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Development of a topical niosomal preparation of acetazolamide: preparation and evaluation

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Abstract

Orally administered acetazolamide has a limited use in glaucoma due to the systemic side effects associated with its use. No topical formulation of acetazolamide is available, mainly because of it having a limited aqueous solubility and poor corneal permeation. To enhance the bioavailability of acetazolamide by the topical route and to improve the corneal permeability of the drug, niosomes of acetazolamide were prepared (employing span 60 and cholesterol) by different methods. Transmission electron microscopy (TEM) of the selected formulation was carried out to study the morphology. Niosomes were also prepared in the presence of dicetyl phosphate and stearylamine to obtain negatively and positively charged vesicles, respectively. It was found that the reverse-phase evaporation method (REV) gave the maximum drug entrapment efficiency (43.75%) as compared with ether injection (39.62%) and film hydration (31.43%) techniques. Drug entrapment efficiency varied with the charge and the percent entrapment efficiency for the REV method was 43.75, 51.23 and 36.26% for neutral, positively charged and negatively charged niosomes, respectively. Corneal permeability studies, however, showed that the percent permeation and the apparent permeability coefficient for the charged niosomes were less than for the neutral ones. A bioadhesive niosomal formulation of acetazolamide was also prepared and compared with the positively charged formulation, considering that both of them would have a prolonged stay in the cul-de-sac because of their expected interactions with mucin. The formulations were also compared based on their intraocular pressure (IOP)-lowering capacity. The positively charged niosomes (REV2), although showing good corneal permeability and pharmacodynamics, were however found to be inappropriate in terms of the corneal cell toxicity. The bioadhesive coated formulation (REV1bio) compared well with REV2 and also showed a much lesser toxicity. Further, the IOP-lowering effect of the developed formulations was compared with that of a marketed formulation of dorzolamide 2%, a topical carbonic anhydrase inhibitor. The developed niosomal formulations of acetazolamide showed a comparable physiological effect (33% reduction of IOP in REV1bio and 37% reduction in dorzolamide) with a duration of up to 6 h (the duration being 3 h for dorzolamide). Results of the study indicate that it is possible to develop a safe (as indicated by corneal toxicity studies) and physiologically active topical niosomal formulation of acetazolamide relative in efficiency to the newer local carbonic anhydrase inhibitor, dorzolamide. The developed formulations can form a cost effective treatment plan, which is especially important in the treatment of glaucoma, a chronic ailment affecting middle-aged to old patients.

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Introduction

Acetazolamide, a carbonic anhydrase inhibitor, is a potent ocular hypotensive agent used to relieve the acute symptoms of open angle glaucoma, delay the onset of blindness in persons with advanced glaucoma and reduce intraocular pressure (IOP) preoperatively (Khaw & Cordiero 2000). Despite this, the use of acetazolamide for the treatment of glaucoma is limited as it is administered orally (no topical formulation being available) and, considering the large distribution of carbonic anhydrase enzyme in the various organs of the body, it causes a wide array of systemic side effects (Epstein & Grant 1977; Gamm 1984). The constraints in the development of a topical formulation of acetazolamide (Kaur et al 2002; Singla et al 2002) are its very low solubility ($0.7\,mg\,mL^{-1}$) in aqueous tear fluid and in water and its limited corneal penetration ($\log P = 0.3$) (Parasampuria 1993). Moreover, the degradation of acetazolamide increases many fold on the basic side (the highly soluble

sodium salt of the drug gives a solution of pH > 9), the pH of maximum stability being 4.5. Several attempts have been made to improve the topical delivery of acetazolamide (Flach et al 1984; Friedman et al 1985; Manners et al 1993; Loftsson et al 1994; El-Gazayerly & Hikal 1997; Kaur et al 2000, 2002, 2004b). Many of these systems (e.g., soluble polymers, ocuserts, gels) prolong ocular bioavailability but cannot control drug penetration through the cornea. Consequently the drug concentration at the site of action might remain inadequate. For a carbonic anhydrase inhibitor to be effective, more than 99.99% inhibition of carbonic anhydrase is required to decrease the aqueous flow and achieve a lowering of IOP (Maren 1987). Therefore, it is necessary to develop more effective (in terms of a high concentration reaching the iris/ciliary body), safe and more acceptable therapeutic systems (Durrani et al 1992).

In the formulation of newer topical ocular dosage forms, great attention is now being devoted to new drug delivery systems that can ensure a localized effect, have the convenience of a drop and at the same time increase the corneal permeability of poorly permeable drugs. For this purpose, vesicular systems (in particular, liposomes) have been investigated by several groups. Further, vesicles consisting of one or more surfactant bilayers enclosing aqueous spaces (called niosomes) have been considered of particular interest as they offer several advantages over liposomes with respect to chemical stability, lower cost and availability of materials (Saettone et al 1996; Uchegbu & Vyas 1998; Kaur et al 2004a).

The aim of this study was to formulate a niosomal preparation of acetazolamide using different methods and to evaluate them by in-vitro corneal permeation studies using porcine cornea. Based on drug entrapment efficiency, corneal permeability and IOP-lowering effect, a most suitable niosomal formulation of acetazolamide is presented. Further, the safety of the developed formulation is also established and its efficacy is compared with that of newer carbonic anhydrase inhibitors (e.g. dorzolamide) launched in the market. The latter are costly and the experience with these molecules is limited (few years) in comparison with acetazolamide, whose safety profile has been established over half a decade of use.

Materials and Methods

Materials

Acetazolamide (Shallaks Pharmaceutials Pvt. Ltd, New Delhi, India), span 60 (Loba Chemie, Mumbai, India), cholesterol (Loba Chemie, Mumbai, India), stearylamine (Sigma, USA), dicetyl phosphate (Sigma, USA) and dorzolamide (Dorzox; Cipla, Mumbai, India) were used. All other chemicals and reagents were of analytical grade.

Preparation of niosomes

Different methods of preparation were employed to prepare niosomes of acetazolamide.

Film hydration (FH) method

Niosomes containing acetazolamide were prepared by adopting the procedure of Azmin et al (1985). Span 60 and cholesterol in a molar ratio of 1:1 were dissolved in chloroform. The solvent was evaporated using a rotavapor apparatus and the dry film thus formed was hydrated with a 0.5% w/v solution of acetazolamide in a 2% boric acid solution containing 20% acetone at 60°C.

Ether evaporation (EE) method

In this case, the ether injection method was used. Lipids (span 60 and cholesterol in a 1:1 molar ratio) were dissolved in ether and a (0.5%) solution of the drug, as described above, was prepared. The ether containing lipids was then slowly injected (at a constant rate) into the aqueous phase (maintained at 60°C) using a 16-gauge needle (Baillie et al 1985).

Reverse-phase evaporation (REV) technique

This method was first reported by Szoka & Papahadjopoulos (1978). Span 60 and cholesterol in equimolar ratio (1:1) were dissolved in a mixture of ether and chloroform (1:6). Aqueous phase (as described under hydration method) containing acetazolamide 0.5% w/v was added such that the organic-to-aqueous-phase ratio was 3:1. The mixture was then sonicated for 5 min using a probe sonicator (Misonix Sonicator 3000; USA). The stable emulsion so formed was dried down to a semi-solid gel in rotary evaporator at 60°C until a semi-solid gel-like structure was formed. The gel was then shaken vigorously on a vortex mixer and the resultant viscous dispersion was diluted with boric acid.

pH and charge of niosomal formulations

In all the above methods, stearylamine (SA) and dicetylphosphate (DCP) were added when preparing positively and negatively charged niosomes, respectively. Boric acid solution (2%), pH 5, was chosen as a vehicle for all the preparations, as the pH of maximum stability for acetazolamide is 4.5 (Parasampuria 1993). By using boric acid solution, the pH of all the formulations was maintained at 4–4.5 (Table 1). The pH of acetazolamide suspension was 4.30 ± 0.50. Further, a 2% solution of boric acid is isotonic with tears and is recommended as a suitable vehicle for aqueous eye drops (Riegelman & Sorby 1966). It may be noted that the unentrapped drug was not removed from the niosomal preparations of acetazolamide and hence they contained 5 mg mL^{-1} of the drug.

Bioadhesive coated niosomes

These were prepared by incubating the reverse-phase evaporation vesicles (REVs) at 37°C in Carbopol 934P (0.05%) for 5 min.

Preparation of acetazolamide suspension

A 0.5% w/v suspension of acetazolamide was prepared in 2% boric acid solution containing 1% Tween 80 as a dispersing agent, by stirring on a water bath shaker for

Table 1 Comparative study of the different methods of niosome preparation

Method	Formulation code	Surfactant (mole fraction)	Cholesterol (mole fraction)	Stearylamine	Dicetyl phosphate	Vesicle type	Mean particle size (μm) (n=3)	%E±s.d. (n=4)[b]	pH (n=3)
Film hydration	FH1	50	50	—	—	MLV	30.0[a] ± 0.7	31.43 ± 0.4	4.20 ± 0.63
	FH2	47.5	47.5	5	—			37.28 ± 0.32	4.30 ± 0.54
	FH3	47.5	47.5	—	5			23.67 ± 0.21	4.20 ± 0.45
Ethanol evaporation	EE1	50	50	—	—	SUV	1.20 ± 0.24	39.62 ± 0.69	4.15 ± 0.72
	EE2	47.5	47.5	5	—			43.04 ± 0.95	4.10 ± 0.66
	EE3	47.5	47.5	—	5			29.64 ± 0.2	4.24 ± 0.91
Reverse-phase evaporation	REV1	50	50	—	—	LUV	2.50 ± 0.56	43.75 ± 0.25	4.20 ± 0.43
	REV2	47.5	47.5	5	—			51.23 ± 0.15	4.20 ± 0.44
	REV3	47.5	47.5	—	5			36.26 ± 0.96	4.32 ± 0.32

[a]Sonication was done for a suitable time to reduce the average particle size to 5–10 μm, the optimum size for ocular instillation. [b]All values are significantly different from one another.

3 h. Before use, the suspension was vortexed for 5 min to ensure a uniform dispersion of the drug particles.

Determination of entrapment efficiency of niosomes

The dispersions were each ultracentrifuged at 50 000 *g* for 30 min. The supernatant was analysed spectrophotometrically at 265 nm, using a UV-1601 spectrophotometer (UV-1601; Shimadzu Corp., Japan), for the amount of acetazolamide present. The latter when subtracted from the total drug amount gives the entrapped drug. The amount of entrapped drug was also determined directly by disrupting the niosomes (pellet formed by ultracentrifugation) using 1% isopropyl alcohol and analysing the samples spectrophotometrically. The values obtained by both the methods matched well.

$$\text{Percentage entrapment (\%E)} = [\text{Entrapped drug (mg)}/\text{Total drug added (mg)}] \times 100 \quad (1)$$

Particle size distribution

Particle size was determined using Laser Diffraction Master sizer 2000 (Malvern Instruments Ltd, UK). The particle size of the suspension was determined by optical microscopy.

Transmission electron microscopy (TEM)

The morphology of both the uncoated and the bioadhesive coated niosomes (REV1 and REV1bio, respectively) was studied using TEM.

Corneal permeation studies

For the in-vitro corneal permeability studies of acetazolamide, a membrane diffusion technique was used. The studies were conducted within a jacketed cell (Figure 1), maintained at a constant temperature (35 ± 0.5°C), under mixing conditions using a magnetic stirrer. The cell used was a two-limbed reservoir, on one limb of which cornea was mounted and the other limb was used as the sampling port (volume, 19 mL). The preparation (0.5 mL) to be studied was placed on the cornea. Porcine cornea was used for the studies (obtained from the local slaughter house; cornea was mounted within half an hour of sacrifice of the pigs). The diffusion medium used was freshly prepared glutathione bicarbonated Ringer (GBR) solution equilibrated at 35 ± 0.5°C. The pH of the GBR was maintained at 7.2–7.4 by passing CO_2. This solution closely simulates tears and is referred to as the simulated tear fluid (STF). This medium is known to preserve the integrity of the cornea for up to 6 h (O'Brien & Edelhauser 1977). Samples of the medium were withdrawn at fixed time intervals from the sampling port and were replaced with an equal quantity of fresh GBR to maintain a constant volume. Sink conditions were maintained throughout the study. Samples were analysed spectrophotometrically.

The apparent corneal permeability coefficient (P_{app}) of different formulations was determined according to equation 2 (Schoenwald & Huang 1983; Camber 1985).

$$\text{Papp} = \Delta Q/(\Delta t \times 60 \times A \times Co)\ (\text{cm s}^{-1}) \quad (2)$$

where $\Delta Q/\Delta t$ 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), Co is the initial concentration of drug in the donor cell and 60 represents the conversion of minutes to seconds.

In-vivo studies

Adult male rabbits, 1.5–2.0 kg, were used for the studies. The rabbits were provided with free access to food and water in a temperature-controlled room (18–24°C). All rabbits used in these experiments were normotensive and were housed under proper conditions, in the central animal house of the Panjab University, Chandigarh, India. The animal house is suitably approved by the committee for the purpose of control and supervision of experiments

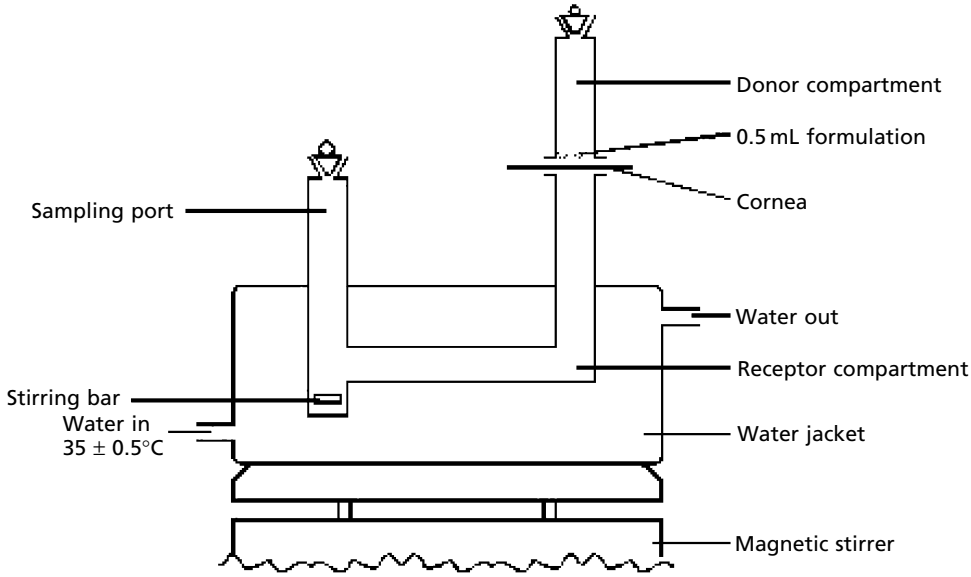

Figure 1 Diffusion cell assembly used for in-vitro corneal permeation studies.

on animals. The experimental protocol was approved by the institutional ethical committee.

Intraocular pressure (IOP) was measured using a Reichert non-contact hand-held pneumatonometer (PT 100). All IOP measurements were carried out by the same operator, using the same tonometer. IOP was measured three times at each interval and the means taken. The rabbits used were accustomed to the experimental procedure. The only restraint was the hand of the investigator lightly laid on the back and shoulders of the rabbit. Rabbits that showed a consistent difference in IOP between the left and the right eye during baseline measurements, or any sign of eye irritation, were excluded from the study.

Formulations were instilled topically into the upper quadrant of the eye and the eye was manually blinked three times; one eye received 30 μL of the formulation and the contralateral eye served as the control. The IOP was measured immediately before giving the drug and at a suitable time interval following the treatment. Each formulation was tested on a group of at least six healthy male rabbits. Each rabbit was given a washout of three days after every treatment.

Change in IOP (ΔIOP) is expressed as $IOP_{dosed\ eye} - IOP_{control\ eye}$ and is reported as the mean ($\pm$ s.e.m.).

Statistical analysis

All the experimental data were subjected to statistical analysis, using one-way analysis of variance followed by Tukey's test. $P < 0.05$, was considered to be statistically significant.

Results and Discussion

The spectrophotometric method used for the analytical determinations was previously confirmed to obey Beer-Lambert's law in the concentration range of 0–20 $\mu g\,mL^{-1}$. When standard drug solutions (concentrations 2.5, 5 and 10 $\mu g\,mL^{-1}$) were assayed repeatedly (n = 3) the relative error (accuracy) and relative standard deviation (precision) was found to be 1.06%, 0.9%, 0.9% (for three different concentrations taken) and 0.01%, respectively. The excipients used in the formulation did not show any interference at this wavelength.

Several methods of niosome preparation were investigated and their efficiency evaluated in terms of the extent of entrapment and the data (Table 1) indicates that the reverse-phase (REV) method showed a maximum (51%) entrapment ($P < 0.05$) for positively charged niosomes. The entrapment efficiency with the ether injection method was found to be more than the film hydration method, which is in accordance with the findings of Baillie et al (1985) who demonstrated that the entrapment efficiency depends on the kind of vesicles formed. It has been reported that the small unilamellar vesicles (SUVs) formed by the ether injection method represent a more efficient use of surfactant than the multilamellar structures formed by the film hydration method and hence represent significantly greater entrapment efficiency (Deamer & Bangham 1976). It may be added here that the significantly higher entrapment shown by REVs in our study could be due to the formation of large unilamellar vesicles (LUVs). It is well documented that LUVs prepared by the REV method possess a number of advantages over multilamellar vesicles (MLVs), including high encapsulation of water-soluble drugs, economy of lipid and reproducible drug release rate. The high encapsulation of water-soluble drug can be explained on the basis of the fact that the surface-to-volume ratio of LUVs is less than that of SUVs. Hence, the large internal aqueous space of LUVs makes it possible to encapsulate a higher percent of drug within the vesicle relative to the amount of

lipid contained in the bilayer (Gould-Fogerite & Mannino 1992). However, it may be noted that preparation of vesicles by REV is comparatively difficult.

The particle size of niosomes prepared by the different methods was also determined and is shown in Table 1. The size of a particle, in ophthalmics, apart from influencing bioavailability (retention in cul-de-sac), also plays an important role in the irritation potential of the formulation. Hence it is recommended that particles in ophthalmic dispersion, in general, should be approximately 10 μm or less in size to minimize irritation to the eye (Hecht 2001). MLVs obtained by the film hydration method were larger in size and hence the sample was sonicated to get a smaller particle size. Optical microscopy of acetazolamide suspension indicated 60% particles to be less than 10 μm, while almost 8% particles were of a size more than 50 μm.

One method of providing vesicles with the necessary site adherence and site retention to achieve carrier and drug targeting in topical ocular therapy is to endow them with the ability to be mucoadhesive. There are a large number of bioadhesive polymers which are being used in ophthalmics (Kaur & Smitha 2002). Carbopol was chosen as a bioadhesive polymer in our study as it acts by forming a three-dimensional microgel structure in aqueous media, which provides interaction with phospholipids. The pK_a of Carbopol polymer is 6.0 ± 0.5 and above this point the carboxylic acid groups are ionized to a great extent, thus reducing H-bonding. For a greater effectiveness, the pH of the solution containing Carbopol should be kept at or below 6 (Ch'ng et al 1985; Park & Robinson 1985; Davies et al 1992). Since the pH of our formulations was maintained near 4.5, Carbopol seemed to be an ideal choice.

TEM photographs of coated (REV1bio) and uncoated (REV) vesicles (Figures 2 and 3) show them to be spherical and unilamellar. The coated vesicles (Figure 3) have a hazy outline, which could possibly be due to the presence of the bioadhesive.

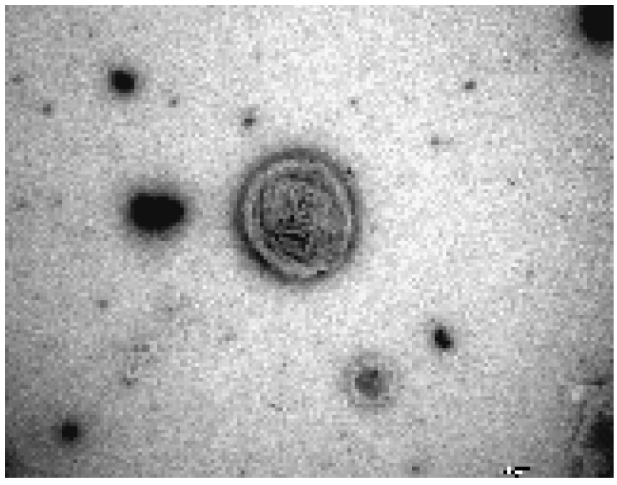

Figure 2 TEM photograph of acetazolamide vesicles (REV1; 60 000×) prepared by reverse-phase method.

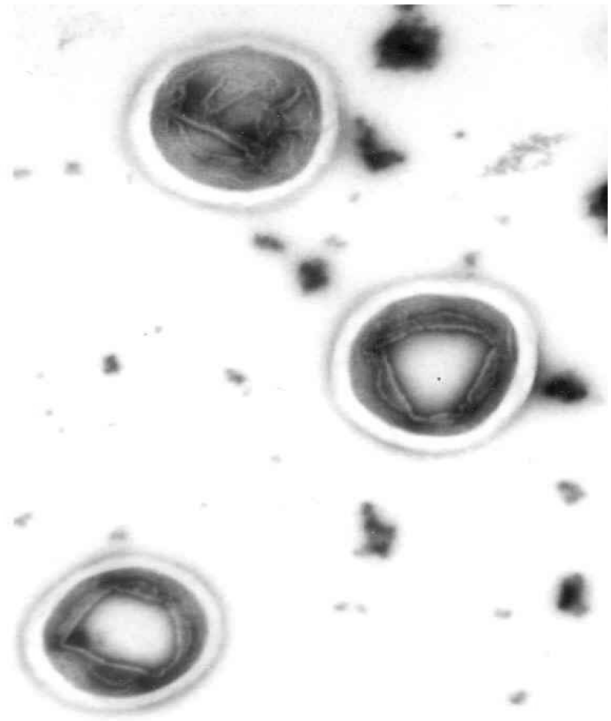

Figure 3 TEM photograph of bioadhesive niosomal formulation of acetazolamide (REV1bio; 40 000×).

It was found that the encapsulation efficiency of niosomes also varied with the composition of the vesicles (Table 1). Positively charged niosomes (those containing stearylamine) showed a higher encapsulation within all the methods, followed by neutral niosomes and then the negatively charged ones (containing dicetylphosphate), when the same surfactant-to-cholesterol ratio was used. This trend is in agreement with that found by El-Gazayerly & Hikal (1997) and also by Singh & Mezei (1984). According to them, this order of entrapment efficiency can be attributed to the strength of the binding forces involved in the interaction of the drug with the phospholipids. Acetazolamide is a weak acid and an electrostatic attraction would occur between the drug anion and the positively charged stearylamine. This attraction probably accounts for the higher encapsulation efficiency.

The data obtained from corneal permeability studies (carried out for 5 h; Figure 4) showed that the cumulative amount permeated in the case of charged niosomes was less than for the neutral niosomes (REV1, FH1, EE1), which gave the highest rate and extent of drug permeation (Table 2), followed by positively (REV2, FH2, EE2) and negatively charged (REV3, FH3, EE3) vesicles, respectively. The release obtained by bioadhesive coated formulation (REV1bio) was comparable with REV1. P_{app} values (Table 2) of various formulations suggest the influence of charge and size on the permeability of the drug through the cornea. Permeability pattern REV > EE > FH was observed, which may be attributed to a smaller size of unilamellar vesicles prepared by both the reverse-phase and ether evaporation method in

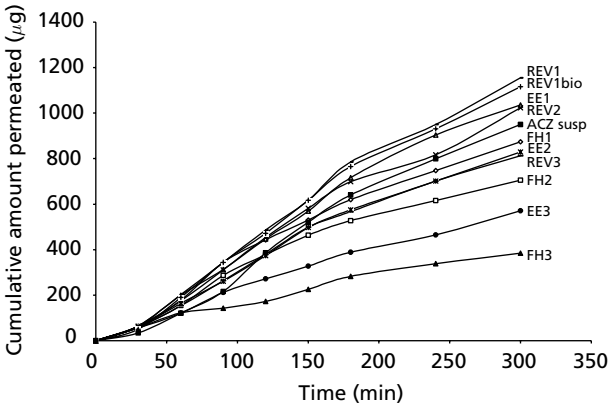

Figure 4 Comparison of in-vitro permeability of niosomal vesicles of acetazolamide prepared by different methods across the porcine corneal membrane.

Table 2 Comparative evaluation of different niosomes on the basis of corneal permeability

Formulation code	Corneal permeability (%) (n = 3)[b]	Apparent permeability coefficient P_{app} ($cm^{-2}s^{-1}$)[a]
FH1	34.97 ± 0.24	7.65×10^{-6}
FH2	28.19 ± 0.76	6.20×10^{-6}
FH3	15.40 ± 0.31	3.25×10^{-6}
EE1	41.42 ± 0.26	9.30×10^{-6}
EE2	33.14 ± 0.85	7.35×10^{-6}
EE3	22.82 ± 0.43	4.85×10^{-6}
REV1	46.22 ± 0.34	1.02×10^{-6}
REV2	40.94 ± 0.19	8.95×10^{-6}
REV3	32.48 ± 0.54	7.25×10^{-6}
REV1bio	44.61 ± 0.18	9.95×10^{-6}
ACZsusp	36.40 ± 0.43	8.25×10^{-6}

REV1bio was prepared by coating REV1 vesicles with Carbopol 934P. [a]All the values are significantly different from one another. [b]All the values are significantly different except REV1, EE1, FH1 from REV1bio, REV2and REV3 (EE2), respectively.

comparison with MLVs prepared by the film hydration method. Further, a higher P_{app} and the total amount permeated shown by the REV formulation can be explained in terms of the high percent entrapment achieved by the REV formulation (43.75 vs 39.62 for EE). Even though the rate of permeation for both types may be the same, the difference in entrapment results in a higher cumulative amount of drug in the endothelial chamber because of the greater amount being released from the LUVs. Furthermore, in all formulations the permeability coefficient (P_{app}) for charged vesicles is less than for neutral vesicles. The charged lipids probably tighten the molecular packaging of the vesicle bilayer (Finkelstein & Weissman 1979), resulting in a slower release of drug. The amount of drug permeated would be a sum total of the rate at which the vesicles permeate through the cornea and the rate and extent of drug release from the vesicles at the surface of the cornea (forming an effective concentration gradient) or after passing through the cornea into the STF. Thus the charged vesicles, especially the positively charged ones, though expected to pass through the cornea at a faster rate (because of the compact packing and hence a smaller size for charged vesicles and a closer interaction of positively charged vesicles with negatively charged mucin glycoproteins in the cornea), may not release the drug as efficiently, hence showing a lower P_{app} and cumulative amount permeated. The permeability coefficient of bioadhesive formulation (9.95×10^{-6}) is comparable with that of the neutral (REV1) formulation (1.02×10^{-5}).

Acetazolamide suspension gave a P_{app} of 8.25×10^{-6} and the percent amount released was 36.4%, which was significantly higher than all the FH niosomal preparations and the charged EE niosomes. However, at initial times (30 and 60 min) the amount permeated was significantly less than the other formulations (Figure 4). The high permeability characteristics shown by the acetazolamide suspension may be due to the presence of Tween 80 (1% w/v) used for dispersing acetazolamide in 2% boric acid solution. It was found that in the presence of 1% Tween 80 the solubility of acetazolamide increased from 0.67 to 0.87 mg mL^{-1} (an increase of almost 30%). Non-ionic surfactants are also reported to act as penetration enhancers (Kaur & Smitha 2002), hence it is probable that in addition to increasing the solubility of acetazolamide (thus presenting an increased concentration of drug at the corneal surface), Tween 80 also helps to improve the permeability of acetazolamide (P_{app} is considerably more than that reported for acetazolamide solution (Duffel et al 1986)).

The physiological effectiveness of the formulations (REV1, REV2 and REV1bio) was determined in terms of their IOP-lowering effect in normotensive rabbits. Table 3 shows the measured drop in the IOP of normotensive rabbits as a function of time after administration of various formulations of acetazolamide. No change in IOP was observed in the untreated eye during the course of measurement in any of the formulations. This clearly indicates that all the formulations exerted a local action within the eye and that the activity shown is not because of any systemic absorption, followed by a subsequent redistribution (Surgue 1996; Ponticello et al 1998).

Acetazolamide suspension and marketed dorzolamide drops (Dorzox, Cipla), containing 2% w/v dorzolamide, were taken as controls. Acetazolamide suspension decreases IOP to a maximum value of 2.3 mmHg during a period of 1.5 h. However, at 30 min only a 3% reduction was obtained compared with an almost 20% reduction with niosomal formulations and also dorzolamide. This indicates that the initial lowering of IOP achieved by the niosomal formulation is probably because of the high penetrability of the niosomes. Further, span 60, a non-ionic surfactant used in their preparation might also enhance the penetration of the unentrapped (free) drug present in these formulations (as mentioned in the Methods section, the unentrapped drug was not removed). A 0.5% w/v suspension of acetazolamide in 2% boric acid solution mixed with empty niosomes showed a 7% higher reduction in IOP ($\Delta IOP = 1.0$ mmHg; $\Delta IOP = 0.3$ mmHg

Table 3 ΔIOP (mmHg) at various time intervals (h) (IOPdosed eye − IOPcontrol eye) for different formulations of acetazolamide

Formulation	ΔIOP (mmHg) at various time intervals (h) (IOPdosed eye − IOPcontrol eye)							
	0.5	1	1.5	2	3	4	5	6
ACZsusp	0.3 ± 0.1	1.3 ± 0.4	2.3 ± 0.3	1.3 ± 0.6	1.0 ± 0.7	0.7 ± 0.2	—	—
REV1	2.0 ± 0.4	1.3 ± 0.2	3.0 ± 0.4	2.3 ± 0.6	1.7 ± 0.6	1.3 ± 0.7	0.7 ± 0.2	—
REV2	2.0 ± 0.5	1.3 ± 0.5	3.3 ± 0.7	2.7 ± 0.9	2.0 ± 0.4	1.3 ± 0.5	1.0 ± 0.4	0.7 ± 0.3
REV1bio	2.0 ± 0.2	1.7 ± 0.6	3.3 ± 0.8	2.7 ± 0.6	2.0 ± 0.5	1.7 ± 0.6	1.0 ± 0.4	0.7 ± 0.1
Dorzolamide	2.0 ± 0.3	3.7 ± 0.2	2.7 ± 0.6	1.3 ± 0.5	0.7 ± 0.2	—	—	—
Plain niosomes	—	—	—	—	—	—	—	—
Plain niosomes + 0.5% w/v acetazolamide suspension[a]	1.0 ± 0.5	1.7 ± 0.2	2.7 ± 0.6	2.0 ± 0.5	1.3 ± 0.2	0.7 ± 0.3	—	—

All values are negative. — indicates that IOP returns to normal. Control IOP was 10 ± 0.7 mmHg (n = 6); no significant difference in baseline IOP was observed between eyes. The baseline IOP did not show any significant change during the course of the study indicating the absence of any systemic side effects. [a]Five milligrams of acetazolamide was suspended per mL of boric acid; it is different from ACZ susp as no Tween 80 was added. All values for all formulations, at each time, are significantly different, except for REV2 and REV1bio.

Table 4 Activity parameters calculated from ΔIOP values given in Table 3

Formulation	Onset time (h)	Peak effective time (h)	Duration (h)	% Lowering of IOP
ACZsusp	0.5 ± 0.1	1.5 ± 0.3	4.0 ± 0.2	23
REV1	0.5 ± 0.4	1.5 ± 0.4	5.0 ± 0.2	30
REV2	0.5 ± 0.5	1.5 ± 0.7	6.0 ± 0.3	33
REV1bio	0.5 ± 0.2	1.5 ± 0.8	6.0 ± 0.1	33
Dorzolamide	0.5 ± 0.3	1.0 ± 0.2	3.0 ± 0.2	37
Plain niosomes + 0.5% w/v acetazolamide suspension	0.5 ± 0.5	1.5 ± 0.6	4.0 ± 0.3	27

with the formulated acetazolamide suspension in 1% Tween 80). Further, the presence of these niosomes also improved the solubility of acetazolamide from 0.67 mg mL^{-1} to 1.16 mg mL^{-1} (73%). This again could contribute towards an improved penetration of the unentrapped drug in the niosomal formulations.

With REV1 there was a 3 mmHg decrease in IOP (7% more than the suspension) and the effect was maintained for up to 5 h. In the case of positively charged REV (REV2) the peak effect was obtained at 1.5 h and maintained for up to 6 h. Similar behaviour was obtained with bioadhesive coated niosomes (REV1bio). A 20% reduction in IOP was observed for all the developed formulations in the first 30 min of instillation (even Dorzox). When compared with a marketed topical formulation of dorzolamide, Dorzox (2%) (Cipla, Mumbai, India), a topical carbonic anhydrase inhibitor available on the market, a more sustained effect was observed with vesicles though the peak effect observed was 4% less ($P < 0.05$) (Table 3, 4). Plain niosomes did not cause any change in IOP of the rabbits and hence the effect of vehicle on the IOP lowering is ruled out.

Drug leakage behaviour of the REV1bio formulation was evaluated, at different time intervals, at ambient conditions (room temperature), under refrigeration (4–8°C) and at accelerated conditions (ICH guidelines) of 40°C and 75% relative humidity (RH). The results (not shown) indicated that the niosomes were more stable when stored under refrigeration temperature; at higher temperatures the rate of drug loss was high (up to 30% in 6 months – in comparison, it was only 13.2% when niosomes were stored in the refrigerator). Further, the corneal toxicity of the developed formulations (ACZsusp, REV2, REV1bio) was also evaluated in rabbit corneal cell lines (SIRC). It was found that approximately 80% of the cells were viable after 24 and 48 h exposure of the cell lines in the REV1 formulation. The cell viability (and hence safety), however, increased significantly ($P < 0.5$) with bioadhesive niosomal formulations (unpublished work), which suggests the cytoprotective role of Carbopol 934P. Similar effects have been reported by Debbasch et al (2002) with Carbopol. The REV2 formulation showed a considerably high toxicity (viability < 40%).

Conclusion

Niosomes of acetazolamide were prepared successfully using different methods, of which the REV method was found to be the most suitable both in terms of entrapment efficiency and corneal permeability. It can be concluded from the study that incorporation of acetazolamide in niosomes can be of considerable value as a means of reducing the side effects of the drug encountered with oral

therapy and for development of effective topical delivery. Moreover, it was found that positively charged niosomes produced a higher entrapment efficiency compared with neutral and negatively charged niosomes but at the same time induction of charge reduced corneal permeability and increased toxicity. Similar effects with charged vesicles have been reported by other workers (Taniguchi et al 1988). Hence, the use of bioadhesive polymers to achieve an intimate contact at the corneal surface can be considered a better approach. The bioadhesive formulation (REV1bio) showed a high IOP-lowering effect, which compared well with that achieved by positively charged niosomes. However, the significant corneal toxicity observed with the REV2 preparation was greatly reduced (unpublished data), establishing the usefulness of bioadhesives in ocular drug delivery systems. The results indicate that using niosomes as an ocular drug carrier system for topical delivery of acetazolamide (0.5% w/v) can produce a peak effect, which compares well with that of a 2% dorzolamide solution, having a longer duration of action (6 h with REV1bio vs 3 h with Dorzox).

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