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Preparation and evaluation of reverse-phase evaporation and multilamellar niosomes as ophthalmic carriers of acetazolamide

Ahmed S. Guinedi, Nahed D. Mortada, Samar Mansour*, Rania M. Hathout

Department of Pharmaceutics, Faculty of Pharmacy, Ain Shams University, Monazamet El Wehda El Afrikia St., El Abbassia, Cairo, Egypt

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Abstract

Niosomes have been reported as a possible approach to improve the low corneal penetration and bioavailability characteristics shown by conventional ophthalmic vehicles. Niosomes formed from Span 40 or Span 60 and cholesterol in the molar ratios of 7:4, 7:6 and 7:7 were prepared using reverse-phase evaporation and thin film hydration methods. The prepared systems were characterized for entrapment efficiency, size, shape and in vitro drug release. Stability studies were carried out to investigate the leaching of drug from niosomes during storage. The intraocular pressure (IOP) lowering activity of acetazolamide niosomal formulations in rabbits was measured using ShiØtz tonometer. Histological examination for the corneal tissues of rabbits receiving niosomal formulations was carried out for assessment of the ocular irritancy of niosomes. The results showed that the type of surfactant, cholesterol content and the method of preparation altered the entrapment efficiency and drug release rate from niosomes. Higher entrapment efficiency was obtained with multilamellar niosomes prepared from Span 60 and cholesterol in a 7:6 molar ratio. Niosomal formulations have shown a fairly high retention of acetazolamide inside the vesicles (approximately 75%) at a refrigerated temperature up to a period of 3 months. Each of the tested acetazolamide niosomes prepared by either method produced a significant decrease in IOP compared to the solution of free drug and plain niosomes. Multilamellar acetazolamide niosomes formulated with Span 60 and cholesterol in a 7:4 molar ratio were found to be the most effective and showed prolonged decrease in IOP. Histological examination of corneal tissues after instillation of niosomal formulation for 40 days showed slight irritation in the substantia propria of the eye which is reversible and no major changes in tissues were observed.

Keywords: Acetazolamide; Niosomes; Non-ionic surfactant vesicles; Ocular irritancy; Ophthalmic; Stability

1. Introduction

Amongst the available carbonic anhydrase inhibitors CAIs, acetazolamide is still the most effective drug for the treatment of glaucoma (Kaur et al., 2002). To obtain the desired lowering in intraocular

* Corresponding author. Tel.: +20 121 603202/2 4051106; fax: +20 2 4051107.
E-mail addresses: samar_holayel@yahoo.com, samarmansour@asu-pharmacy.edu.eg (S. Mansour).

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pressure (IOP), large oral doses of acetazolamide are used, and this usually leads to numerous systemic side effects, the most common of which are diuresis and metabolic acidosis. As a result, topical administration of acetazolamide is usually preferred over systemic administration. In order to overcome the problems of conventional ocular therapy, such as short residence time, loss of drug through nasolacrimal drainage, impermeability of corneal epithelium and frequent instillation; newer topical delivery systems for acetazolamide are being explored by many researchers (Friedman et al., 1985; Tous and Nasser, 1992; Loftsson et al., 1994; El-Gazayerly and Hikal, 1997; Kaur et al., 2000).

Vesicular drug delivery systems used in ophthalmics such as liposomes and niosomes help in providing prolonged and controlled action at the corneal surface and preventing the metabolism of the drug by enzymes present at the tear/corneal surface (Kaur et al., 2004). Drug enclosed in the vesicles allows for an improved partitioning and transport through the cornea. Moreover, vesicles offer a promising avenue to fulfill the need for an ophthalmic drug delivery system that has the convenience of a drop, but will localize and maintain drug activity at its site of action (Kaur et al., 2000).

Non-ionic surfactant based vesicles (niosomes) are formed from the self-assembly of non-ionic amphiphiles in aqueous media resulting in closed bilayer structures (Uchegbu and Vyas, 1998) which can entrap both hydrophilic and lipophilic drugs either in an aqueous layer or in vesicular membrane (Carafa et al., 1998).

Niosomes in topical ocular delivery are preferred over other vesicular systems because: (i) they are chemically stable; (ii) they have low toxicity because of their non-ionic nature; (iii) they handle surfactants with no special precautions or conditions; (iv) they can improve the performance of the drug via better availability and controlled delivery at a particular site; (v) they are biodegradable, biocompatible and non-immunogenic (Carafa et al., 2002).

The non-ionic surfactant vesicles have been reported to be successful, as an ocular vehicle for cyclopentolate. Niosomes, independent of the pH, significantly improved the ocular bioavailability of cyclopentolate, with respect to reference buffer solution. No irritation with the niosomal formulation was observed (Saettone et al., 1996). Vyas et al. (1998) reported that there was about a 2.48 times increase in the ocular bioavailability of timolol maleate encapsulated in niosomes as compared to timolol maleate solution.

The aim of this work was to formulate reverse-phase evaporation (REV) and multilamellar (MLV) niosomal formulations of acetazolamide to be applied topically and to evaluate the in vitro and in vivo performance of these formulations. Stability studies were carried out to investigate the leaching of drug from niosomes during storage. Histological examination of the corneal tissues of rabbits receiving niosomal formulations was carried out for assessment of ocular irritancy of niosomes.

2. Materials and methods

2.1. Materials

Acetazolamide (ACZ), sorbitan monopalmitate (Span 40), sorbitan monostearate (Span 60), cholesterol (CH), polyethylene glycol 400 (PEG 400) and propylene glycol were purchased from Sigma Chemical Co., St. Louis, USA. Acetone, absolute alcohol, chloroform, methanol, diethyl ether, sodium chloride, potassium dihydrogen phosphate and disodium hydrogen phosphate were purchased from Adwic, El-Nasr Chemical Co., Cairo, Egypt, according to the methods of Prolabo, Paris, France. Acetyl uranil (uranyl acetate-2-hydrate) was purchased from Allied Signal, Riedel-dahaen, Germany. Spectra/Por dialysis membrane, 12,000–14,000 molecular weight cut off was purchased from Spectrum Laboratories Inc., Rancho Dominguez, Canada.

2.2. Preparation of acetazolamide reverse-phase evaporation niosomes

REV vesicles containing acetazolamide were prepared by the method of Szoka and Papahodjopoulos (1978). Briefly, mixtures of surfactant (Span 40 or Span 60) and cholesterol, in different molar ratios, viz. 7:4, 7:6 and 7:7, were accurately measured into a long necked quick fit round-bottom flask and dissolved in 9 ml of a chloroform/methanol mixture (2:1, v/v). The organic solvents were slowly evaporated under reduced pressure, using a rotary evaporator (Janke and

Kunkel, model RVO5-ST, IKA Laboratories, Staufen, Germany) at 60 °C such that a thin dry film of the components was formed on the inner wall of the rotating flask. The film was redissolved in 12 ml ether and a solution containing 20 mg drug in 4 ml acetone together with 6 ml phosphate buffered saline (pH 7.4) was added. The mixture was sonicated for 2 min, swirled by hand, and resonicated again for another 2 min in a bath sonicator. The resultant opalescent dispersion was rotary evaporated to disrupt the gel formed immediately. Following addition of 10 ml phosphate buffered saline (pH 7.4); rotary evaporation was continued for an additional 15 min duration to ensure the removal of residual diethyl ether. The niosomal suspension was left to mature overnight at 4 °C. For sterility, all the above mentioned steps were done under aseptic conditions. All glassware was sterilized by autoclaving, phosphate buffered saline was passed through a 0.22 μm membrane filter, and the entire procedure was carried out in a laminar flow hood (Esco, Singapore).

2.3. Preparation of acetazolamide multilamellar niosomes

Acetazolamide MLV niosomes were prepared using the thin film hydration method. Accurately weighed quantities of the surfactant (either Span 40 or Span 60) and cholesterol in different molar ratios, viz. 7:4, 7:6 and 7:7, were dissolved in 9 ml of a chloroform/methanol mixture (2:1, v/v) in a round-bottom flask (Agarwal et al., 2001). Afterwards, 20 mg acetazolamide dissolved in 5 ml of an acetone:methanol mixture (4:1) was added to the lipid solution. The organic solvents were removed under vacuum in a rotary evaporator at 60 °C to form a thin film on the wall of the flask. After removal of the last trace of organic solvent, the film was hydrated by 10 ml of phosphate buffered saline (PBS, pH 7.4) at 60 °C for 1 h (Pillai and Salim, 1999). The resulting niosomal suspension was mechanically shaken for 1 h using a horizontal mechanical shaker (Kotterman, Hanigsen, Germany) at 60 rpm and 40 °C leading to the formation of multilamellar niosomes. The niosomal suspension was left to mature overnight at 4 °C. To maintain sterility of the preparation, all the above mentioned steps were done under aseptic conditions as described previously in Section 2.2.

2.4. Determination of acetazolamide entrapment efficiency

The prepared REV or MLV acetazolamide niosomes were separated from unentrapped acetazolamide by ultracentrifugation at 18,000 rpm for 1 h using a refrigerated centrifuge at 2 °C (Beckman, Fullerton, Canada). The isolated pellets were washed twice, each with 10 ml phosphate buffered saline, and recentrifuged again for 1 h.

The amount of entrapped acetazolamide was determined by lysis of the vesicles with absolute ethanol (Fang et al., 2001). A 100 μl sample of niosomes was mixed with 5 ml of absolute ethanol and covered well with parafilm to prevent evaporation. The solution then was sonicated for 10 min in a bath type sonicator to obtain a clear solution (Law et al., 1994a). The concentration of acetazolamide in absolute ethanol was determined spectrophotometrically at 265 nm using a UV–vis spectrophotometer (Shimadzu, model UV-1601 PC, Kyoto, Japan).

The entrapment efficiency is defined as follows (Ruckmani et al., 2000):

$$\text{Acetazolamide entrapment efficiency (\%)} = \frac{\text{amount of ACZ entrapped}}{\text{total amount of ACZ}} \times 100$$

2.5. Characterization of acetazolamide niosomes

2.5.1. Photomicroscopy and transmission electron microscopy (TEM)

Vesicle dispersions were characterized by photo and transmission electron microscopy for vesicle formation and morphology.

Samples of the niosomal formulations were examined under optical microscope (Carl Zeiss, Berlin, Germany) and photographed at a magnification of 400×, by means of a fitted camera (Panasonic, Japan).

Negatively stained samples of the acetazolamide niosomes were examined by transmission electron microscope (model JEM-100S, Joel, Tokyo, Japan) at 70 kV. A saturated acetyluranil aqueous solution was used as the staining agent (Hu and Rhodes, 2000). Acetyluranil is a good general stain and fixative, particularly in membranes. Many fixation methods for TEM used acetyluranil as a stain to improve the appearance

of structures by increasing their contrast (Harris et al., 1999).

2.5.2. Determination of vesicle size

The vesicle sizes of the prepared niosomal formulations composed of Span 40 or Span 60 and cholesterol in a 7:4 molar ratio were determined by light scattering based on laser diffraction using the Malvern Mastersizer (Malvern Instruments Ltd., Worcestershire, UK) (Arunothayanun et al., 2000).

2.5.3. In vitro release of acetazolamide from niosomes

The release of acetazolamide from niosomes was determined using the membrane diffusion technique (Devaraj et al., 2002; Glavas-Dodov et al., 2002). An accurately measured amount of acetazolamide niosomal formulations, equivalent to 2 mg, was suspended in 1 ml phosphate buffered saline (pH 7.4) and transferred to a glass tube, having a diameter of 2.5 cm, that was previously covered at its lower end by a soaked cellulose membrane (Spectra/Por dialysis membrane 12,000–14,000 Mwt cut off). Then, the glass tube was suspended in the dissolution flask of a USP dissolution apparatus (Pharma Test, Hainburg, Germany) containing 75 ml phosphate buffered saline (pH 7.4). The glass tube was allowed to rotate at a constant speed (50 rpm). The phosphate buffered saline was maintained at a temperature of 37 °C (Gursoy et al., 2004). At predetermined time intervals for 8 h, aliquots were withdrawn and the drug contents were determined spectrophotometrically at 267 nm. The results were the mean values of three runs. The obtained data were analyzed to determine the order of release.

2.6. Physical stability of acetazolamide niosomes

Physical stability studies were carried out to investigate the leaching of drug from niosomes (in a liquid form) during storage. The optimized acetazolamide MLV and REV niosomal formulations, composed of Span 60 and cholesterol in a 7:4 molar ratio, were sealed in 20 ml glass vials and stored at refrigeration temperature (2–8 °C) for a period of 3 months. Samples from each batch were withdrawn at definite time intervals, the residual amount of the drug in the vesicles was determined as described previously in Section 2.4 after separation from unentrapped drug (Shahiwala and Misra, 2002).

2.7. In vivo study on acetazolamide niosomes

Selected acetazolamide niosomal formulations composed of Span 60 and cholesterol in a 7:4 or 7:6 molar ratio were tested for their intraocular pressure lowering activity on normotensive albino rabbits and the results were compared with that of plain niosomes and acetazolamide solution (1%) prepared using polyethylene glycol 400 (7%, v/v) and propylene glycol (53%, v/v) (Parasrampuria and Gupta, 1990). All niosomal preparations used in this study were adjusted to a concentration of 1% acetazolamide. The experimental procedures conform to the ethical principles of the Egyptian Research Institute of Ophthalmology, Giza (Egypt) on the use of animals (Monem et al., 2000).

Adult albino normotensive rabbits weighing 2.5–3 kg were used in the experiments. The rabbits were kept in individual cages with food and water. The animals were maintained in a 12-h light/12-h dark cycle in a temperature controlled room at 20–24 °C (Winum et al., 2004). The rabbits were divided into seven groups, each consisting of six rabbits, namely: groups I–IV received REV or MLV acetazolamide niosomes composed of Span 60 and cholesterol in a 7:4 and 7:6 molar ratio, group V received acetazolamide solution, 1%. Finally, groups VI and VII received plain REV and MLV niosomes, respectively. The intraocular pressure (IOP) was measured as a function of time using a standardized tonometer (ShiØtz, Germany) (Monem et al., 2000). A single 50 μl dose of 1% acetazolamide preparation was instilled onto the corneal surface of the rabbit's eye (Kaur et al., 2000). IOP was measured first immediately before drug administration, then after 30 min and then every hour for a period of 8 h. The right eye was left as a control in all the experimental rabbits. The ocular hypotensive activity is expressed as the average difference in IOP between the treated and control eye to minimize the diurnal, seasonal and individual variations commonly observed in rabbits (Winum et al., 2004).

2.8. Assessment of ocular irritancy of niosomes

Six New Zealand rabbits weighing 2.5–3 kg were used in this experiment. The left eye of each rabbit received multilamellar niosomal formulations, composed of Span 60 and cholesterol in a 7:4 molar ratio,

once every day for a period of 40 days. The right eye was left as a control in all the experimental rabbits. The rabbits were killed after 40 days. The eyes were separated, fixed, cut vertically, dehydrated, cleared, impregnated in soft and hard paraffin, sectioned at 8 μm thicknesses with the microtone and stained with haematoxylin and eosin. Corneal histological examination was completed after photographing the stained sections using optical microscopy.

3. Results and discussion

3.1. Entrapment efficiency

To obtain the highest encapsulation efficiency, several factors, including the inclusion of cholesterol, the structure of the surfactant and the method of preparation, were investigated and optimized.

3.1.1. Effect of cholesterol content

Data in Table 1 reveals that incorporation of cholesterol into niosomes was found to increase the encapsulation efficiency of acetazolamide up to an optimum concentration of cholesterol. The percentage entrapment efficiency of acetazolamide in niosomes increased from 16.81% to 18.49% for REV niosomes and from 18.21% to 20.74% for multilamellar niosomes composed of Span 40 and cholesterol in a 7:4 and 7:6 molar ratio, respectively. Also the percentage entrapment efficiency of acetazolamide in niosomes increased from 16.66% to 26.27% for REV niosomes and from 21.48% to 32.21% for multilamellar niosomes composed of Span 60 and cholesterol in a 7:4 and 7:6 molar ratio, respectively. The one-way ANOVA showed a significant difference between all pairs at $P < 0.001$. These results can be explained by the fact that an increase in cholesterol content resulted in an increase of microviscosity of the membrane indicating more rigidity of the bilayers (Manosroi et al., 2003). Cholesterol has the ability to cement the leaking space in the bilayer membranes (Agarwal et al., 2001). It is obvious from Table 1 that further increase of cholesterol content, reaching a 7:7 molar ratio, for niosomes composed of Span 40 or Span 60 and cholesterol reduced the entrapment efficiency for both REV and MLV niosomes. This could be due to the fact that cholesterol beyond a certain level starts disrupting the regular bilayered structure leading to loss of drug entrapment (Redziniak and Perrier, 1996).

3.1.2. Effect of the structure of the surfactant

Data in Table 1 reveals that the entrapment efficiencies for niosomes prepared using Span 60 were superior to those prepared using Span 40. This can be explained by many facts: (a) Span 60 has the highest phase transition temperature (Yoshioka et al., 1994). (b) The length of alkyl chain of surfactant is a crucial factor in permeability. Long chain surfactant produces high entrapment (Hao et al., 2002). Span 60 has a longer saturated alkyl chain (C_{16}) compared to Span 40 (C_{14}), so it produces niosomes with higher entrapment efficiency. (c) The longer alkyl chain influences the HLB value of the surfactant mixture which by its turn directly influences the drug entrapment efficiency (RajaNaresh et al., 1994,). The lower the HLB of the surfactant the higher will be the drug entrapment efficiency and sta-

Table 1
Encapsulation efficiency and T_{8h} of acetazolamide niosomes

Niosomal formulation composition (molar ratio)	Reverse-phase evaporation niosomes		Multilamellar niosomes	
	Encapsulation efficiency[a] (% ± S.D.)	T_{8h}[b]	Encapsulation efficiency[a] (% ± S.D.)	T_{8h}[b]
Sp.40:CH (7:4)	16.81% ± 0.60	65.71% ± 1.09	18.21% ± 0.31	58.40% ± 4.55
Sp.40:CH (7:6)	18.49% ± 1.22	55.39% ± 2.30	20.74% ± 2.62	46.80% ± 2.69
Sp.40:CH (7:7)	11.10% ± 0.27	56.86% ± 1.73	13.01% ± 0.64	56.50% ± 2.93
Sp.60:CH (7:4)	16.66% ± 0.73	54.47% ± 3.34	21.48% ± 0.91	50.61% ± 3.16
Sp.60:CH (7:6)	26.27% ± 1.96	48.19% ± 1.83	32.21% ± 0.34	44.15% ± 0.36
Sp.60:CH (7:7)	17.36% ± 0.48	49.61% ± 0.85	21.36% ± 0.92	50.55% ± 1.66

Sp.40: Span 40; Sp.60: Span 60; CH: cholesterol.
[a] Each value is an average of three determinations.
[b] T_{8h}: % acetazolamide released after 8 h.

bility as in the case of niosomes prepared using Span 60.

3.1.3. Effect of the method of preparation

By further inspection of Table 1, it is obvious that entrapment efficiency percentages of acetazolamide entrapped in multilamellar niosomes prepared by thin film hydration method were higher than those in niosomes prepared by the reverse-phase evaporation method. This may be attributed to the fact that acetazolamide is practically insoluble in water and in the aqueous tear fluid, where the solubility of acetazolamide at pH 7 is approximately 1.01 mg/ml despite the fact that its partition coefficient, log *P*, is −0.3 (Parasrampuria, 1993), so multilamellar vesicles are more capable of loading a higher mass of a hydrophobic drug than are REV vesicles (Morella et al., 2002), which have a high aqueous volume to lipid ratio (7 l/mole lipid).

3.2. Characterization of acetazolamide niosomes

3.2.1. Photomicroscopy and transmission electron microscopy

The photomicrographs of acetazolamide REV and multilamellar niosomes composed of Span 60 and cholesterol in a 7:4 molar ratio are shown in Fig. 1a and b, respectively. They reveal that the niosomes are spherical in shape and exist in disperse and aggregate collections.

Negative stain transmission electron micrographs of acetazolamide REV and multilamellar niosomes composed of Span 60 and cholesterol in a 7:4 molar ratio are shown in Fig. 2a and b, respectively. They reveal the presence of well identified, nearly perfect spheres having a large internal aqueous space in the case of REV unilamellar and oligolamellar vesicles, while they reveal the presence of well identified perfect spheres without large internal aqueous space in the case of multilamellar vesicles. It is also to be noticed that the multilamellar niosomes have a higher particle size than do REV niosomes of the same composition and molar ratio.

3.2.2. Determination of vesicle size

The mean particle diameters of REV and MLV niosomes, composed of Span 40 and cholesterol in a 7:4 molar ratio were 3.46 and 5.06 μm, respectively, while the mean particle diameters of REV and MLV niosomes composed of Span 60 and cholesterol in a 7:4 molar ratio were 3.61 and 5.4 μm, respectively. It is obvious that multilamellar niosomes are larger in size than their corresponding unilamellar and oligolamellar REV niosomes. Also, the results reveal that the niosomes prepared using Span 60 are larger in size than niosomes prepared using Span 40. Span 60 has a longer saturated alkyl chain compared to Span 40 as mentioned previously and it was reported that surfactants with longer alkyl chains generally gave larger vesicles (Manosroi et al., 2003). This would account for the higher entrapment efficiencies obtained with Span 60 multilamellar niosomes.

3.2.3. In vitro release of acetazolamide from niosomes

Results of an in vitro study on the release of acetazolamide from REV and MLV niosomal vesicles prepared

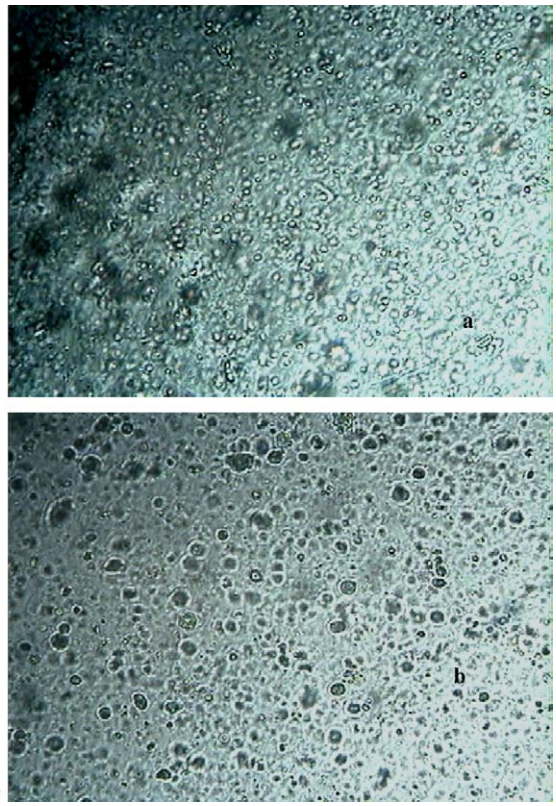

Fig. 1. Photomicrograph of acetazolamide-loaded niosomes composed of Span 60 and cholesterol in a 7:4 molar ratio. (a) Reverse-phase evaporation niosomes; (b) Multilamellar niosomes (400×).

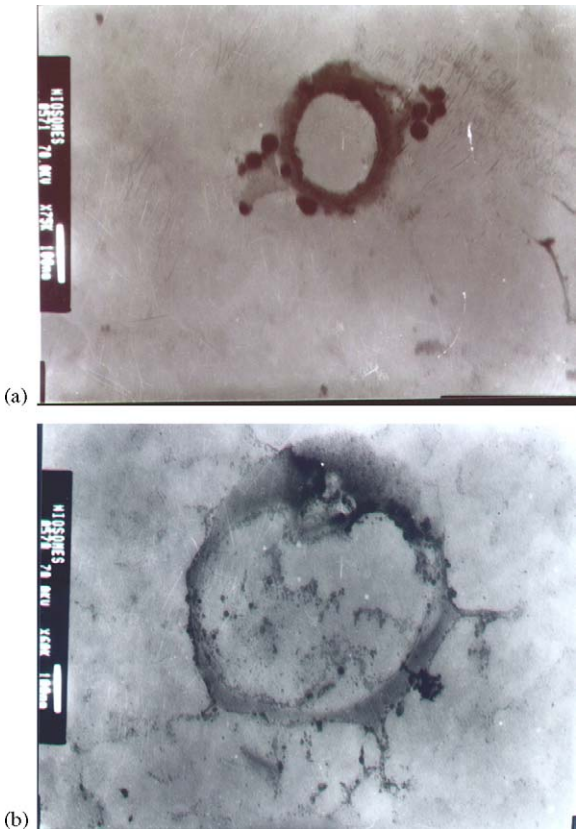

Fig. 2. Negative stain electron micrograph of acetazolamide-loaded niosomes composed of Span 60 and cholesterol in a 7:4 molar ratio. (a) Reverse-phase evaporation niosomes; (b) Multilamellar niosomes.

using Span 40 or Span 60 and cholesterol in the molar ratios 7:4, 7:6 and 7:7 are shown in Figs. 3–6. The percentages of drug released after 8 h (T_{8h}) from the niosomal vesicles are shown in Table 1.

From the results it is obvious that the increase of cholesterol molar ratio from 7:4 to 7:6 markedly reduced the efflux of the drug, which is in accordance with its membrane stabilizing ability (Betageri and Parsons, 1992). Cholesterol is known to abolish the gel to liquid phase transition of niosome systems (Cable, 1989), resulting in niosomes that are less leaky (Rogerson et al., 1987), but increasing the cholesterol beyond a certain molar ratio (7:7) starts disrupting the bilayered structure leading to loss of drug entrapment levels in this case (Redziniak and Perrier, 1996). By reviewing the data in Table 1, T_{8h} for the niosomal

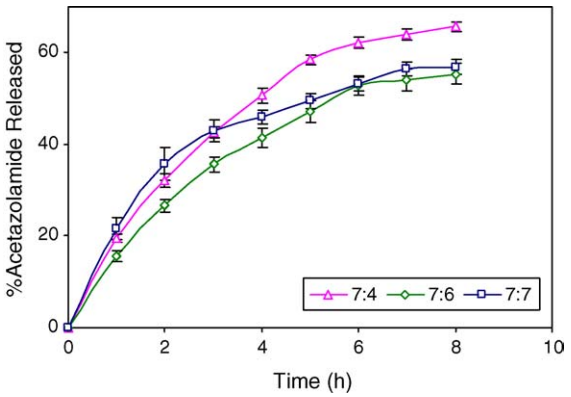

Fig. 3. Effect of cholesterol content on the release of acetazolamide from REV niosomes composed of Span 40 and cholesterol in different molar ratios.

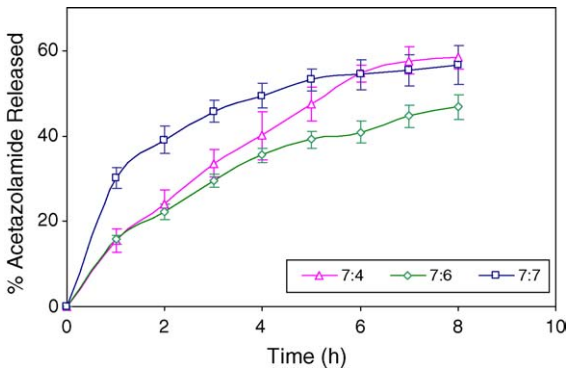

Fig. 4. Effect of cholesterol content on the release of acetazolamide from multilamellar niosomes composed of Span 40 and cholesterol in different molar ratios.

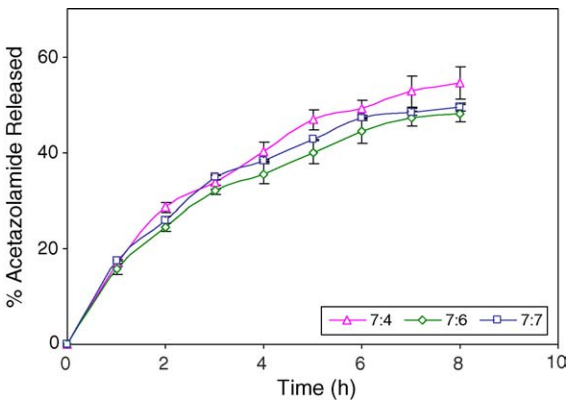

Fig. 5. Effect of cholesterol content on the release of acetazolamide from REV niosomes composed of Span 40 and cholesterol in different molar ratios.

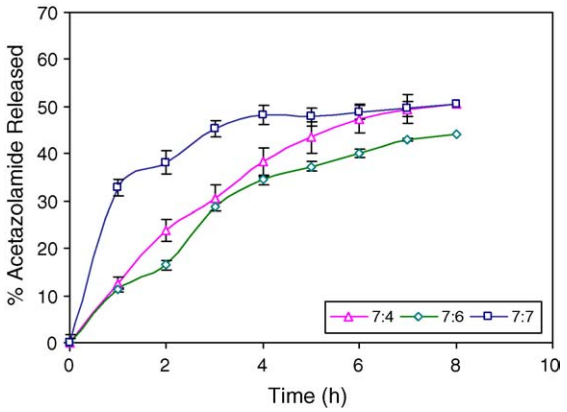

Fig. 6. Effect of cholesterol content on the release of acetazolamide from multilamellar niosomes composed of Span 60 and cholesterol in different molar ratios.

formulations composed of Span 40 or Span 60 and cholesterol can be arranged in the following decreasing order: 7:4 > 7:7 > 7:6 molar ratios for all REV and MLV niosomal vesicles except for MLV niosomes composed of Span 60 and cholesterol where the ranking was 7:7 > 7:4 > 7:6.

By comparing the release data of acetazolamide REV niosomes with that of multilamellar niosomes, it is clear that the release is slower from multilamellar niosomes. This may be attributed to the fact that multilamellar vesicles consist of several concentric spheres of lipid bilayers separated by aqueous compartments rather than one to three lipid bilayers in REV niosomes (Yu et al., 1998). Therefore, the diffusion of acetazolamide entrapped in the hydrophobic regions of the multilamellar vesicles would be expected to occur over a prolonged period of time.

By further inspection of the data we can conclude that niosomal formulations prepared using Span 60 yielded a lower rate of release compared to Span 40 corresponding ones, and this is in accordance with a previous study done by Ruckmani et al. (2000). This can be explained by the fact that niosomes exhibit an alkyl chain length-dependent release. The higher the chain length, the lower the release rate (Devaraj et al., 2002).

It is to be noted that the in vitro release results are consistent with those of the entrapment efficiency, as the multilamellar niosomes composed of Span 60 and cholesterol (7:6) molar ratio with the highest entrapment efficiency (32.21%) showed the lowest drug release percent after 8 h (T_{8h} = 44.15%). This trend is also seen with REV niosomes. The comparative release data indicate that, by encapsulation of drug into niosomes, it is possible to sustain and control the release of the drug for a longer duration (Ruckmani et al., 2000).

Linear regression analysis for the release data was done to determine the proper order of release. Zero-, first- and Higuchi diffusion controlled model equations were applied to all in vitro release results. From the results shown in Table 2, one can conclude that the drug is released from niosomes by a diffusion controlled mechanism. Similar results were obtained by Law et al. (1994b), Arica et al. (1995) and Glavas-Dodov et al. (2002).

3.3. Physical stability of acetazolamide niosomes

Physical stability studies were carried out to investigate the leaching of drug from niosomes during storage at refrigerator temperatures as shown in Fig. 7a and b. The percentages of acetazolamide retained after a period of 3 months in REV and MLV niosomes composed of Span 40 and cholesterol in a 7:4 molar ratio were 73.44% and 74.15%, respectively, while the percentages of acetazolamide retained after a period of 3 months in REV and MLV niosomes composed of Span 60 and cholesterol in a 7:4 molar ratio were 76.11% and 77.12%, respectively. Also the results indicate that approximately 90% of acetazolamide was retained in niosomal formulations for a period of 45 days. Similar results were obtained by Shahiwala and Misra (2002) and Ruckmani et al. (2000). By inspection of Fig. 7a and b, there was no significant difference in physical stability results between reverse-phase evaporation and multilamellar niosomes stored at refrigerator temperature.

3.4. In vivo study on acetazolamide niosomes

Acetazolamide REV and MLV niosomes prepared with Span 60 and cholesterol in 7:4 and 7:6 molar ratios were selected for in vivo studies as they showed higher entrapment efficiency than those counterparts prepared using Span 40 and cholesterol. Also the 7:4 molar ratio exhibited higher T_{8h} values and the 7:6 molar ratio exhibited the highest entrapment efficiency

Table 2
Determination of the order of release of acetazolamide from different niosomal formulations using the correlation coefficient parameter (r)

Niosomal formulation	Zero order	First order	Diffusion	Order of release
Span 40:CH (7:4)[a]	0.956	0.979	0.986	Diffusion
Span 40:CH (7:6)[a]	0.963	0.980	0.990	Diffusion
Span 40:CH (7:7)[a]	0.939	0.966	0.976	Diffusion
Span 40:CH (7:4)[b]	0.980	0.989	0.994	Diffusion
Span 40:CH (7:6)[b]	0.987	0.989	0.990	Diffusion
Span 40:CH (7:7)[b]	0.999	0.998	1.000	Diffusion
Span 60:CH (7:4)[a]	0.971	0.986	0.993	Diffusion
Span 60:CH (7:6)[a]	0.972	0.985	0.994	Diffusion
Span 60:CH (7:7)[a]	0.960	0.975	0.988	Diffusion
Span 60:CH (7:4)[b]	0.952	0.968	0.980	Diffusion
Span 60:CH (7:6)[b]	0.962	0.980	0.989	Diffusion
Span 60:CH (7:7)[b]	0.884	0.897	0.936	Diffusion

[a] Reverse-phase evaporation niosomes.
[b] Multilamellar niosomes.

among the prepared niosomal formulations. The values of the reduction in IOP (mmHg) in normotensive rabbits after instillation of a single dose of each selected acetazolamide REV and MLV noisome type as a function of time were compared to acetazolamide solution and plain niosomes results, as shown in Figs. 8 and 9, respectively.

From the results it is obvious that the instillation of plain niosomes showed little insignificant effect on ocular hypotensive activity. The IOP lowering activity of acetazolamide solution reached a maximum value of not more than −3.7 mmHg after 1 h of drug instillation. This effect markedly decreased and abolished completely during the time of experiment, whereas each tested niosomal formulations produced a significant lowering in IOP compared to acetazolamide solution or plain niosomes. One-way ANOVA revealed significant differences between all pairs at $P < 0.01$. The IOP lowering activity of acetazolamide REV and MLV niosomes in a 7:4 molar ratio reached values of −7.15 and −6.9 mmHg after 1 h of drug instillation, respectively, and reached values of −6.10 and −7.75 mmHg after 4 h of drug instillation, respectively. The increased ocular bioavailability of acetazolamide entrapped in niosomes may be attributed to the fact that niosomes may promote absorption of acetazolamide by preferentially modifying the permeability characteristics through the cornea. Surfactants (the chief constituents of these niosomes) act as penetration enhancers as they can remove the mucus layer and break junctional complexes (Kaur et al., 2004). This lowering effect produced with acetazolamide vesicular systems was prolonged for the time of the experiment (8 h).

The results showed the superiority of both REV and MLV acetazolamide niosomes composed of Span 60 and cholesterol in a 7:4 molar ratio over the 7:6 molar ratio. This could be attributed to the higher cholesterol content leading to a decreased permeability of the drug from the lipid bilayer of the 7:6 molar ratio niosomes which lowers its bioavailability.

The results also revealed the more prolonged effect obtained with acetazolamide multilamellar niosomes than reverse-phase evaporation niosomes. This prolonged effect is due to the numerous lipid bilayers found in multilamellar vesicles which play a very important role in retarding drug release over a prolonged period of time. Gross examination of the rabbit eyes during this study showed no signs of abnormal lachrymation or increased blinking upon instillation of the prepared niosomal formulations.

3.5. Assessment of ocular irritancy of niosomes

Fig. 10a and b show the histological photomicrographs of control corneal tissues and corneal tissues following instillation of multilamellar niosomal formulations, composed of Span 60 and cholesterol in a 7:4 molar ratio, once every day for a period of 40 days. By comparing the two photomicrographs we can observe that the cornea treated with niosomal formulation showed slight oedema of the substantia propria especially in the deep stroma where the collagen

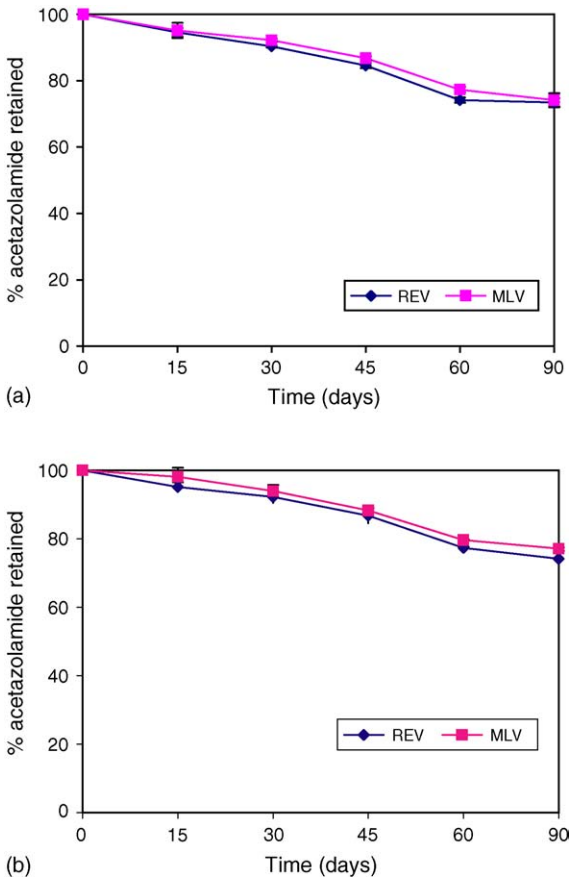

Fig. 7. (a) Percentage of acetazolamide retained in niosomal formulations composed of Span 40 and cholesterol in a 7:4 molar ratio after storage at refrigeration temperature. (b) Percentage of acetazolamide retained in niosomal formulations composed of Span 60 and cholesterol in a 7:4 molar ratio after storage at refrigeration temperature.

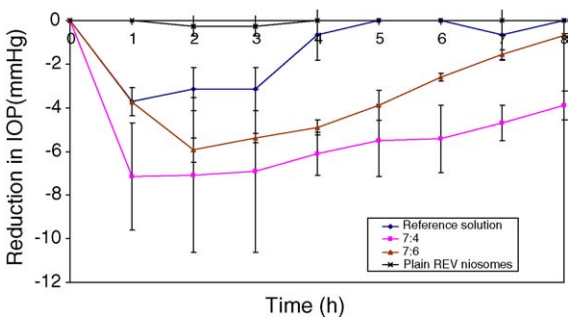

Fig. 8. Reduction in IOP after topical administration of acetazolamide reverse-phase evaporation niosomes composed of Span 60 and cholesterol in 7:4 and 7:6 molar ratios compared to acetazolamide solution 1% and plain REV niosomes.

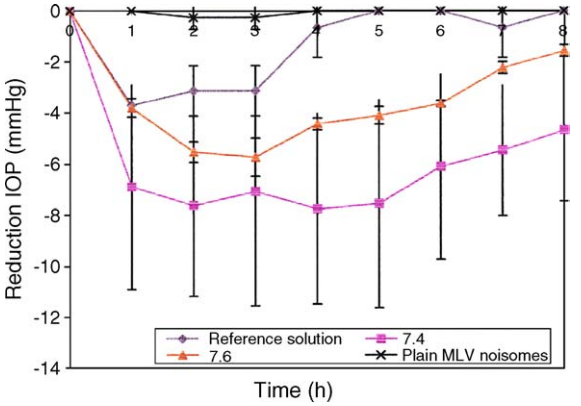

Fig. 9. Reduction in IOP after topical administration of acetazolamide multilamellar niosomes composed of Span 60 and cholesterol in 7:4 and 7:6 molar ratios compared to acetazolamide solution 1% and plain MLV niosomes.

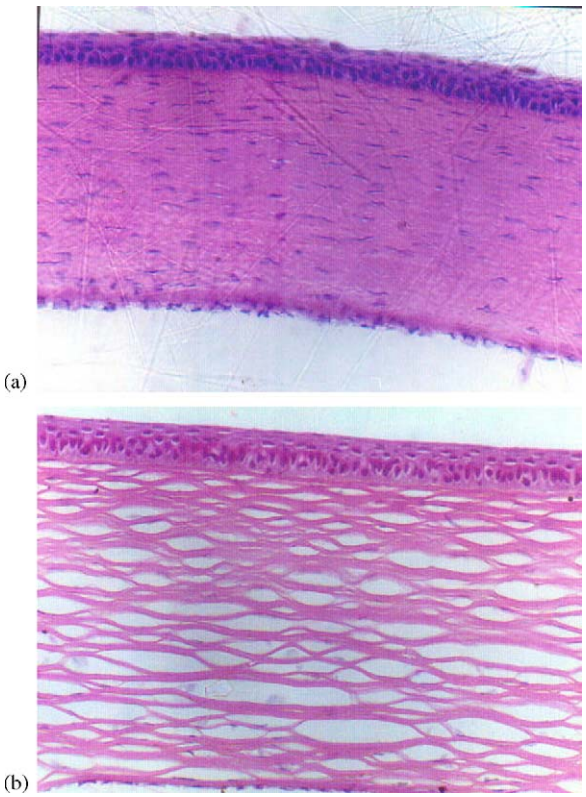

Fig. 10. (a) Histological corneal examination of the control eye. (b) Histological corneal examination of the eye treated with Span 60 multilamellar niosomes after 40 days of instillation once every day.

fibers were separated from each other. This change may be due to slight irritation caused by the non-ionic surfactant. This type of irritation is reversible where the slight oedema clears over time (Hughes et al., 2004). As a conclusion, no major changes are observed, and therefore the preparation is considered safe to use for short and long term treatment. This slight irritation may be due to the fact that the irritation power of surfactants decreases in the following order: cationic > anionic > ampholytic > non-ionic, and so the niosomes prepared with non-ionic surfactants, Span 40 and Span 60, are preferred for ocular delivery (Van Abbe, 1973).

4. Conclusions

The results of this study show that cholesterol content, the type of surfactant and the method of preparation altered the entrapment efficiency and drug release rate from niosomes. Higher entrapment efficiency was obtained with multilamellar niosomes prepared from Span 60 and cholesterol in a 7:6 molar ratio. The in vivo evaluation of acetazolamide niosomes in comparison to acetazolamide solution and plain niosomes showed that acetazolamide multilamellar niosomes composed of Span 60 and cholesterol were found to most effectively prolong a decrease in the IOP. This formula retained 77.12% of acetazolamide in niosomes after storage at refrigerated temperature for a period of 3 months. Slight irritation could be observed in corneal tissues after long treatment with these niosomes which is reversible and abolished by time. Both REV and MLV niosomes can markedly promote absorption of acetazolamide and they may be promising candidates as ophthalmic carriers of acetazolamide.

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