Research Article

DEVELOPMENT AND CHARACTERIZATION OF CO-SOLVENT BASED ANTI-GLAUCOMIC OPHTHALMIC VESICLES OF ACETAZOLAMIDE

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ABSTRACT

The purpose of the study was to formulate topically effective ophthalmic acetazolamide vesicular formulations. Ethanolic and ethereal injection methods were used for the preparation of bi-lamellar and multi-lamellar acetazolamide vesicles consisting of span-60, PEG-400 and PG (co-solvents and penetration enhancers) with or without tween-80 (an edge activator) and non-ionic surfactant. Reverse phase evaporation (REV) method was used to prepare niosomes (control) and 1% w/v acetazolamide suspension for comparison purpose to evaluate ex-vivo corneal permeability performance. The prepared vesicles were evaluated for their abundance, size, shape, lamellarity and number of vesicles/mm³ by optical microscopy, entrapment efficiency, drug content, zeta potential, pH, DSC studies, ex-vivo corneal permeability studies, sterilization studies, in-vivo studies, stability studies and safety studies. Bi-lamellar and multi-lamellar vesicles entrapped greater amounts of drug than uni-lamellar REVs niosomes. Physical stability study indicated that approximately 93% and 94% of acetazolamide was retained in selected vesicular formulations up to a period of 4 months at 4°C. The intraocular pressure (IOP) lowering activity of selected acetazolamide vesicular formulations was determined and compared with Dorzox[®], a marketed formulation. Acetazolamide vesicles revealed more prolonged effect than marketed formulation. The selected vesicular formulations F1 and F2 exhibited greater lowering in IOP and a more prolonged effect than the other formulations prepared by ethanolic injection and ethereal injection methods without co-solvents. The selected vesicular formulation F1 composed of PEG 400PG (1:7) ratio and formulation F2 composed of PEG-400 without PG showed the maximal response, which reached a value of -3.3 ± 0.4 mmHg after 1 hour of topical administration.

Keywords: Ophthalmic vesicles, Acetazolamide, Niosomes

INTRODUCTION

Drug delivery in ocular therapeutics is a challenging problem and is a subject of interest to scientists working in the multidisciplinary areas pertaining to the eye. Current trends in ocular therapeutics and drug delivery suggest that the existing dosage forms will be replaced by novel drug delivery systems that offer improved biopharmaceutical properties.

Acetazolamide (the most effective carbonic anhydrase inhibitor, CAI) is used orally in large doses for the reduction of intra-ocular pressure (IOP) in patients suffering from glaucoma, the third most prevalent cause of visual impairment and blindness in the United States, after cataracts and macular degeneration¹ and in the promotion of diuresis in instances of abnormal fluid secretion². 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.

The 2 major problems that hinder the topical effectiveness of acetazolamide are its poor aqueous solubility (0.7 mg/mL)^{3,4} and low permeability coefficient of $4.1 \times 10^{-6} \text{ cm/s}$. Topical formulations of acetazolamide solution (in the form of sodium salt) were initially unsuccessful because of its limited ocular penetration, which caused an insufficient amount of the drug to reach the ciliary body. Other significant attempts have been made to formulate effective acetazolamide topical preparations (eg. contact lenses containing acetazolamide; topically active

surfactant gel preparation of acetazolamide; aqueous acetazolamide solution using 2-hydroxypropyl- β -cyclodextrin; polymeric suspensions of acetazolamide containing viscolyzers and penetration enhancers. Recently our research team Kaur and Smitha successfully prepared topically effective formulations of acetazolamide using cyclodextrins in combination with bioadhesive polymers, penetration enhancers, and co-solvents.

The various drug delivery systems mentioned above offer numerous advantages over conventional drug therapy, yet 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. In order to overcome these problems, the researchers conceived the concept of vesicular drug delivery systems for ocular therapy.

The objective of the present study was to formulate topical acetazolamide vesicular formulations by ethanolic injection and ethereal injection methods, niosomes by reverse-phase evaporation (REV) method (NS control) and 1% w/v acetazolamide suspension for the purpose of comparison. A comparison study was performed between REV-NIO, ACZ-SUS and vesicular formulations to evaluate the ex-vivo corneal permeability performance. The factors influencing the encapsulation of acetazolamide into vesicles were investigated. Characterization of the prepared vesicles regarding physical morphology, particle size, entrapment efficiency, drug content, pH, zeta potential, DSC, ex-vivo corneal permeability, sterilization of vesicles and safety studies were performed. Stability study was performed to investigate the leak out of the

drug from vesicles during storage. The intra-ocular lowering activity of selected acetazolamide vesicular formulations was evaluated.

MATERIALS AND METHODS

Acetazolamide (ACZ; Gift sample from F.D.C. Limited, Mumbai.); Acetone (Sisco Research Laboratories Pvt. Ltd.); Cholesterol (Hi-media Pvt. Ltd.); Chloroform (Merck Specialities Pvt. Ltd.); Diethyl ether (Thermo Fisher Scientific India Pvt. Ltd.); Dorzox[®] (Cipla Pvt. Ltd.); Ethanol (Changshu Yangyuan Chemical China); Glutathione bicarbonate ringer (GBR); Glutathione, oxidized (Hi-media Pvt. Ltd.); Iso-propyl alcohol (Merck Specialities Pvt. Ltd.); Methanol (Thermo Fisher Scientific India Pvt. Ltd.); Polyethylene glycol-400 (Loba Chemie Pvt. Ltd.); Propylene glycol (Loba Chemie Pvt. Ltd.); Span-60 (Central Drug House Pvt. Ltd.); Triple distilled water (Millipore); Tween-80 (Central Drug House Pvt. Ltd.).

Preparation of Co-solvent Based Vesicles

Ethanol Injection Method

Span-60 in a weight of 80 mg was dissolved in 10 ml ethanol. Aqueous phase (containing suitable quantities of PEG-400/PG along with ACZ 1% w/v or Tween-80 20 mg and ACZ 1% w/v) was prepared. The aqueous phase was maintained at 60°C and was magnetically stirred. Organic phase was slowly injected into preheated aqueous phase maintained at 60°C, using a 16 gauge needle (Figure 1).

Ether Injection Method

Span-60 in a weight of 80 mg was dissolved in 10 ml of ether. Aqueous phase (containing edge activator i.e. 20 mg Tween-80 along with ACZ 1% w/v) was prepared. The aqueous phase was maintained at 60°C at magnetic stirrer. Organic phase was slowly injected in to a preheated aqueous phase maintained at 60°C, using a 16 gauge needle.

Figure 1: Flow diagram of preparation of vesicles by Ethanol Injection Method.

Optimization of Addition of Co-solvents

The amount(s) of co-solvent(s) added in the final formulations were decided after carrying out the preliminary optimization. Amounts were based upon the solubility of drug in the respective co-solvents. Various batches of vesicles were prepared and were then evaluated on the basis of % drug entrapped. The formula showing the best % entrapment was selected as the final formulation.

Similar approach was applied to the combination of co-solvents. Range of combinations (5-33%) was selected and the formulations were evaluated for percent entrapment (Table 2). Concept of combination of co-solvent was applied keeping in mind the additive/synergistic effects that we may obtain.

Preparation of Niosomal Formulation (control) Reverse Phase Evaporation (REV) Method

Span 60 and cholesterol in a weight ratio of 2:1 (a total of 150 mg) were dissolved in a mixture (12 ml) of ether and chloroform (2:1). Aqueous phase (containing suitable quantities of acetone and distilled water along with ACZ 1.0 w/v) was added such that the organic to aqueous phase ratio was 3:1. The mixture was then sonicated for 5 minutes using probe sonicator. A stable emulsion so formed was dried down in a rotary evaporator at 60°

C till a semi solid gel like structure was formed. Gel was then shaken vigorously on the vortex mixer and the resultant viscous dispersion was diluted suitably with distilled water to make up the volume to 4 ml (volume of aqueous phase used initially). Un-entrapped drug was not removed and hence the formulation contained 10 mg/ml of ACZ. The preparation was similar to one, reported by us, to be a successful carrier system for acetazolamide except that it contained 1% w/v ACZ instead of 0.5% w/v⁵. This formulation was taken as a control for comparing the presently prepared vesicular system.

Formulation of Control Drug Suspension (ACZ suspension)

A 1% w/v suspension of acetazolamide was prepared in distilled water containing 1% Tween-80 as a dispersing agent, by stirring on magnetic stirrer for 3h. Before use the suspension was vortexed for 5 min, to ensure a uniform dispersion of the drug particles.

CHARACTERIZATION OF VESICLES

Preliminary Studies

Vesicles were evaluated for shape, size, lamellarity, and abundance, immediately after preparation and at one month period from the time of preparation.

Morphology and Structure of Vesicles

The morphological characters (viz. shape uniformity and lamellarity) of prepared vesicles were monitored employing microscopy (eclipse i90, Nikon).

Number of Vesicles per Cubic Millimeter

Vesicles were suitably diluted with water, and the number of vesicles per cubic mm was counted by microscopy using haemocytometer. The vesicles in 80 small squares were counted and no. of vesicles/mm³ was calculated using the following formula⁶.

$$\text{Total number of vesicles / cubic mm} = \frac{\text{Total number of vesicle counted} \times \text{Dilution factor} \times 4000}{\text{Total number of squares counted}}$$

Entrapment Efficiency of the Vesicles

The entrapment efficiency of the vesicles was analyzed by dialysis method using cellophane membrane. 1 ml of vesicular dispersion was poured in dialysis bag in a beaker containing 100ml methanol as a sink media, for 45 min. and the sample was analyzed spectrophotometrically at wavelength of 265 nm (amount of free drug was present in sink media). Separately dialysis bag was ruptured and the amount of the entrapped drug was determined experimentally by disrupting the vesicles using isopropyl-alcohol (IPA) and analyzing the samples spectrophotometrically.

$$\text{Percentage entrapment} = \frac{\text{Entrapped drug (mg)} \times 100}{\text{Total drug added (mg)}}$$

Mass balance equation was confirmed after obtaining the values for entrapped (vesicles in dialysis bag) and un-entrapped ACZ (drug in methanol, the sink media).

Drug Content

IPA was chosen as a suitable solvent for disrupting the prepared vesicles. 1 ml of the aqueous dispersion was disrupted using sufficient quantity of IPA and the absorbance was recorded at 265 nm.

Vesicle Shape and Type Microscopy

Selected vesicles were observed under optical microscope, (Nikon, eclipse i90; magnification 400X) a thin layer of diluted vesicular dispersion was spread over the glass slide covered with a cover slip, and observed optically for structural attributes such as lamellarity, uniformity of size and shape.

Vesicle Size and Size Distribution

The size and size distribution of vesicles was determined by dynamic light scattering method (DLS), using a computerized inspection system (Malvern Zeta master, ZEM 5002, Malvern, U.K.).

Zeta Potential

Zeta potential of the vesicular dispersions was measured using Malvern's zetasizer. The measurements were done at 25°C and the electric field strength was around 23.2 V/cm. The zetasizer measures the zeta potential based on the Smoluchowski equation.

$$\zeta = \frac{U_E \eta}{\epsilon}$$

Where ζ is zeta potential, U_E is electrophoretic mobility, η is viscosity of the medium and ϵ is dielectric constant.

pH of the Formulations

pH of the formulations was measured using deluxe pH meter 101E, at 25°C.

Differential Scanning Calorimeter (DSC)

For thermal analysis, sample of the pure drug, all the excipients and vesicles were scanned using DSC and the thermo grams so generated were observed /evaluated for any significant shift or disappearance or appearance of new peaks.

Differential Scanning Calorimeter (DSC) was used to assess any interactions between the drug and the excipients added in the formulation. Samples were weighed in a hermetic aluminium pan and heated from 25-300°C at a rate of 10°C/min.

Ex-vivo corneal permeability studies

Influence of the various components of the formulations on the *ex vivo* permeability of acetazolamide through pig cornea was investigated using an in-house built glass diffusion cell. GBR was used as the diffusion medium considering that it acts as simulated tear fluid (STF). This solution is reported to preserve the integrity of the excised cornea for up to 6 hrs^{7,8}. It is prepared by mixing equal parts of solutions A & B as given below just prior to use.

Part A

Sodium chloride - 12.4 g/L
Potassium chloride - 0.72 g/L
Sodium dehydrogenate phosphate monohydrate - 0.21 g/L
Sodium bicarbonate - 4.91 g/L

Part B

Calcium chloride dehydrate - 0.23 g/L
Magnesium chloride hex hydrate - 0.32g/L
Glucose - 1.80 g/L
Oxidized glutathione - 0.18 g/L

Solutions were stored in refrigerator and used within 3 weeks of preparation. The final solution was equilibrated at 35 ± 0.5°C, and the pH of the solution was adjusted between 7.2-7.4.

Preparation of Cornea

Enucleated whole eye bulb of pigs were obtained from a local slaughter house. For preparation, the bulb was always placed with the corneal side facing upwards so as to avoid the contamination of epithelial surface or physical trauma to the tissue. Starting at a distance of approximately 2 mm from the corneal rim, the sclera was incised until the vitreous body was reached. The cornea was incised circularly at that distance. The whole interior part of the eye was lifted, lens and iris still attached to the corneal part. This was important in order to avoid corneal wrinkling. The iris/ciliary body and lens were carefully removed, using forceps, taking care that the cornea remained well shaped (maintained its curvature). The later was washed in glutathione bicarbonate-ringer solution (simulated tear fluid; STF) before mounting it on diffusion cell assembly. To ensure that the cornea remains in its place, the rim of the donor compartment was lightly coated with elfy (Prime Products, Delhi) before mounting the cornea. Then the receptor compartment was placed over it and two compartments were fixed together using hooks (on the assembly) and rubber bands.

Figure 2: Diffusion Cell Assembly

Method: For the in vitro corneal permeability studies, membrane diffusion technique was used. Studies were conducted within a jacketed cell, maintained at a constant temperature ($37 \pm 0.2^\circ\text{C}$), under mixing conditions using a magnetic stirrer. The cell used was a two-limbed reservoir (Figure 2); on one limb of which cornea was mounted and the other limb was used as the sampling port (volume = 19 ml). The preparation (0.2ml) to be studied was placed on the cornea. The cornea was mounted within half an hour of sacrifice of the animals. Diffusion medium used was freshly prepared GBR solution equilibrated at $37 \pm 0.2^\circ\text{C}$, pH 7.4. Aliquots of the medium were withdrawn after a fixed time interval from the sampling port and were replaced with equal quantity of fresh GBR to maintain a constant volume. Sink conditions were maintained throughout the study. Samples were analyzed spectrophotometrically at a 265 nm λ_{max} . The apparent corneal permeability coefficient (P_{app}) of different formulations was determined according to the following equation:

$$P_{\text{app}} = \frac{\Delta Q}{\Delta t \cdot 60 \cdot A \cdot C_0} (\text{Cms}^{-1})$$

Where $\frac{\Delta Q}{\Delta t}$ is the steady state slope of the linear portion of the plots of the amount of drug in the receiving chamber (Q) vs. time (t). A is the exposed corneal surface area (1.327 cm^2), C_0 , the initial concentration of drug in the donor cell and 60 represents the conversion of minutes to seconds.

Sterilization

Final vesicular formulations were sterilized both by autoclaving at 121°C (15 psi pressure) for 20 minutes and by filtration through membrane filters ($0.45 \mu\text{m}$ membrane). The formulations were evaluated post sterilization in terms of their % entrapment, % drug content, particle size, aggregation, and abundance of vesicles.

Pharmacodynamic Studies

Animals

Adult male rabbit weighing 1.0-1.5 kg were used for the studies. The rabbits were provided with food and water *ad libitum* in a temperature-controlled room ($18-24^\circ\text{C}$). They were exposed to the normal (ambient) light and dark cycles. All rabbits used in these experiments were normotensive.

Method

Intraocular pressure (IOP) was measured using Reichert's PT100 pneumatonometer. All IOP measurements were carried out by the same operator using the same pneumatonometer. IOP was measured three times at each interval and the means taken. The animals used, were accustomed, to the experimental procedure. The only restraint was the hand of the investigator lightly packed on the back and shoulders of the rabbit.

Formulations were instilled topically in to the lower quadrant of the eye and the eye was manually blinked three times, one eye received $30 \mu\text{l}$ of the formulation, and the contralateral eye served as the control. The IOP was measured immediately prior to administering the formulation, and at 15, 30, 60, 90, 120, 180, 240, 300, 360 min following the treatment. Each formulation was tested on a group of at least three ($n=4$) healthy male rabbits. Each animal was given a washout of three days after every treatment.

Change in IOP at any time t (ΔIOP_t) is expressed as :

$$\Delta\text{IOP} = \text{IOP at time } t - \text{IOP at } 0 \text{ time}$$

ΔIOP is reported as the mean ($\pm\text{SD}$) of the values from three rabbits.

Stability Studies

The formulations were evaluated for stability in terms of their leakiness, total drug content, aggregation behavior, and abundance. The formulations were kept in 30 ml stoppered glass vials and stored under two different conditions (refrigerator i.e. $2-8^\circ\text{C}$ and ambient room temperature i.e. $25^\circ\text{C} \pm 2.0$). Aliquots samples from the formulations were withdrawn at definite time intervals (0, 1, 2, 3 and 4 months) and analyzed

spectrophotometrically for the extent of entrapment at 265 nm. Reduction in the percent entrapment with respect to the initial sample (0 month; 100% entrapment) is reported for the developed vesicles. The samples were also evaluated microscopically to observe the extent of aggregation and in terms of their abundance using haemocytometer.

In-vivo Safety Studies

Since presently reported vesicular systems developed and reported by us was a novel system so it was necessary to evaluate them for safety. We employed in vivo dermal/ocular irritation (OECD guidelines) study in rabbits. Details of these tests are given below.

Acute Dermal Irritation/Corrosion Test as per OECD Guideline 404

According to OECD guideline 405, before considering in vivo eye irritation/corrosion test, preferably a study of the in vivo dermal effects of the substance should be conducted and evaluated in accordance with OECD Testing Guideline 404.

According to these guidelines a special attention was given to possible improvements in relation to animal welfare concerns and to the evaluation of all existing information on the test substance in order to avoid unnecessary testing in laboratory animals.

Principle of the in vivo test

The substance to be tested was applied in a single dose to the skin of an experimental animal; untreated skin areas of the test animal serve as the control. The degree of irritation/corrosion was read and scored at specified intervals.

Selection of animal species

The albino rabbits were the preferable laboratory animal and healthy young male rabbits (6 month; average weight- 1.4-1.7 kg) were used.

Preparation of the animals

Approximately 24 h before the test, fur of the animals was removed using hair removing cream from the dorsal area of the

trunk of the animals. Care was taken to avoid abrading the skin, and only animals with healthy, intact skin were used.

Test Procedure

Application of the test substance

The test substance (0.5 ml of vesicular dispersion) was applied to a gauze patch (approximately 6 cm²) which was then applied to the skin with non-irritating tape for a period of 4 h. The test patch was attached to the skin in such a manner that there was good contact and uniform distribution of the substance on the skin. The care was taken that the access of the animal to the patch and/or its ingestion or inhalation was not possible.

A small shaved skin area adjacent to test area served as control. At the end of the exposure period, gauze patch was removed, and examined after 1h of patch removal.

Initial test (in vivo dermal irritation/corrosion test using one animal)

The in vivo test was performed initially using one animal and the response was graded.

Confirmatory test (in vivo dermal irritation test with additional animals)

When the initial test on one animal showed the absence of irritation/corrosion or negative response was confirmed using two additional animals, each with one patch, for an exposure period of four hours.

Clinical observations and grading of skin reactions

All animals were examined for signs of erythema and edema and the responses scored at 60 minutes, and then at 24, 48 and 72 hours after the patch were removed. For the initial test in one animal, the test site was also examined immediately after the patch was removed.

RESULTS AND DISCUSSION

Characterization of vesicles

Preliminary Studies

The developed vesicles (F1-F4; F6 and F7) were compared morphologically with F5 (NS control) in terms of size, shape, abundance and lamellae 1 month after preparation, to evaluate stability on keeping, as shown in table 1. None of the selected formulations showed any aggregation upon storage.

Table 1: Evaluation of Morphological Characteristics of the Vesicles Prepared With Span-60 in Combination With Cholesterol (for Niosomal preparation) or Edge Activator

Formulation	Abundance	Size	Shape	Lamellar	No. of vesicles /mm ³
F1	very abundant	Small	Round, regular	bilamellar, multi-lamellar	40,500
F2	very abundant	Small	round, regular	bilamellar, multi-lamellar	81,000
F3	very abundant	Large	round, irregular	bilamellar, multi-lamellar	75,500
F4	very abundant	Large	round, irregular	bilamellar, multi-lamellar	74,500
F5	Abundant	Small	round, irregular	Uni-lamellar	39,500
F6	very abundant	small	round, regular	Bilamellar	66,500
F7 (Ether inj.)	very abundant	Medium	round, regular	Bilamellar	68,100

Abundant 30,000-40,000 vesicles/mm³; Very abundant- >40,000 vesicles/mm³.

Entrapment efficiency of developed formulations

Percent entrapment of various vesicular formulations is listed in table 2 and their particle size distribution is shown in Figure 3. Results indicate that the formulations prepared using PEG-400 alone or in combination with PG had significantly better entrapment efficiencies. It may be noted from the results that formulation-1 (F1) and formulation-2 (F2) showed a

significantly higher entrapment of almost 67 and 69% which was almost 13-15% more than the other similarly prepared formulations (F3, F4) and was more than 2.5 times that of NS control (F5). Further it was observed that preparation of vesicles (using tween-80 as EA; F6) improved the entrapment by almost 2 times in comparison to NS control. However use of co-solvent EAs had significantly better influence which was compromised upon incorporation of tween-80 (F3; F4).

In general, entrapment efficiency of vesicles prepared by ethanol injection was higher as compared to those prepared by ether injection method and reverse phase evaporation method.

Drug Content

Actual amount added for all practical purposes was 10.0 mg/ml (100%) and the drug content of the developed formulations was not found to be significantly different from added amount except F5, as shown in table 2. This is expected considering that method (REV) of preparation of NS (F5) may result in significant drug losses.

Table 2: Entrapment efficiency and Drug content of various formulations

Formulations	%ENT $\pm$ SD ^a	%T.D.C. $\pm$ SD ^b
F1	64.0 $\pm$ 3.05 [#]	97.17 $\pm$ 1.58
F2	66.5 $\pm$ 2.9 [#]	96.4 $\pm$ 1.86
F3	51.8 $\pm$ 2.7 [*]	97.13 $\pm$ 1.02
F4	48.2 $\pm$ 4.1 [*]	93.92 $\pm$ 5.33
F5	26.01 $\pm$ 2.19	81.42 $\pm$ 4.81 [*]
F6	51.83 $\pm$ 2.6 [*]	96.78 $\pm$ 2.23
F7 (Ether inj.)	52.85 $\pm$ 2.9 [*]	97.24 $\pm$ 2.57

a- ENT= Entrapment efficiency; All values are significantly different (p<0.001) from one another except those marked similarly.

b- All values are not significantly different from 100% value i.e. 10 mg/ml (p>0.05) except * (p<0.001).

Vesicle size and Size Distribution

Particle size is an important parameter in in-process control and particularly in quality assurance, because the physical stability of vesicular dispersions depend on particle size and particle size distribution. Particle size of the ophthalmic preparation should be less than 10 μ m in order to avoid irritation to the eyes⁹. Also with smaller particle size easy penetration through the cornea is observed¹⁰. The smaller ganciclovir liposomes (200 nm) probably could penetrate the cornea more easily than the larger (1000 nm) acyclovir liposomes. Average particle size of vesicles developed by us as shown in table 3 fall in nano range and hence are not only appropriate for ocular use, but are also expected to cross the corneal barrier and reach up to the internal eye tissues. The particle size of the developed formulation was measured with particle size analyzer (Malvern™) zetasizer. The particle size of the developed vesicles was found to lie in a narrow range, with formulation F1 and F2 having a small average particle size of 250 nm and 162 nm respectively. (especially the developed formulation F2 showed an average size of 162 nm with 10% vesicles smaller than 80 nm while 90% of the vesicles were equal to 490 nm). While the other two vesicular formulations viz. F3 and F4 showed a very large particle size (16 μ m and 15 μ m), which was unsuitable for ocular use; so for all subsequent studies F3 and F4 were not included. Presence of Tween 80 in the formulations seems to contribute a large particle size. Since PEG 400 and Tween 80 have hydrophilic nature (HLB 11.6 and 16 respectively); this may be responsible for an increase in particle size. However, the ether injection method resulted in a fairly particle size even with Tween 80. Even the niosomal formulation, F5 showed a large particle size of 13.4 μ m and was used as an internal control for the presently developed vesicular formulation of ACZ.

Figure 3: Particle size of formulations F1, F2, F3 and F4.

Vesicle Shape and Type Optic Microscopy

Formulations F1 and F2 were examined microscopically (optical microscope, Nikon eclipsed i90; magnification 400X). Optical inspection indicated the vesicles to be small in size, round in shape, bilamellar and multilamellar (Figure 4) with no aggregation or irregularities being observed in the systems. The particles were uniformly distributed as shown in Figure 4.

F1

F2

Figure 4: Microscopic pictures of formulations F1 and F2.

Zeta Potential

In dispersion, the Brownian motion results in frequent collision between the particles. Such interactions are mainly responsible for stability of dispersion system. When attraction predominates, the particles adhere after collision and tend to aggregate. When repulsion predominates, the particles rebound after collision and remain individually dispersed. As we know that, zeta potential is defined as the difference in potential between the surface of tightly bound layer and electroneutral region of solution¹¹, thus,

a high (+ve) or low (-ve) zeta potential value signifies greater interparticle distance or in other words repulsion between vesicles and hence a good stability of the system.

Zeta potential of the formulations F1-F4 was measured employing Malvern's zetasizer at 25 °C. Observed zeta-potential of the optimized formulations is shown in Table 3. The negative value of zeta-potential obtained for formulations indicates a low scope of coalescence of vesicular system and sufficient stability of the nanodispersion.

Table 3: Particle Size, Abundance, %Transmittance and Zeta potential of Various Formulations of Acetazolamide (n=5)

Formulation	Particle size $\pm$ sd ^a	%Transmittance	Zeta potential
F1	250 $\pm$ 20.5 nm [*]	39.7	-4.36
F2	162 $\pm$ 11.1 nm [*]	25.2	-8.84
F3	16.8 $\pm$ 5.9 μ m	35.2	-2.74
F4	15.4 $\pm$ 2.7 μ m	37.1	-4.55
F5	13.4 $\pm$ 3.97 μ m	36.8	-3.38
F6	6.0 $\pm$ 2.8 μ m [#]	35.7	-3.89
F7 (ether inj.)	535 $\pm$ 23.1 nm [#]	37.0	-3.10

a-All values are significantly different ($p < 0.001$) from one another expect for those marked similarly.

pH of the formulations

Human tears normally have a pH of about 7.2 with a good buffering capacity. Despite the sensitivity of cornea, un-buffered solutions with pH value between 3.5 and 10.5 are usually tolerated, with little discomfort in the eye. Outside this pH range, irritation of the eye usually occurs accompanied with increased lacrimation, particularly so with alkaline solutions. It is expected that the unbuffered solutions will be buffered to the physiological pH, within minutes of instillation, by the lacrimal fluid. Moreover, since the volume of instilled drop is very small (0.03 ml), a small amount of tears can buffer the instilled

solution. At the same time, stability of the drug is an important consideration and the pH of the formulations can be compromised keeping the stability of the drug into consideration⁹. The pH of maximum stability of acetazolamide is reported to be 4.5³.

Maintaining the pH between 4 to 6, also ensures that ACZ will be present in an unionized form which in addition to better stability would also show a better permeation through biological membranes. pH of the developed disperse systems is shown below in table 4

Table 4: pH of the Various Formulations of Acetazolamide

Formulation	F1	F2	F3	F4	F5	F6	F7	F8
pH	5.31	5.72	6.16	5.90	5.02	5.7	5.5	5.06

Determination of Drug-Excipient Interactions Using DSC

Successful formulation of a stable and effective dosage form depends on the careful selection of excipients. The latter can facilitate administration, promote a consistent release and bioavailability of the drug and protect it from degradation. Drug-excipient compatibility/interaction studies are thereby conducted towards the final stages of preformulation to eliminate "surprise" stability or compatibility problems. Hence samples of pure acetazolamide, all excipients (span 60,

Polyethylene glycol 400, Propylene glycol) and their physical mixtures were scanned using differential scanning calorimeter (DSC) and thermograms so generated were analyzed for any significant shift in the peak, disappearance of a peak, or appearance of any new peak(s), with respect to the vesicular formulations.

In case of acetazolamide the drug peak was observed at 261.57°C corresponding to the melting point of acetazolamide (263°C), which is accompanied by decomposition as indicated

by an exothermic peak at 259.26°C. Further, the endothermic peak of propylene glycol (Figure 5 b) was observed at 153.90°C

and endothermic peak of PEG-400 (Figure 5 c) was observed at 160.99°C. As shown below in Table 5.

5 (a)

5 (b)

5 (c)

5 (d)

5 (e)

5 (f)

5 (g)

5 (h)

Figure 5: DSC of Acetazolamide (a); Propylene glycol (b); Polyethylene glycol 400 (c); B- Unloaded (Blank formulation F1) (d); C-Unloaded (Blank formulation F2) (e); B-Loaded (formulation F1) (f); C-Loaded (formulation F2) (g); B-Unloaded+drug (h); C-Unloaded+drug (i); Physical mixture B (components of form. F1) (j); Physical mixture C (components of formulation F2) (k); Span 60 vesicles (l); Polyethylene glycol 400+Propylene glycol (m).

Figure 5d and 5e indicated broadening of peak thus signifying the development of vesicles. Furthermore the peak shifted to a much lower transition temperature, (with respect to the constituents i.e. Span 60, PEG 400 or PG) indirectly indicating the fluid nature of vesicles. The drug loaded vesicles (Figure 5f and 5g) did not show any peak corresponding to that of ACZ indicating that either the drug is completely incorporated into the respective vesicles or probably the free drug is in a solubilised form due to presence of co-solvents. This was indicated by the fact that the DSC of unloaded vesicles along

with ACZ, also showed an absence of the drug peak, both in Figure 5h and 5i which may be due to the interaction (i.e. solubilisation) of drug with free propylene glycol (PG) and polyethylene glycol 400 (PEG 400). Similar results were also seen in DSC of physical mixtures (Figure 5j and 5k). The peaks observed by a combination of propylene glycol (PG) and polyethylene glycol 400 (PEG 400) (Figure 5m) were found to disappear from blank vesicles (Figure 5d and 5e) indicating the incorporation of PEG 400 and propylene glycol (PG) into the vesicles.

Table 5: Endothermic Peaks Shown by Drug, Co-solvents, Various Vesicles and Their Different Combinations

Drug/co-solvents/formulations	Peak (Endothermic)
Acetazolamide	261.57°C
Propylene glycol	153.90°C
Polyethylene glycol 400	160.99°C
Propylene glycol + Polyethylene glycol 400-Fig 12 m	140.39°C, 155.95°C
F1 (Blank)-Fig 12 d	77.31°C, 114.53°C
F2 (Blank)-Fig 12 e	85.52°C, 97.32°C
F1 (Loaded)	94.77°C, 104.37°C, 116.37°C
F2 (Loaded)	94.38°C, 105.68°C
F1 (Blank + Drug)	87.77°C, 99.40°C, 112.19°C, 134.80°C
F2(Blank + Drug)	36.52°C, 55.77°C, 97.44°C
F1 (Physical mixture)	59.82°C, 103.77°C, Several peaks near 141.13°C.
F2 (Physical mixture)	52.13°C, 76.45°C, 116.31°C
Span 60 (Blank vesicles)	92.89°C

Ex-vivo Corneal Permeability Studies

Ex-vivo corneal permeability data of various formulations is summarized in Table 6. Linear permeability plot with correlation coefficient (r^2) in the range 0.969-0.996 were obtained for log % permeated vs time (Figure 6) in case of developed formulations F1, F5, F8 (suspension). An aqueous suspension of ACZ in 1% w/v Tween 80 (F8) was taken as control in this study. Results indicate a considerable improvement in effect when free drug is incorporated into vesicles. This indicates first order release kinetics of developed systems. It may however, be noted that data also fitted the zero order kinetics i.e. % permeated vs time plots showed r^2 0.980-0.999; thus indicating a controlled release pattern. Table 7 shows the total amount permeated, % permeation, steady state flux and apparent permeability coefficient (P_{app}) of all the

formulations. A one way ANOVA followed by Tukey's test was applied on the data. Broadly speaking formulations F1 and F2 were significantly better than all other formulations in terms of % amount permeated at each time interval. F1 and F2 show an asymptot from 3h onwards as shown by absence of any significant difference between amount released at subsequent times; again indicating a prolonged effect.

F1 and F2 also showed significantly better (more than 2 times) results ($p < 0.001$) in terms of % amount permeated, steady state flux and the apparent permeability coefficient of ACZ when entrapped within these vesicles. However, there was no significant difference between F1 and F2 in terms these parameters. (Table 7). The apparent permeability coefficient of ACZ from the suspension form was significantly less than F1 and F2 ($p < 0.001$) again confirming their efficiency.

Table 6: Ex-vivo Corneal Permeability Data of the Various Formulations in Terms of the Percent

Form.	0.25h	0.5h	1h	1.5h	2h	2.5h	3h	4h	5h
F1	13.0±0.9 ^a	16.6±1.7 ^a	20.4±0.8 ^a	24.9±1.4 ^a	29.6±1.2 ^a	34.9±3.9 ^a	^a 40.0±4.6 ^a	^{a,b} 44.8±3.3 ^a	^b 51.5±3.6 ^a
F2	13.1±0.1 ^a	17.4±0.6 ^a	23.0±4.5 ^a	27.4±3.4 ^a	33.0±3.9 ^a	35.9±4.8 ^a	^a 40.6±4.3 ^a	^{a,b} 47.7±1.4 ^a	^b 52.8±3.2 ^a
F5	6.7±0.3 ^{##}	7.6±0.4 [*]	10.7±0.3 [*]	14.2±0.4 ^{##}	17.4±0.4 [*]	20.4±0.7 ^{##}	24.1±1.0 [*]	27.2±0.2 ^{##}	30.8±0.6 [*]
F6	7.3±0.4 [*]	8.9±0.2 [*]	10.9±0.4 [*]	13.4±0.3 ^{##}	15.9±0.6 [*]	18.1±0.8 ^{##}	21.0±0.7 [*]	25.9±0.6 [*]	31.1±0.7 [*]
F7	5.6±0.4 [*]	7.0±0.3 [*]	9.1±0.1 [*]	10.9±0.3 [*]	12.1±0.4 [*]	14.5±0.1 ^{##}	17.6±0.6 [*]	22.0±0.1 [*]	27.5±0.5 [*]
F8	3.2±0.1	4.5±0.2	6.3±0.2 [*]	7.6±0.1 [*]	9.7±0.2 ^{##}	11.5±0.1 ^{##}	14.0±0.4 [*]	16.1±0.3	21.1±0.3

Amount Permeated (µg) at Each Interval (h)

2 ml of each formulation was taken. Actual T.D.C. was taken as 100%.

At each time interval all formulations are significantly ($p < 0.001$) different from one another except for those marked similarly. For every formulation, the % amount permeated at each time interval was significantly ($p < 0.001$) different from preceding time interval except for those marked similarly, on the top left hand side of each numeric value.

Table 7: Comparison of Various Formulations in Terms of Total Amount Permeated, % Permeation, Steady State Flux and Apparent Permeability Coefficient P_{app} from In vitro Permeation Studies Using Pig Cornea

Formulations	Total amount permeated in 5h(µg)	% Permeation	Steady state flux (µg/min/cm ²)	Apparent permeability coefficient P_{app} (cm/sec) X 10 ⁻⁶
F1	999.2±69.9 ^a	51.5±3.6 ^a	2.4±0.2 ^a	17.09±67.0 ^a
F2	1019.3±62.6 ^a	52.8±3.2 ^a	2.5±0.2 ^a	17.5±60.5 ^a
F5	501.1±9.0 [*]	30.8±0.6 [*]	1.21±0.03 [*]	10.25±7.7 [*]
F6	545±13.0 [*]	31.1±0.7 [*]	1.32±0.03 [*]	9.17±0.12 [*]
F7(Ether inj.)	489±8.0 [*]	27.5±0.5 [*]	1.19±0.02 [*]	8.72±0.21 [*]
F8(ACZ _{-sus})	422.1±5.0 [*]	21.1±0.3	1.03±0.02 [*]	7.9±2.8 [*]

All formulations are significantly ($p < 0.001$) different from one another, except for those marked similarly, in terms of their total amount permeated, % permeation, steady state flux and apparent permeability coefficient.

Figure 6: % Corneal permeation of various formulations with reference to Time in hours

In-vivo Studies

The physiological effectiveness of the formulations was determined in terms of their IOP lowering effect in normotensive rabbits. Table 8 shows the measured drop in IOP of the normotensive rabbits as a function of time after instillation of various formulations of acetazolamide. No significant change in IOP was observed in the untreated eye during the entire course of measurement in any of the

formulations. This clearly indicates that all the formulations elicited a localised action within the eye and that the activity shown is in no case related to systemic absorption, followed by a subsequent redistribution^{12,13}.

One way ANOVA followed by Dunnet's test was carried out for all the formulations to compare each formulation with dorzolamide at different time interval.

Table 8: Comparison of Reduction in Intraocular Pressure vs Time of Various Formulations in Normotensive Rabbits (n=4)

Time	DORZOX	F1	F2	F6 ^b	F7 (Ether inj.)
0h	0.0	0.0	0.0	0.0	0.0
0.25h	*-0.5±0.2	*-0.7±0.5	*-0.7±0.4	*-0.8±0.2	-1.4±0.4 ^a
0.5h	*-0.8±0.1	*-1.9±0.4 ^a	-2.7±1.0 ^{a*}	-1.1±0.6	*-1.5±0.2 ^a
1h	*-1.7±0.2 ^a	*-2.2±0.5 ^{a*}	-3.3±0.4 ^{a*}	*-1.0±1.2	-1.0±0.2 ^a
1.5h	*-1.6±0.2 ^a	*-2.2±0.7 ^a	*-2.9±0.2 ^{a*}	-0.4±0.8	*-0.8±0.3 ^a
2h	*-1.3±0.2 ^a	-2.7±0.5 ^{a*}	-2.5±0.2 ^{a*}	-0.6±0.7	*-0.7±0.3 ^a
3h	*-1.2±0.2 ^a	-2.4±0.3 ^{a*}	-2.0±0.3 ^{a*}	-0.6±0.5	*-0.6±0.3
4h	*-0.8±0.2	-2.0±0.4 ^{a*}	*-1.4±0.2 ^{a*}	-0.5±0.8	*-0.9±0.7 ^a
5h	-0.5±0.1	-1.4±0.5	-0.9±0.3	-0.3±0.7	*-0.8±0.4 ^a
6 h	-0.1±0.0	-0.6±0.6	-0.2±0.2	-0.3±0.3	-0.1±0.3

Results are expressed as mean±S.D., n=4.

b- Values for F6 at all time points are not significantly different from one another (p>=0.05).

*- Values are significantly higher than Dorzox at respective time point (p<0.001).

On comparison of ΔIOP values for each formulation at different time points; and amongst different formulations at each time point, (marked on top left hand side), those marked similarly were not significantly different (p>=0.001) from one another.

Reduction in IOP was highest with F2. A maximum reduction of 3.3 mm Hg was observed at 1h and the value was twice of that that achieved with DORZOX[®] that was 1.7 mm Hg at 1 h. It may however be noted that Dorzox[®] contains 2% w/v dorzolamide vis-à-vis our formulations which contains 1.0%

w/v ACZ. So the array of systemic side effects, if any (no change in IOP observed in the contralateral eye indicates low systemic absorption), is expected to be low with ACZ formulation developed by us.

F1 showed a maximum IOP reduction of 2.7 mm Hg at 2h and the value was 2.2 mm Hg at 1h. In case of formulation F6 and F7 (Ether inj.) a maximum reduction of 1.1 mm Hg and 1.4 mm Hg was achieved, respectively was observed at 0.5 h.

Figure 7: Showing change in intraocular pressure of various developed formulations at different interval of time (h).

Sterilization Studies

The vesicular formulations F1 and F2 were sterilized in quadruplicate (four different batches F1a-d; F2a-d), and respective particle-size, T.D.C., entrapment, abundance and aggregation were analyzed for all the formulations after

sterilization, by two different methods i.e. Steam sterilization (autoclave) and membrane filtration. In autoclave, the formulations were maintained at 121°C for 20 minutes and in filtration the formulation was passed through Millipore filter of 0.45µm. All data of evaluation are given in table 9 below.

Table 9: Sterilization of Elastic Vesicular Formulations by Autoclave and Filtration (n=4)

	Initial					Autoclave					Filtration				
	Particle size ^a	% T.D.C. ^b	% Ent ^c	Abu	Agg	Particle size	% T.D.C. ^a	% Ent	Abu	Agg	Particle size	% T.D.C. ^b	% Ent	Abu	Agg
F1a	235 nm	95.12	68.77	++	-	257 nm	93.74	67.77	++	+	217 nm	93.08	64.65	++	-
F1b	254 nm	96.73	64.30	++	-	280 nm	91.22	61.42	++	-	247 nm	93.10	61.21	++	-
F1c	242 nm	95.38	65.57	++	-	277 nm	88.13	64.23	++	-	289 nm	91.14	60.39	++	-
F1d	274 nm	97.29	67.21	++	-	285 nm	90.21	64.56	++	-	293 nm	94.22	59.11	++	-
F2a	182 nm	99.11	68.72	+++	-	224nm	96.28	65.11	+++	-	215 nm	93.10	66.29	+++	-
F2b	160 nm	99.78	69.39	+++	-	220nm	91.78	67.02	+++	-	205 nm	95.28	62.23	+++	-
F2c	174 nm	97.03	66.34	+++	-	212nm	92.15	61.43	+++	-	220 nm	91.11	64.89	+++	-
F2d	154 nm	97.91	67.54	+++	-	210nm	94.67	59.11	+++	-	227 nm	92.65	62.33	+++	-

Abu- Abundance; Agg- Aggregation; ++ No. of vesicles/mm² are >40,000 and <90,000; +++ No. of vesicles/mm² are >60,000 and >90,000; - Nil.

^aThe T.D.C. was significantly (p<0.05) reduced for formulation F1 and F2 upon autoclaving but ^bthere was no significant change upon filtration.

Results indicate suitability of both the methods for sterilization with filtration being highly indicated. This establishes the proposed application of the formulation for ocular use.

All the formulations have been compared in terms of their activity parameters (onset time, peak effective time, duration of action and % lowering of IOP) as shown in table 10.

Surprisingly, the F2 formulation showed a maximum IOP lowering effect of 29.2% followed closely by F1 (23.9%), while Dorzox[®] showed almost half the activity (15.0%). Significantly higher effect shown by F2 in view of the fact that F2 did not show a significantly better entrapment and in vitro permeability characteristics in comparison to F1, may be because of a better membrane stability of the formed vesicles, such that they form a

more intimate contact with the corneal epithelium and hence permeate to a larger extent. Use of a combination of surfactants has been reported to enhance the stability of the micellar membrane of invertebrate¹⁴. Further PG is proposed to be a better permeation enhancer. It may however be noted that formulation F2 showed a significant more prolonged effect.

The formulations F6 (10.09%) and F7 (14.3%) showed comparatively lower IOP reduction. It can be explained on the basis lower percent entrapments observed in these cases.

All the developed formulations, however showed a prolonged effect with an immediate onset (as observed at 15 minutes) and the effect was apparent even up to 6h.

Table 10: Summary of Activity Parameters of Various Formulations

Formulation	Peak effect* (h)	Peak iop lowering effect** (%)
F1	2.0 (0.5h – 4.0h)	23.9* (2)
F2	1.0 (0.5h – 3.0h)	29.2* (1)
F6	0.5	10.09 (0.5)
F7	0.5	14.3* (0.5)
DORZOX	1.0 (1h – 3h)	15.0* (1)

All the values for % ΔIOP were significantly different (p<0.001) except those marked similarly.

*Values in parenthesis indicate the duration for which ΔIOP was not significantly different (p<0.001) from peak ΔIOP value. **Value in parenthesis indicate the time at which peak effect is observed. The values are calculated with respect to zero time IOP in each group.

Stability Studies

The formulations showing significantly high entrapment and promising pharmacodynamic results (F1 and F2) were evaluated for leakiness, aggregation behavior and T.D.C, upon storage. The formulations were kept in 30 ml stoppered glass vials and stored under two different conditions (refrigerated and ambient

condition). Aliquot samples from the formulations were withdrawn at definite time intervals (0, 1, 2, 3 and 4 months) and analyzed spectrophotometrically for the extent of entrapment. Reduction in the percent entrapment with respect to the initial sample (0 month; 100% entrapment) is reported for the vesicles. The samples were also evaluated microscopically to observe the extent of aggregation

Table 11: Results of stability studies for formulation F1

Condition	Drug Leakage (% Loss)					Drug Aggregation Behaviour				
	0 mon	1 mon	2 mon	3 mon	4 mon	0 mon	1 mon	2 mon	3 mon	4 mon
Ambient Cond	-	3.73	7.52	10.78	14.56	-	+	++	++	++
Refrigerator	-	0.82	2.10	6.65	7.18	-	+	+	++	++

Table 12: Results of Stability Studies for Formulation F2

Condition	Drug Leakage (% Loss)					Drug Aggregation Behaviour				
	0 mon	1 mon	2 mon	3 mon	4 mon	0 mon	1 mon	2 mon	3 mon	4 mon
Ambient Cond	-	2.91	4.84	6.53	7.81	-	+	++	++	++
Refrigerator	-	0.89	2.18	5.31	6.03	-	+	+	++	++

- (indicates no change)

Drug leakage and fusion behavior of the formulations F1 and F2 were evaluated, at different time intervals, at ambient conditions (room temperature) and under refrigeration (4°C). Extent of aggregation and drug leakage upon storage of formulation F1 and F2 at ambient temperature or under refrigeration was sufficiently low. From the above data shown in table 11 it was noticed that maximum drug loss for formulation F1 was up to 14.56 % at ambient temperature, while it was significantly lower (7.81%) for F2. The stability or storage of formulations F1 and F2 were better in refrigerator than at ambient temperature because % drug leakage and drug aggregation at refrigerator were sufficiently lower than at an ambient temperature.

Safety Studies

Acute Dermal Irritation/Corrosion Test as per OECD Guideline 404

The score for dermal irritation/corrosion study are compiled. The interpretation of scores clearly indicates a non-irritant/non-corrosive nature of developed vesicles when applied to dermal tissues, and hence they may be considered safe for dermal application. The study was conducted in accordance with OECD guidelines as a preamble to guideline 405 for ocular irritancy.

Acute Eye Irritation/Corrosion Test as per OECD Guideline 405

The scores for developed formulation(s) again established that developed vesicular systems are safe for ocular use. There were no signs of irritation/corrosion in any of the ocular tissues. Score for F1 and F2 was 0. Hence, the developed vesicles may be considered safe for ocular use.

CONCLUSION

It was observed that the formulations prepared by ethanol injection method using co-solvent (F2) showed better entrapment efficiencies (66.5%) and corneal permeation (52.8%). Another formulation, F1 in which a combination of co-solvents was employed, also yielded sufficient percent entrapment (64.0%) and corneal permeation (51.5%). The particle size of formulations F1 (250 nm) and F2 (162 nm) were in nanometer range. A corresponding niosomal formulation was prepared, by reverse evaporation method, and evaluated in terms

of its particle size (14 µm), % entrapment efficiency (26%) and % corneal permeation (31%), establishing the importance of the presented system. We also compared the developed system by similarly prepared system where the co-solvents was replaced with tween 80 as an edge activator to result in elastic vesicles. Later did not compare with formulations F1 and F2.

The developed formulations (F1 and F2) could be sterilized successfully both by autoclave and filtration methods. Both the formulations were also stable (in terms of % percent drug loss and aggregation behavior). With more than 90% of the drug retaining/entrapped within the vesicles even after storage of up to 4 months in case of formulation F2. The developed formulations were found to be safe for ocular use. Comparing the formulations F1 and F2 with marketed formulation Dorzox[®], indicates significantly better % lowering of IOP with the developed formulations.

The present work establishes the safety, stability and sterility of the developed formulations indicating the scope of attaining economically viable preparations; however the clinical effectiveness needs to be confirmed in humans.

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