concluded that both ACZ NE formulae had higher bioavailability than either Azopt eye drops or Cidamex tablets where NE 18a showed the highest therapeutic activity with AUC 1.8 and 2 times higher than that of Azopt drops and Cidamex tablet respectively followed by NE 18b which showed AUC 1.2 and 1.4 times higher than that of Azopt drops and Cidamex tablet respectively. Higher AUC obtained by NE formulation is probably because of the penetration enhancement effect of NEs which could revealed to removal of mucous layer and disruption of tight junctional complexes of corneal tissue by NEs [8, 77].

Although, the effect of NE 18b on ACZ permeability is less pronounced than in NE 18a which is consistent with the results obtained from ex- vivo permeation study, it shows greater AUC than that observed in case of brinzolamide (Azopt®) which is known to be intrinsically more permeable than ACZ [3].

With respect to the duration of IOP reduction, it is evidenced from Fig.5 that both NE formulae showed more prolonged effect than that shown by Azopt® or Cidamex®. This result was consistent with the calculated MRT (Table 9) as NE 18a had MRT 3.4 and 2.4 times higher than those of Azopt® and Cidamex® respectively. NE 18b showed MRT 1.85 and 1.3 times higher than that shown by Azopt® and Cidamex® respectively.

The more prolonged IOP lowering effect exhibited by NE 18a over NE 18b could be correlated to higher viscosity of the former NE which increases the ocular residence time. Also, higher oil content in NE 18a could result in a more significant contact time and area with the cornea.

It was found that no change in IOP was observed in the untreated eye during the course of measurement in any of the two NEs. Thus, indicating that ACZ NEs exerted a local action within the eye and the observed IOP lowering activity is not because of any systemic absorption followed by subsequent redistribution.

The results of this bioavailability study lead to a conclusion that formulation of ACZ as NE resulted in an improvement of the therapeutic efficacy of the drug. This improvement is associated with a decrease or disappearance of the systemic side effects of ACZ due to the local action exerted by ACZ NEs.

CONCLUSION

ACZ NEs were prepared successfully using IPM, oleic acid and peanut oil at 39% water content. ACZ release rate from NEs were dependent on the type of surfactant and cosurfactant used in NE preparation. NE 4, NE 12 and NE 18 showed highest drug release among other NEs. These NEs were prepared at higher water content of 59%. All prepared NEs showed acceptable physicochemical properties, thermodynamic stability and they were non irritant to rabbits' eyes. NEs improved ACZ permeation across goat corneas with 6 times improvement in permeation coefficient of ACZ when incorporated in NE 18a. NE 18a showed fast onset of IOP lowering activity and prolonged effect as well as enhanced drug bioavailability compared to ACZ tablets and brinzolamide eye drops. Thus, ACZ in NE 18a offers an intensive treatment of glaucoma with a decrease in number of instillation per day and a decrease or disappearance of systemic side effects of ACZ with an improvement in patient compliance.

CONFLICT OF INTEREST

None to declare

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