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Preparation and evaluation of acetazolamide liposomes as an ocular delivery system

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Abstract

The aim of this work was to formulate a liposomal preparation of acetazolamide to be applied topically, and to evaluate the in vitro and in vivo performance of the prepared liposomes. Acetazolamide liposomes were prepared using the reverse evaporation technique. Neutral, positively-charged, and negatively-charged liposomes were evaluated for their entrapment efficiency, drug release, and in vivo activity. Drug release from liposomes was studied using the membrane diffusion technique. Carbonic anhydrase inhibition activity was determined by the pH stat method, and compared to spectrophotometric determination of acetazolamide. The prepared liposomes were tested for their effect on the intraocular pressure (IOP) in rabbits. The percent entrapment efficiency was 29.27, 41.06 and 49.58% for negatively-charged, neutral and positively-charged liposomes, respectively. The proportion of drug released after 9 h was 13.36, 33.8 and 26.7%, for negatively-charged, neutral and positively-charged liposomes, respectively. There was a good correlation between the percent of inhibition of carbonic anhydrase activity and the amount of drug released from the liposomes as determined spectrophotometrically. Two acetazolamide formulations produced a marked decrease in the IOP. © 1997 Elsevier Science B.V.

Keywords: Liposome; Acetazolamide; Topical carbonic anhydrase inhibitors; Glaucoma; Intraocular pressure; Rabbit

1. Introduction

Acetazolamide is used orally for the reduction of IOP in patients suffering from glaucoma. It is used in the pre-operative management of closed-angle glaucoma, or as an adjunct therapy in the treatment of open-angle glaucoma. It is also used

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in the treatment of various forms of epilepsy and to prevent or ameliorate the symptoms of acute high altitude sickness. To obtain the desired lowering in IOP, large oral doses of acetazolamide are used, and this usually leads to systemic side effects, the most common of which are diuresis and metabolic acidosis.

Acetazolamide is practically insoluble in water and in the aqueous tear fluid, and this limits its ocular bioavailability. The solubility of acetazolamide at pH 7 is approximately 1.01 mg/ml (Parasrampuria and Gupta, 1991), and its partition coefficient, log $P = -0.3$ (Jack, 1992). Several attempts have been made to formulate topically active acetazolamide in order to minimise its systemic side effects. These included surfactant-gel preparations (Tous and Nasser, 1992), contact lenses (Friedman et al., 1985) and aqueous solutions containing cyclodextrins (Loftsson et al., 1994).

Liposomes have gained considerable attention for ocular drug delivery. They have been primarily investigated as a modality to enhance corneal drug absorption. This is achieved through their ability to come in intimate contact with the corneal and conjunctival surfaces, thereby increasing the probability of ocular drug absorption. The administration of drugs entrapped in liposomes to the eye was first studied by Smolin et al. (1981) who showed that liposome-associated iodoxuridine is superior to the solution form of the drug in the treatment of herpes simplex keratitis in rabbits. Iodoxuridine liposomes were shown to promote the corneal permeation of the drug (Dharma et al., 1986). Different liposomal formulations of atropine and atropine sulfate were studied. It was found that atropine entrapped in multilamellar lipid vesicles with positive surface charge displayed the most prolonged effect; whereas, preparations containing atropine sulfate were shorter-acting than similar preparations containing atropine (Meisner et al., 1989). The influence of mucoadhesive polymers on the in vitro release and in vivo ocular bioavailability of pilocarpine nitrate entrapped in liposomes was studied (Durrani et al., 1992). The in vitro and in vivo efficacy of dexamethasone sodium phosphate liposomes as an ocular system was studied, and the delivery of the drug was evaluated in rabbit eyes. Positively charged liposomal formulations of the drug provided the highest drug concentration at the anterior segments of the eye, thus proving useful for the therapy of eye inflammations such as iritis and choroiditis (Al-Muhammed et al., 1996). The aim of this work was to formulate a liposomal preparation of acetazolamide to be applied topically, and to evaluate the in vitro and in vivo performance of the prepared liposomes.

2. Materials and methods

2.1. Materials

Acetazolamide, egg phosphatidylcholine, cholesterol, stearylamine, dicetyl phosphate, Tris[hydroxymethyl]aminomethane hydrochloride, chloroform, acetone, and diethyl ether were obtained from Sigma (St. Louis, MO). Carbonic anhydrase was obtained from Calbiochem-Novabiochem, (La Jolla, CA), and proparacaine HCl, 0.5% solution, from Solvay Animal Health, (Mendota Heights, Minnesota).

2.2. Preparation of liposomes

Acetazolamide liposomes were prepared using the reverse evaporation technique (Szoka and Papahadjopoulos, 1978). Table 1 represents the molar ratios of the lipid components used in the different liposomal formulations. The lipid components were weighed and dissolved in 6 ml chloroform. The organic solvent was removed using a rotary evaporator (Buchi RE-111 Rotavapor,

Table 1
Acetazolamide liposomal formulations expressed as molar ratios of lipid components

Formula	PC	Chol	SA	DP
I	9	1	—	—
II	7	2	—	—
III	7	2	1	—
IV	7	2	—	1

PC: Phosphatidylcholine, Chol: Cholesterol, SA: Stearylamine, DP: Dicetylphosphate.

Brinkmann, Westbury, NY) to produce a thin film of lipid. The lipid film was redissolved in 10 ml ether, and the drug solution in acetone together with 6 ml phosphate buffered saline (PBS), pH 7.4, were added. The mixture was sonicated for 1 min, swirled by hand, and resonicated for another minute. Then, the ether was evaporated on the rotary evaporator. The liposomes were allowed to equilibrate at room temperature, and 10 ml of PBS was added to the liposomal suspension which was kept overnight in the refrigerator. On the following day, the liposomal suspension was filtered through nylon membrane filter, 5 μm pore size, under vacuum. The filtrate was centrifuged at 3000 $\times$ g and 4°C for 1 h. The supernatant was separated from the liposomal pellet that was then resuspended in PBS.

2.3. Determination of acetazolamide entrapment efficiency in liposomes

The concentration of free acetazolamide was determined in the supernatant by measuring the UV absorbance at 265 nm. The entrapment efficiency was determined by difference from the original concentration of drug added, applying the following formula:

$$\%E = (TD - UED) \times 100/TD$$

where %E is the percent encapsulation efficiency, TD is the total drug concentration, and UED is the concentration of unencapsulated drug.

2.4. Drug release from liposomes

The release of acetazolamide from the different liposomal formulations was determined using the membrane diffusion technique (Benita et al., 1986). Briefly, 0.5 ml of the liposomal suspension containing 5 mg acetazolamide together with 0.5 ml of PBS (pH 7.4) were placed in a sac of semi-permeable membrane (Spectrapor No. 2). The sac was placed in 25 ml of PBS (pH 7.4) maintained at 37°C and stirred with a magnetic stirrer. Aliquots of the release medium were withdrawn every hour for 9 h, and the samples were replaced with fresh PBS to maintain a constant volume. The concentration of acetazolamide was determined spectrophotometrically.

2.5. Determinafion of carbonic anhydrase inhibition activity

Inhibition of carbonic anhydrase was assessed using the pH stat assay (Surgue et al., 1990). This assay measures the rate of hydration of CO_2 by determining the rate at which a standard solution of NaOH has to be added to Tris–HCl buffer, pH 8.6, to maintain a constant pH as CO_2 is bubbled into the buffer. Enzymatic activity is proportional to the volume of 0.025 N NaOH that is required to maintain the pH at a value of 8.3. Twenty-five microlitres of buffer-diluted enyme, containing the equivalent to 0.5 U of carbonic anhydrase activity, were added to 4 ml of 0.02 M Tris–HCl buffer, pH 8.6, maintained at 2°C. A mixture of CO_2 air (5/95) was bubbled into the assay vessel at a rate of 150 ml/min. The pH stat end point was set at pH 8.3, and the volume of 0.025 N NaOH added over a 3 min period in order to maintain pH 8.3 was measured. Carbonic anhydrase inhibitors depress the reaction, and this depression is measured as a decrease in the volume of NaOH required to maintain the pH. Results were expressed as IC_{50} values, which were obtained from a semilog plot of percent inhibition against log concentration (Ponticello et al., 1987).

2.6. In vivo studies

Twelve New Zealand albino rabbits were used. The animals were divided into four groups, and all the animals received four treatments: the blank liposomes (control), formula II, formula III and the drug solution, in a Latin Square design. A 3 day washout period was allowed between treatments. Digilab pneumatonometer was used to measure the IOP in conscious rabbits. A 0.5% solution of proparcaine HCI was used as a local anesthetic. The resting IOP level was measured in all the animals before drug administration. The dose of acetazolamide administered was 1 mg either in the liposomal formulation or in the solution form. A single 50 μl drop was instilled into the experimental eye at time 0, and the IOP was measured at 0.5, 1, 2, 3, 4 and 5 h after drug administration.

2.7. Treatment of data

A one way ANOVA (SigmaStat, Jandel, San Rafael, CA) was used to analyse the IOP data at each time point. Significance was considered at $p < 0.05$.

3. Results and discussion

The reverse evaporation method yielded liposomes which upon evaluation with optical microscope revealed large unilamellar vesicles with a particle size in the range of 300–500 nm. The encapsulation efficiency of acetazolamide in liposomes varied with the composition and the ratio of the lipid components used. Positively-charged liposomes showed the highest encapsulation efficiency ($49.58 \pm 1.95\%$), followed by neutral liposomes ($41.06 \pm 2.70\%$), then negatively-charged ones ($29.27 \pm 2.16\%$), using the same phosphatidylcholine–tocholesterol ratio. This order of entrapment efficiency can be attributed to the strength of the binding forces involved in the interaction of the drug with phospholipids. Acetazolamide is a weak acid, and an electrostatic attraction would occur between drug anion and the positively charged stearylamine. This attraction would account for the higher encapsulation efficiency when compared to the negatively-charged liposomes, where such interaction would not be possible.

Fig. 1 shows the release profiles of acetazolamide from the different liposomal formulations Table 2. Neutral liposomes gave the highest rate and extent of drug release, followed by positively-charged liposomes, then negatively-charged ones. Drug release was reduced in the case of charged liposomes compared to the neutral ones. This is consistent with literature reports that the charged lipids serve to tighten the molecular packaging of the vesicle bilayer (Finkelstein and Weissman, 1979) resulting in decreased drug release from charged liposomes. The IC_{50} of acetazolamide liposomal formulation, determined using the pH stat method, was 12.452 nM, which is comparable to the previously determined value of 10.8 nM by Surgue et al. (1990). A good correlation ($r =$ 0.998) was obtained between the carbonic anhydrase inhibition activity of acetazolamide as determined by the pH stat method and the concentration of drug released from liposomes as measured spectrophotometrically (Fig. 2).

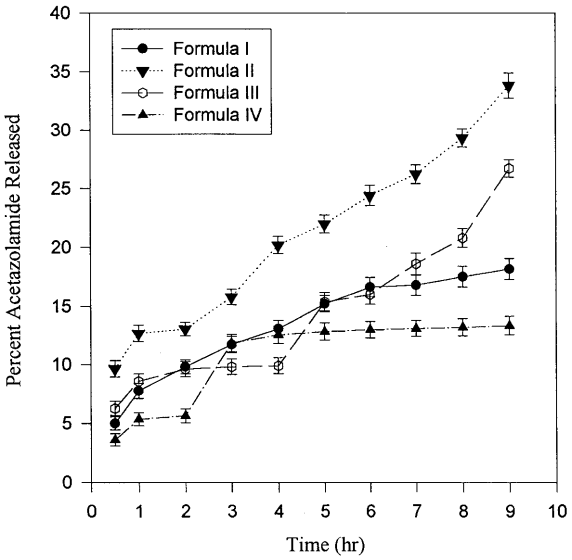

Fig. 1. Release of acetazolamide from liposomes in PBS.

The instillation of a 50 μl drop of a 2% liposomal suspension of acetazolamide formula II significantly lowered the IOP of normotensive rabbits after 2 h of drug administration, while formula III significantly lowered the IOP at 1, 2 and 4 h compared to the control blank liposomes. Acetazolamide solution showed a significant reduction in IOP at 0.5 and 1 h when compared to control (Fig. 3).

The extended ocular hypotensive effect produced by formula III (positively-charged) can be attributed to the fact that positively-charged liposomes have a higher binding affinity to the corneal surface than neutral or negatively-charged vesicles. Hence, positively-charged liposomes can enhance and maintain cornea-liposome interaction to a greater extent. This finding is in accordance with the previous work of Guo et al. (1989, 1990) who confirmed the importance of positive charge to corneal retention of liposomes, presumably, as a result of association with the polyanionic corneal and conjunctival mucoglyco-

Table 2
Comparison of the different acetazolamide liposomal formulations

Formula	Encapsulation efficiency (%) ± S.D.	Rate of release (mg/h) ± S.D.	Total release at 9 h (%) ± S.D.
I	28.21 ± 1.32	0.036 ± 0.001	18.18 ± 0.95
II	41.06 ± 2.70	0.066 ± 0.002	33.87 ± 1.05
III	49.58 ± 1.95	0.038 ± 0.001	26.76 ± 1.04
IV	29.27 ± 2.16	0.024 ± 0.001	13.36 ± 0 80

proteins. Thus, the corneal surface becomes saturated with liposomes. Such an effect accounts for the sustained reduction in IOP produced by the positively-charged acetazolamide liposomes, which lasted up to 4 h after instillation (Fig. 4).

The penetration of drug molecules into the eye from a topically applied preparation is a complex phenomenon. The rate of drug penetration depends on the physicochemical properties of the drug, such as, solubility (Hanna, 1980) and particle size (Schoenwald and Stewart, 1980), as well as the properties of the vehicle (Kupferman et al., 1981). In the liposomal dosage form, the drug is encapsulated in lipid vesicles that can cross cell membranes. Thus, liposomes as drug carriers can change the rate and extent of drug absorption (Singh and Mezei, 1984). The release of a drug from liposomes will increase its local concentration at the corneal surface; however, after release from the vesicles, molecules rely on passive diffusion to cross the corneal barrier. Thus, the longer the contact time at the corneal surface is, the more the amount of drug released. Hence, a more pronounced reduction in IOP was produced by positively-charged acetazolamide liposomes. Positively charged liposomes were previously shown to bind to the cornea more strongly than neutral

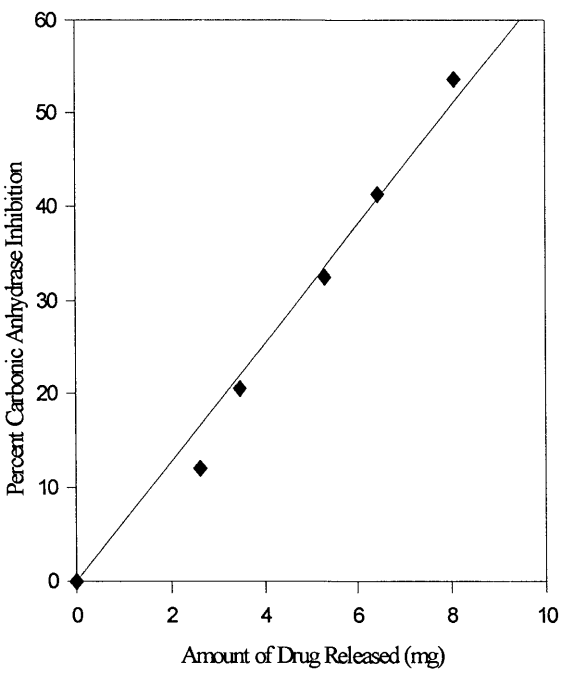

Fig. 2. Relation between the amount of acetazolamide released from liposomes determined spectrophotometrically and the corresponding enzymatic activity.

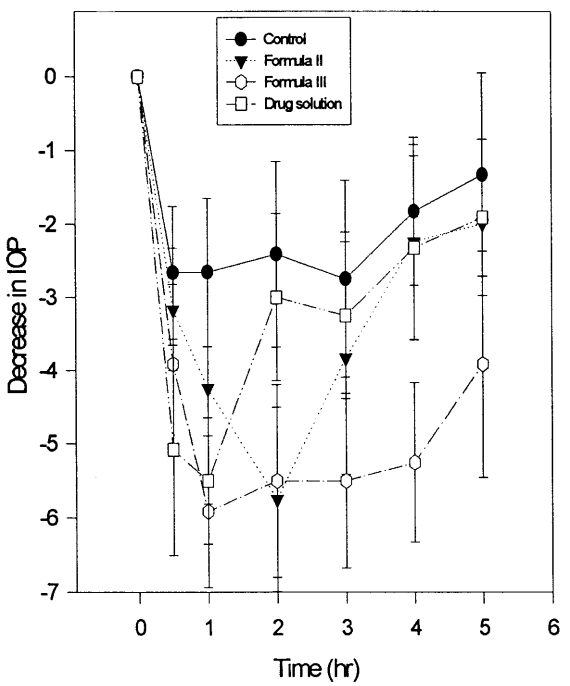

Fig. 3. Effect of test formulations on IOP in rabbits

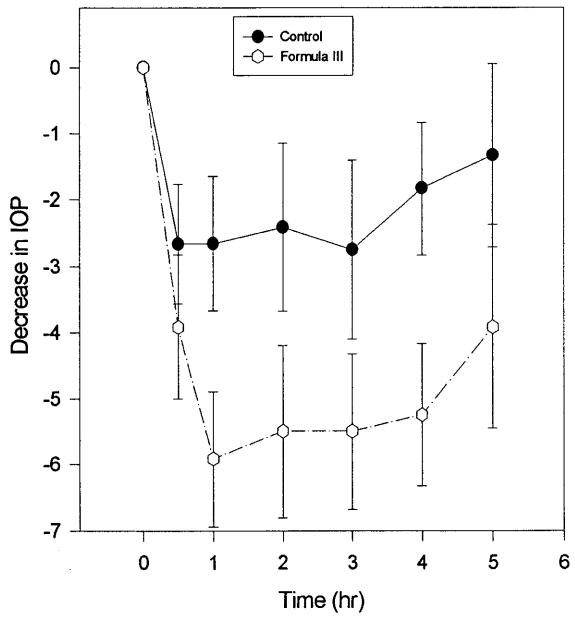

Fig. 4. Effect of formula III versus control on IOP in rabbits

or negatively-charged liposomes (Schaffer et al., 1983). Corneal epithelium does not offer the majority of resistance to penetration of lipophilic drugs (Meisner et al., 1989). Thus, enhanced corneal attachment is possibly the mechanism by which positively-charged acetazolamide liposomes produced a pronounced reduction in IOP.

4. Conclusion

Positively-charged acetazolamide liposomes produced higher entrapment efficiency than either negatively-charged or neutral liposomes. Positively-charged liposomes also produced a strong and sustained reduction in IOP in rabbits. The incorporation of acetazolamide in a liposomal preparation for ocular drug delivery can be of considerable value as a means to minimise the side effects encountered with the oral administration of the drug.

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