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Study of the extent of ocular absorption of acetazolamide from a developed niosomal formulation, by microdialysis sampling of aqueous humor

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Abstract

Acetazolamide, a carbonic anhydrase inhibitor is used orally (no topical formulation being available in the market) for the reduction of intraocular pressure (IOP) in patients suffering from glaucoma. Two major reasons responsible for the failure to develop a topically effective formulation of acetazolamide are its low solubility (0.7 mg/ml) and its low permeability coefficient. It is generally recognized that topical acetazolamide formulation possessing efficacy similar to that achieved upon oral administration would be a significant advancement in the treatment of glaucoma. In order to enhance the bioavailability of acetazolamide by topical route and to improve the corneal permeability of the drug, the niosomes of acetazolamide were prepared (by reverse phase evaporation method) and coated with Carbopol for the latter's bioadhesive effect. The pharmacodynamic studies showed 33% fall in IOP with the developed formulation, and the effect was sustained for 6 h after instillation. The effect compared well with a four times higher concentration of dorzolamide (Dorzox®), a topical CAI available in the market. In the present study, the aqueous humor disposition of the drug from the developed bioadhesive coated niosomal formulation (ACZREVbio) is compared with the aqueous suspension of the drug (containing 1% (w/v) Tween 80 as a dispersing agent) at similar concentrations.

The concentration of acetazolamide absorbed in the aqueous humor at various times from the control suspension and from ACZREVbio was determined by microdialysis in male albino rabbits. Microdialysis provides a complete concentration versus time profile and hence is an important advance to the regional sampling of tissues. The peak concentration of drug absorbed in the aqueous humor from the ACZREVbio formulation (14.94 μg/ml) was almost two times of that obtained with the equivalent amount of acetazolamide control suspension (6.93 μg).

The results show a significant broadening of peak from 80 to 120 min with the concentration of more than 13 μg being maintained at these times, for the bioadhesive coated niosomal formulation (ACZREVbio). An important observation was the fact that a high drug concentration of 12.02 μg reached immediately, i.e., 20 min after instillation of ACZREVbio indicating a high penetration being achieved, while a meagre concentration of only 3.53 μg is obtained at 60 min after instillation of the control suspension. The aqueous humor disposition indicates peaks and troughs in drug concentration which may be related to the decrease in aqueous humor formation, such that the drug concentration/volume increased at these points.

Keywords: Acetazolamide; Topical delivery; Niosomal formulation; Bioadhesive coating; Ocular pharmacokinetics; Microdialysis

1. Introduction

A major problem in ocular therapeutics is the attainment of an optimal drug concentration at the site of action. Acetazolamide, a carbonic anhydrase inhibitor is used orally (no topical formulation being available in the market) for the reduction of IOP in patients suffering from glaucoma. The peripheral inhibition of carbonic anhydrase produces a wide array of side effects which most of the patients are unable to tolerate and hence they discontinue the therapy (Epstein and Grant, 1977). The constraints in development of topical formulation of acetazolamide (Kaur et al., 2002; Singla et al., 2002) are its very low solubility (0.7 mg/ml) in aqueous tear fluid and in water and its limited corneal penetration (log $P = 0.3$) (Parasrampuria, 1993). Moreover, the degradation of acetazolamide increases many folds 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. In the formulation of newer topical ocular dosage forms, a great

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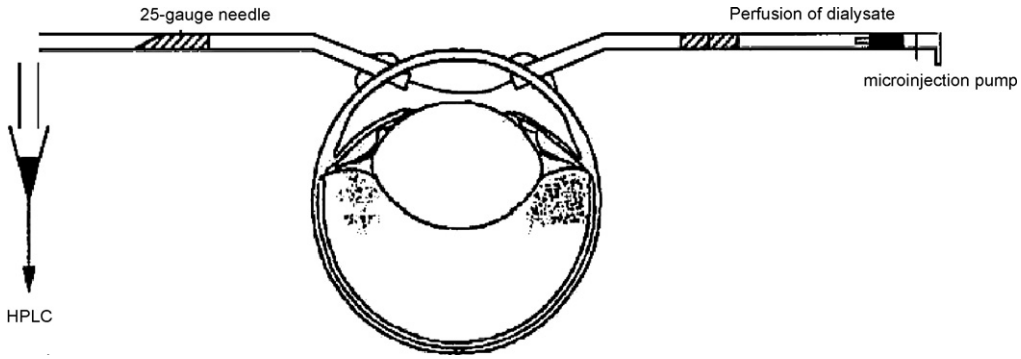

Fig. 1. Schematic diagram of the microdialysis method for determination of drug levels in aqueous humor.

attention is now being devoted to new drug delivery systems that can ensure a localized effect, have 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 bilayer 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 (Kaur et al., 2004; Saettone et al., 1996; Uchegbu and Vyas, 1998). Further, these vesicular systems can be used in combination with mucoadhesive polymers to obtain a controlled as well as prolonged effect. Prolonging the drug contact time with the surface of the eye can also increase the penetration through the cornea, hence, increasing the accessibility of drug to the aqueous humor (Liaw and Robinson, 1993; Sasaki et al., 1995; Kaur and Smitha, 2002). This will reduce the amount of drug and the dose frequency necessary for therapeutic effect (Rosenlund, 1996). Reduction in dose will help reduce the incidence of systemic side effects.

Microdialysis has been employed as an analytical tool for regional sampling of fluids of brain, blood, liver, muscle, kidney, joint and ocular tissue (Ben-Nun et al., 1989). Microdialysis has been used in vitreous (Waga et al., 1991; Ben-Nun et al., 1989), retina (Louzada-Junior et al., 1992) and aqueous humor (Ohtori et al., 1998; Rittenhouse et al., 1998; Rittenhouse et al., 1999) in order to estimate the ocular bioavailability of regionally administered ophthalmics (Fig. 1). Determination of aqueous humor samples traditionally has been conducted with paracentesis sampling of multiple animals at each time point. In order to obtain a sufficient sample pool to characterize pharmacokinetics reliably, a large number of animals is required. Microdialysis provides an important advance to the regional sampling of the tissues, as a complete concentration versus time profile can be obtained in individual animals (Rittenhouse et al., 1999). The process of microdialysis is controlled by diffusion. The driving force for mass transport is the concentration gradient between the ECF and the fluid in the probe lumen. If the concentration of the compound is higher in the ECF, some portion of it will diffuse in the probe lumen, some fraction will diffuse out of the probe.

The aim of this study was to establish that the niosomal preparation of acetazolamide developed by us and previously reported to possess a better corneal penetration and IOP lowering effect (Aggarwal et al., 2004) penetrated well through the cornea and was available at sufficiently high concentrations in the aqueous humor. The concentration of the drug absorbed in the aqueous humor was determined using the microdialysis technique.

2. Materials and methods

2.1. Materials

Acetazolamide (Shallak, India); Carbopol 934P (BF Goodrich, USA); span 60 (Loba Chemie, Mumbai, India); cholesterol (Loba Chemie, Mumbai, India); ketamine HCl injection, USP (Dodge Animal Health, USA); pentobarbital (Sigma–Aldrich, USA); xylazine (Lloyd Labs, USA); all other chemicals and reagents were of analytical grade.

2.2. Method

The niosomal vesicles (ACZREV), of acetazolamide (0.5%, w/v) were prepared by reverse phase evaporation method as reported earlier. In order to increase the retention time of the drug in the eye, the vesicles were coated with 0.05% Carbopol 934P (ACZREVbio) (Aggarwal et al., 2004).

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

Corneal Permeation and Pharmacodynamic studies (IOP lowering effect) have been reported earlier (Aggarwal et al., 2004).

2.2.1. Microdialysis

This method was used to assay aqueous humor of the rabbit eyes, into which the developed formulations were instilled.

2.2.1.1. Animals. New Zealand albino rabbits weighing between 5 and 6 lb (approximately 2.5 kg) were used for the study. The rabbits were kept under anesthesia throughout the experiment by intramuscularly injecting ketamine hydrochloride (3.5 mg/kg) and xylazine (3.5 mg/kg), every 40 min. The animals were sacrificed by an overdose of pentobarbital (100 mg/kg) into the marginal ear vein at the completion of the experiment. These animals do not recover from the anesthesia

during the entire length of the experiment making this a non-survival surgery.

2.2.1.2. Method. Pupils of the rabbits were dilated with 1% tropicamide solution before the probe implantation. A linear probe was implanted into the aqueous humor using a 25G needle. The needle was inserted across the cornea, just above the corneoscleral limbus, so that it traversed through the center of the anterior chamber to the other end of the cornea. The sample-collecting end of the linear probe (made of polyacrylonitrile membrane and 0.5 mm in diameter) was inserted carefully into the bevel end edge of the needle (Fig. 1). The needle was slowly retracted leaving the probe with the dialysis membrane in the middle of the anterior chamber. The outlet of the probe was then fixed to prevent any disturbance during sample collection. The probes were perfused with pH 7.4 isotonic phosphate buffer saline (IPBS) at a flow rate of 2 μl/min using the CMA/100 microinjection pump. Three rabbits were arranged in series and were attached to the same microdialysis pump. After probe implantation, the animals were allowed to stabilize for 2 h. This duration has been shown to be sufficient for the restoration of intraocular pressure and replenishment of the aqueous humor originally lost during probe implantation (Tak et al., 2001). After 2 h, 30 μl of the formulation was instilled into the eye. The experiment was continued for 6 h after instillation of the formulation. Samples were collected every 20 min, throughout the study, and were analyzed using HPLC with UV detector (λ_{max}, 265 nm). At the end of the experiment, euthanasia was performed under deep anaesthesia, with an intravenous injection of sodium pentobarbital through the marginal ear vein.

2.2.1.3. Relative recovery. To determine the in vitro relative recovery of drugs through the membrane, three probes connected to one microinjection pump were bathed in (IPBS) solution containing known concentration of drug and perfused with IPBS at 2 μl/min. Dialysate was collected every 20 min over 2 h; 30 μl of this dialysate was injected into the HPLC column and drug levels were determined. All these measurements were made using the same microinjection pump, HPLC system, and IPBS except dialysis probes. Relative recovery is expressed as a ratio of drug concentration in dialysate and that in the solution bathing the dialysis membrane.

The recovery of acetazolamide is calculated according to the following equation:

$$\text{Recovery} = \frac{C_{\text{out}}}{C_i}$$

C_{out} is the concentration in the outflow solution and C_i is the known concentration of the drug solution bathing the dialysis membrane.

After determining the recovery for the compound, dialysate concentrations were transformed into the actual anterior chamber concentrations using the following equation:

$$C_{i'} = \frac{C_{\text{out}'}}{\text{recovery}}$$

$C_{i'}$ is the drug concentration in the aqueous humor and $C_{\text{out}'}$ is the concentration of the dialysate.

There was no considerable variation (1–2%) in the recovery of the probes with time over the experimental time period, but for all experiments the average of the recovery determined before and after the experiment was considered for assessing the intraocular fluid concentrations.

2.3. Statistical analysis

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

3. Results and discussion

The niosomes of acetazolamide were prepared using reverse phase method, as by this method large unilamellar vesicles are formed, having large aqueous space. Hence, the entrapment of drugs dissolved in the aqueous phase will be more in these vesicles compared to small unilamellar and multilamellar vesicles formed by other methods (Gould-Fogerite and Mannino, 1992; Aggarwal et al., 2004). Further, the niosomes were coated with bioadhesive polymer Carbopol 934P in order to increase the residence time of the formulation in the eye. Carbopol acts by forming 3D microgel structure in aqueous media, which provides interaction with phospholipids. It is also reported to increase the penetration of drugs (Lehr et al., 1994). The pH of acetazolamide formulations was adjusted between 4 and 5 (the pH of maximum stability of acetazolamide being 4.5) (Parasrampuria, 1993) using a 2% boric acid solution. The entrapment efficiency of the developed reverse phase vesicles was found to be 43.75% (Aggarwal et al., 2004). Fig. 2 shows the release studies of the different formulations where acetazolamide suspension is taken as control. The release obtained by bioadhesive coated formulation (ACZREVbio) was comparable to ACZREV.

The physiological effectiveness of the formulations (ACZREV and ACZREVbio) was determined in terms of their IOP lowering effect in normotensive rabbits. ACZREVbio formulation showed a significantly better effect both in terms of the %lowering of effect and the duration of action (Aggarwal et

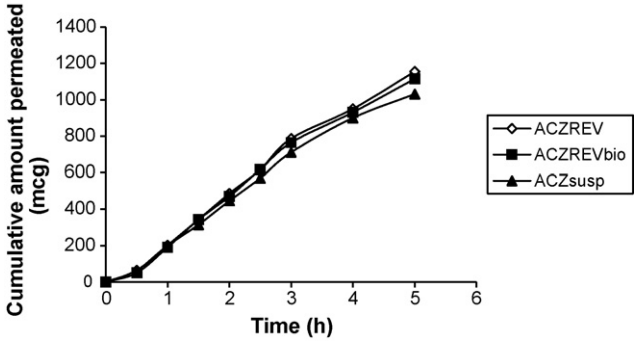

Fig. 2. A plot of cumulative amount vs. time.

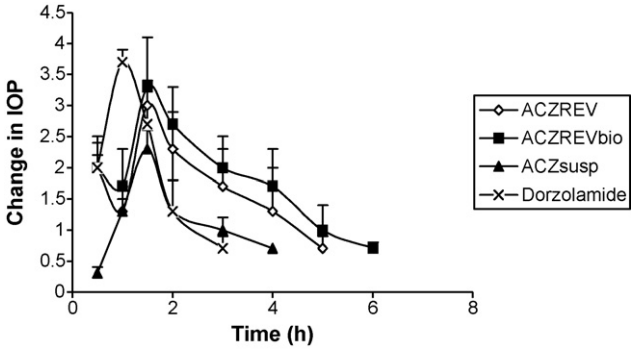

Fig. 3. Change in IOP vs. time graph for various formulations of acetazolamide.

al., 2004). No change in IOP was observed in the untreated eye during the course of measurement in any of the formulations, thus indicating that these formulations exerted a local action within the eye and that the observed IOP lowering activity 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 decreased 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 in comparison to an almost 20% reduction in niosomal formulations and also dorzolamide at this time. This indicates that the initial lowering of IOP achieved by the niosomal formulation is probably because of the high penetrability of the niosomes as is observed in case of the tailor made newer CAI dorzolamide claimed to be highly permeable. With ACZREV there was a maximum of 3 mmHg (30%; peak effect) lowering of IOP (7% more than the suspension) and the effect was maintained for upto 5 h. In case of bioadhesive coated niosomes (ACZREVbio) the peak effect was obtained at 1.5 h and the effect was maintained for upto 6 h. The %lowering of IOP was 33% vis-a-vis 30% obtained with the uncoated formulation ACZREV. When compared with marketed topical formulation of dorzolamide, Dorzox® (2%) (Cipla, Mumbai, India) a topical carbonic anhydrase inhibitor available in the market, a more sustained effect was observed with vesicles though the peak effect (37% lowering of IOP) observed was 4% less ($P < 0.05$) (Fig. 3; Aggarwal et al., 2004).

Table 1 and Fig. 4 (microdialysis studies), clearly suggest that the absorption or permeation of acetazolamide from the niosomal formulation (ACZREVbio) into the aqueous humor, is far better than that from the acetazolamide suspension ($P < 0.01$). The C_{max} of 14.94 μg/ml achieved with niosomal acetazolamide formulation is almost two times (6.93 μg/ml) that obtained upon instillation of the suspension containing same amount of acetazolamide.

Minimal plasma concentrations that satisfactorily lower IOP have been reported as 5–20 μg/ml (Friedland et al., 1977; Alm et al., 1982; Lehmann et al., 1969; Berson et al., 1980) by different group of workers. It has also been reported that upon i.v. administration of 8 mg/kg of acetazolamide there was a decrease in IOP by 18 ± 4% and the concentration of acetazolamide in aqueous humor was 0.276 ± 0.071 μg/ml (Duffel et al., 1986).

Table 1
Concentration time profile of Acetazolamide, from the developed bioadhesive niosomal (ACZREVbio) formulation and its suspension, in the aqueous humor of rabbits

Time (min)	Concentration (μg) ± S.D. (ACZsusp)	Concentration (μg) ± S.D. (ACZREVbio)
20	0	12.02 ± 4.69
40	0.05 ± 1.53	9.40 ± 7.05
60	3.53 ± 1.19	3.79 ± 0.95
80	1.61	13.59 ± 7.25
100	6.93 ± 4.81	14.94 ± 0.65
120	1.91	13.31 ± 3.76
140	3.35 ± 2.28	0.86 ± 0.82
160	3.15	5.47
180	1.25	1.98 ± 0.25
200	0.64 ± 0.2	
C_{max} (μg/ml)	6.93	14.94
T_{max} (min)	100	100
AUC	537.231	1230.0116

The concentration of 14.94 μg/ml, achieved by us, in aqueous humor, upon topical administration is almost 65 times more than that achieved with a fairly high i.v. dose. We also report a significantly high concentration achieved with acetazolamide suspension, for the first time. The suspension has earlier been shown by us to permeate well (Fig. 2) and also to be pharmacodynamically effective (Aggarwal et al., 2004). The present results show a significant broadening of peak from 80 to 120 min with the concentration of more than 13 μg being maintained at these times, for the bioadhesive coated niosomal formulation. An important observation was the fact that a high drug concentration of 12.56 μg reached immediately, i.e., 20 min after instillation of the niosomal formulation indicating a high and fast penetration being achieved; while an effective concentration is observed only at 60 min (3.53 μg; four times less than the concentration at 20 min for ACZREVbio) after instillation of the control suspension.

Further a 2% dorzolamide solution is reported to show a maximum concentration of 7.8 μg/ml at 2 h after instillation (Ponticello et al., 1998). Considering that dorzolamide is a tailor made molecule with a much better solubility and penetration, a two times higher C_{max} shown by a four times less concentration of acetazolamide in ACZREVbio formulation (0.5%

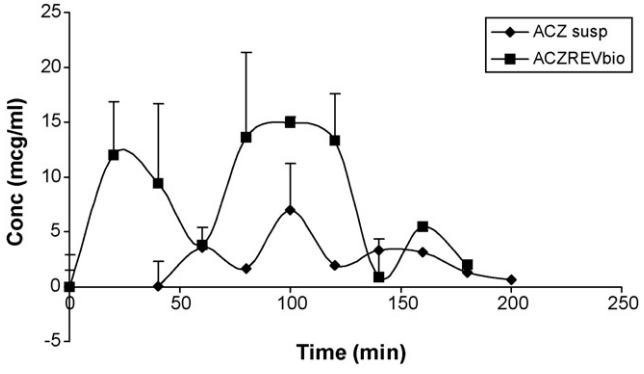

Fig. 4. Comparison of suspension and biaodhesive niosomal formulation of acetazolamide using microdialysis.

acetazolamide in comparison to 2% dorzolamide) developed by us is a remarkable achievement. Eventhough, acetazolamide is a weaker inhibitor of CAII and CAIV (Ponticello et al., 1998) but the high concentration achieved in the aqueous humor by the developed niosomal formulation seems to effectively inhibit the carbonic anhydrase enzyme (as indicated by a significant IOP lowering which compares well with dorzolamide, Fig. 3). Further, it is expected that the concentration of acetazolamide in iris-cilliary body would be much higher (as also indicated for dorzolamide by Ponticello et al., 1998) and since CAII and CAIV are majorly present in these tissues they are thus expected to be completely and effectively inhibited.

It may be noted from Table 1 and Fig. 4 that the aqueous humor concentrations are significantly high even upto 6 h after instillation indicating a sustained effect. The aqueous humor concentration versus time plot of acetazolamide (Fig. 4), however shows several peaks and troughs. These can be attributed to the inhibition of aqueous humor production by the drug. Once the drug enters the aqueous chamber in a sufficient quantity it is expected to inhibit the aqueous humor secretion, thus resulting in a high drug concentration/ml of the aqueous humor. The acetazolamide suspension of the drug also shows several peaks (though of much lower intensity), again probably due to the sustained delivery of the drug from the suspended particles.

4. Conclusion

Antiglaucoma therapy requires a continuous and chronic administration of the drug. Many patients cannot tolerate oral treatment with acetazolamide because of the systemic side effects associated with its use (Epstein and Grant, 1977). Development of a topically effective formulation of acetazolamide has not been possible because of its unfavourable partition coefficient, solubility, low stability and a low permeability coefficient (Michael and Kass, 1989; Kaur et al., 2002; Parasrampuria, 1993). These problems were effectively overcome by us in the presented research work, by the incorporation of acetazolamide into niosomes (Aggarwal et al., 2004). Coating these niosomes with a bioadhesive produced significantly better effect, which compared well with a 2% dorzolamide solution. The developed formulation showed a longer duration of action (6 h with ACZREVbio vis-a-vis 3 h with Dorzox®). The ocular pharmacokinetics (determined using microdialysis studies) of the developed formulation (ACZREVbio) and the control drug suspension, as presented here, confirm these claims. The peak concentration of drug absorbed into the aqueous humor from niosomal formulation (Fig. 4) was significantly more than the suspension ($P < 0.01$). The present study can thus be considered a successful extension and also a confirmation of suitability of using topical niosomal formulation(s) of acetazolamide for the control of elevated IOP.

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