Liposomes as an Ocular Delivery System for Acetazolamide: In Vitro and In Vivo Studies

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ABSTRACT

The purpose of this study was to formulate topically effective controlled release ophthalmic acetazolamide liposomal formulations. Reverse-phase evaporation and lipid film hydration methods were used for the preparation of reverse-phase evaporation (REVs) and multilamellar (MLVs) acetazolamide liposomes consisting of egg phosphatidylcholine (PC) and cholesterol (CH) in the molar ratios of (7:2), (7:4), (7:6), and (7:7) with or without stearylamine (SA) or dicetyl phosphate (DP) as positive and negative charge inducers, respectively. The prepared liposomes were evaluated for their entrapment efficiency and in vitro release. Multilamellar liposomes entrapped greater amounts of drug than REVs liposomes. Drug loading was increased by increasing CH content as well as by inclusion of SA. Drug release rate showed an order of negatively charged > neutral > positively charged liposomes, which is the reverse of the data of drug loading efficiency. Physical stability study indicated that approximately 89%, 77%, and 69% of acetazolamide was retained in positive, negative, and neutral MLVs liposomal formulations up to a period of 3 months at 4°C. The intraocular pressure (IOP)-lowering activity of selected acetazolamide liposomal formulations was determined and compared with that of plain liposomes and acetazolamide solution. Multilamellar acetazolamide liposomes revealed more prolonged effect than REVs liposomes. The positively charged and neutral liposomes exhibited greater lowering in IOP and a more prolonged effect than the negatively charged ones. The positive multilamellar liposomes composed of PC:CH:SA (7:4:1) molar ratio showed the maximal response, which reached a value of –7.8 ± 1.04 mmHg after 3 hours of topical administration.

KEYWORDS: Acetazolamide, multilamellar liposomes, reverse-phase evaporation liposomes.

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INTRODUCTION

Drug delivery in ocular therapeutics is a challenging problem and is a subject of interest to scientists working in the multidisciplinary areas pertaining to the eye. Current trends in ocular therapeutics and drug delivery suggest that the existing dosage forms will be replaced by novel drug delivery systems that offer improved biopharmaceutical properties.[1]

Acetazolamide (the most effective carbonic anhydrase inhibitor, CAI) is used orally in large doses for the reduction of intraocular pressure (IOP) in patients suffering from glaucoma. This treatment leads to unpleasant systemic side effects such as central nervous system (CNS) depression, renal failure, diuresis, vomiting, anorexia, and metabolic acidosis. So, its oral use has become unpopular and several scientists have sought to replace oral CAIs with topical CAIs to abolish systemic side effects.[2]

The 2 major problems that hinder the topical effectiveness of acetazolamide are its poor aqueous solubility (0.7 mg/mL) and low permeability coefficient of 4.1×10^{-6} cm/s.[3] Topical formulations of acetazolamide solution (in the form of sodium salt) were initially unsuccessful because of its limited ocular penetration, which caused an insufficient amount of the drug to reach the ciliary body.[2] Other significant attempts have been made to formulate effective acetazolamide topical preparations (eg, contact lenses containing acetazolamide[4]; topically active surfactant gel preparation of acetazolamide[5]; aqueous acetazolamide solution using 2-hydroxypropyl-β-cyclodextrin[6]; polymeric suspensions of acetazolamide containing viscolyzers and penetration enhancers.[7] Recently Kaur and Smitha[8] successfully prepared topically effective formulations of acetazolamide using cyclodextrins in combination with bioadhesive polymers, penetration enhancers, and cosolvents.

The various drug delivery systems mentioned above offer numerous advantages over conventional drug therapy, yet they are not devoid of pitfalls including poor patient compliance and difficulty of insertion, as in contact lenses, and tissue irritation, as well as damage and toxicological complications caused by penetration enhancers.[1] In order to overcome these problems, the researchers conceived the concept of vesicular drug delivery systems for ocular therapy. Kaur et al[1] recommended the incorporation of CAIs in vesicular delivery systems to enhance the bioavailability of these agents by improving their corneal penetration.

Niosomes have been reported as a possible approach to improve the low corneal penetration and bioavailability characteristics of acetazolamide.[9] Also, topical ocular formulation of acetazolamide using large unilamellar liposomes as a vehicle has been reported in the literature. These vesicles are composed of phosphatidylcholine (PC) and cholesterol (CH) in the molar ratios (9:1) and (7:2), respectively, with or without positive and negative charge inducers.[10]

Liposomes offer advantages over most ophthalmic delivery systems in being completely biodegradable and relatively nontoxic. A potential advantage of liposomes is their ability to make intimate contact with corneal and conjunctival surfaces, thereby increasing the probability of ocular drug absorption. Liposomes offer a promising avenue to fulfill the need for an ophthalmic drug delivery system that not only has the convenience of a drop but that can localize and maintain drug activity at its site of action for a longer period of time thus allowing for a sustained action. In addition, liposomes can be used to protect drug molecules from the metabolic enzymes present at the tear/corneal epithelium interface.[1]

The objective of the present study was to formulate topical acetazolamide reverse-phase evaporation (REVs) and multilamellar (MLVs) liposomal formulations in different molar ratios. A comparison study was performed between REVs and MLVs liposomes to evaluate the in vitro and in vivo performance of these formulations. The factors influencing the encapsulation of acetazolamide into liposomes were investigated. Characterization of the prepared liposomes regarding physical morphology, particle size, and in vitro drug release was performed. Stability study was performed to investigate the leak out of the drug from liposomes during storage. The intra-ocular lowering activity of selected REVs and MLVs acetazolamide liposomal formulations was evaluated.

Materials and Methods

Materials

Acetazolamide and L-phosphatidylcholine, type X-E, from dried egg yolk, cholesterol, stearylamine (SA), and dicetyl phosphate (DP) were obtained from Sigma Chemical Co (St Louis, MO). Acetone, absolute alcohol, chloroform, methanol, diethyl ether, sodium chloride, potassium dihydrogen phosphate, and disodium hydrogen phosphate were from Adwic El-Nasr Pharmaceuticals Chemical Co (Cairo, Egypt) and were prepared according to the methods of Prolabo (Paris, France). The Spectra/Por dialysis membrane, 12 000 to 14 000 molecular weight cutoff, was obtained from Spectrum Laboratories Inc (Rancho Dominguez, CA).

Methods

Preparation of Reverse-Phase Evaporation (REVs) Liposomes

Acetazolamide unilamellar and oligolamellar (REVs) liposomes were prepared using the reverse-phase evaporation technique.[11] The lipid components (200 mg) including the egg PC and CH, either alone or mixed with the charge inducing agents, stearylamine or dicetyl phosphate, were accurately weighed into a round-bottom flask and dissolved in chloroform: methanol mixture (2:1, vol/vol). A thin lipid film was formed on the inner side of the flask by evaporating the organic solvents under vacuum using a rotary evaporator at 40°C (Janke and Kunkel, model RVO5-ST, IKA Laboratories, Staufen, Germany). The lipid film was redissolved in ether, in which the reversed-phase vesicles would be formed. The drug solution (20 mg) in acetone together with 6 mL phosphate-buffered saline (PBS, pH 7.4) was added. The system was sonicated for 4 minutes in a bath type sonicator. The mixture was then placed on the rotary evaporator and the organic solvent was removed under reduced pressure. The liposomes were allowed to equilibrate at room temperature, and 10 mL PBS was added to the liposomal suspension, which was kept in the refrigerator overnight. All the above-mentioned steps were performed under aseptic conditions. All glassware was sterilized by autoclaving; PBS was passed through a 0.22-μm membrane filter, and the entire procedure was performed in a laminar flow hood (Esco, Singapore).

Preparation of Multilamellar Liposomes

Multilamellar vesicles containing acetazolamide were prepared using the lipid film hydration technique.[12] Neutral MLVs were composed of PC and CH mixed in different molar ratios; viz, PC:CH (7:2), (7:4), (7:6), and (7:7). Stearylamine or dicetyl phosphate was added to impart either a positive or a negative charge respectively to the last 3 molar ratios, so that the final molar ratios of PC:CH:SA or PC:CH:DP in the charged vesicles were 7:4:1, 7:6:1, and 7:7:1. The lipid components (200 mg) (PC and CH, either alone or mixed with SA or DP) were dissolved in chloroform:methanol mixture (2:1, vol/vol) in a round-bottom flask. Then, 20 mg acetazolamide dissolved in acetone:methanol mixture (4:1, vol/vol) was added to the lipid solution. The organic solvents were slowly removed using rotary evaporator (Janke and Kunkel, model RVO5-ST, IKA Labs) at 40°C such that a very thin film of dry lipids was formed on the inner surface of the flask. The dry lipid film was slowly hydrated with 10 mL of PBS (pH 7.4). The liposomal suspension was mechanically shaken for 1 hour using mechanical shaker (Kottermann GmbH, Hanigsen, Germany). The liposomal suspension was left to mature overnight at 4°C, to ensure full lipid hydration. For sterility, all of the above mentioned steps were done under aseptic conditions as previously described.

Separation of Free Drug

Free unentrapped drug was separated from acetazolamide (REVs) and (MLVs) liposomes by centrifugation at 20 000*g* for 1 hour at 4°C using a refrigerated centrifuge (Beckman Coulter Inc, Fullerton, CA). The pellets formed were washed twice with 10 mL PBS and recentrifuged again for 1 hour.

Determination of Entrapment Efficiency

The percentage of drug encapsulated was determined after lysis of the prepared liposomes with absolute alcohol and sonication for 10 minutes.[13] The concentration of acetazolamide in absolute alcohol was determined spectrophotometrically at 265 nm using a UV-visible spectrophotometer (model UV-1601 PC, Schimadzu, Kyoto, Japan). The entrapment efficiency expressed as entrapment percentage was calculated through the following relationship[14]:

$$\text{Entrapment Efficiency Percentage} = \frac{\text{Entraped drug}}{\text{Total drug}} \times 100 \quad (1)$$

Characterization of Acetazolamide Liposomes: Photomicroscopic Analysis

The physical morphology of samples of acetazolamide (REVs) and (MLVs) liposomes composed of PC:CH (7:4) molar ratio were examined under a photomicroscope (Carl Zeiss, Berlin, Germany) for morphological evaluation. The liposomes were photographed at a original magnification ×400, using a fitted camera (Panasonic, Tokyo, Japan).

Particle Size Measurement

The mean particle size and size distribution of freshly prepared neutral, positively charged, and negatively charged (REVs) and (MLVs) liposomal dispersion with lipid components in the molar ratio of (7:4) were determined using laser diffraction particle size analyzer (Malvern Instruments Ltd, Worcestershire, UK), which consists of a He-Ne laser (5 mW) and a small-volume sample holding cell with stirrer, so that the sample, diluted with distilled water, was stirred to keep the sample in suspension all over the measurement.

In Vitro Drug Release Studies

The release of REVs and MLVs acetazolamide from liposomal formulations was determined using the membrane diffusion technique.[15] In brief, acetazolamide liposomal suspension equivalent to 2 mg acetazolamide was suspended in 1-mL PBS (pH 7.4) in a glass cylinder having a length of 7 cm and diameter of 2.5 cm. This cylinder was fitted, before addition of liposomal suspension, with a presoaked dialysis membrane (Spectra/Por dialysis membrane, 12 000-14 000 molecular weight cutoff) and was suspended in the dissolution flask of the United States Pharmacopeia (USP) dissolution tester (Pharma test, Hainburg, Germany) containing 75 mL PBS (pH 7.4) and maintained at a temperature of 37°C. The glass cylinder was adjusted to rotate at a constant speed (50 rpm). Samples were collected every 1 hour over a period of 8 hours and assayed spectrophotometrically for drug content at 267 nm.

Differential Scanning Calorimetry Measurements

Differential scanning calorimetry (DSC) experiments were performed with differential scanning calorimeter (model TA-50 WSI, Schimadzu) calibrated with indium. Samples of acetazolamide, empty and drug-loaded multilamellar liposomes composed of PC:CH (7:4 or 7:7) molar ratio or PC:CH:SA (7:4:1) molar ratio were submitted to DSC analysis. The analyses were performed on 40-μL or 1-mg samples sealed in standard aluminum pans. Thermograms were obtained at a scanning rate of 10°C/min. Isotonic PBS buffer (pH 7.4) was employed as reference. Each sample was scanned between zero and 400°C. The temperature of maximal excess heat capacity was defined as the phase transition temperature.

Stability Study

Physical stability study was performed to investigate the leak out of the drug from liposomes during storage. Neutral positively charged and negatively charged (REVs) and (MLVs) acetazolamide liposomes with lipid components in the molar ratio of (7:4) were sealed in 20-ml glass vials and stored in refrigerator at 4°C for a period of 3 months. Samples from each liposomal formulation were withdrawn at definite time intervals. The residual amount of the drug in the vesicles was determined after separation from unentrapped drug as described previously under the separation of free drug.

In Vivo Studies

Adult male rabbits, each weighing 3 to 3.5 kg were used in the experiments. The rabbits were fed balanced diet pellets and maintained on 12 h/12 h light/dark cycle in a temperature-controlled room, at 20°C to 24°C before the experiment.[16] The experimental procedures conform to the ethical principles of the Egyptian Research Institute of Ophthalmology (Giza, Egypt) on the use of animals.[17]

Selected liposomal formulations were tested for their intraocular pressure (IOP)-lowering activity on normotensive rabbits, and the data were compared with that of plain

liposomes and acetazolamide solution (1%). The IOP was measured using a standardized Schiotz tonometer (Winters, Eichtabelle, Germany).[17]

The rabbits were divided into 9 groups, each consisting of 6 rabbits: Group I received plain liposomes; Group II received 1% acetazolamide solution (reference solution); Groups III, IV, and V received neutral, negatively charged and positively charged REVs liposomes, composed of PC:CH (7:4) molar ratio or PC:CH:DP or PC:CH:SA (7:4:1) molar ratio, respectively; Groups VI, VII, and VIII were administered neutral, negatively charged, and positively charged MLVs liposomes, composed of PC:CH (7:4) molar ratio or PC:CH:DP or PC:CH:SA (7:4:1) molar ratio, respectively; Group IX received positively charged liposomes composed of PC:CH:SA (7:7:1) molar ratio. All liposomal preparations used in this study were freshly prepared, washed from free drug, and adjusted at a concentration of 1% acetazolamide.

A single 50-μL dose of 1% acetazolamide preparation was instilled onto the corneal surface of rabbit's eye.[7] The rabbits received the drug preparations in one eye (right eye), and the contralateral eye (left eye) received no drug and remained as a control, in this way minimizing the diurnal, seasonal, and individual variations commonly observed in the rabbits.[16] IOP in both eyes of each rabbit was first measured immediately before drug administration (zero reading),[17] 30 minutes after instillation of the different drug formulations, and then every hour for a period of 8 hours. All the measurements were done 3 times at each time interval and the means were reported. All measurement periods began during the same hour on each day, and all measurements were done by the same investigator with the same tonometer.

The ocular hypotensive activity is expressed as the average difference in IOP between the treated and control eye of the same rabbit, according to the following equation[7]:

$$\Delta\text{IOP} = \text{IOP } \textit{Treated eye} - \text{IOP } \textit{Control eye} \quad (2)$$

Results and Discussion

Entrapment Efficiency

By inspection of Table 1 it is obvious that acetazolamide encapsulation efficiency varied with the lipid composition, method of preparation, and the type of charge inducer used in the prepared liposomes.

Concerning the effect of cholesterol content on the encapsulation efficiency of acetazolamide in the prepared liposomes, results showed that the percentage entrapment efficiency of acetazolamide increased by increasing cholesterol content. The percentage entrapment efficiency of acetazolamide into reverse-phase evaporation liposomes was 11.66%, 15.49%, 27.88%, and 35.61% for the molar ratios 7:2, 7:4, 7:6, and 7:7 (PC:CH), respectively, and the values recorded for mutilamellar liposomes were 12.23%, 23.59%, 30.78%, and 39.73% for the molar ratios 7:2, 7:4, 7:6, and 7:7 (PC:CH), respectively. The 1-way analysis of variance (ANOVA) showed a significant difference between all pairs at $P < .001$. This increase in entrapment efficiency occurs because by increasing cholesterol concentration in the lipidic bilayer, the latter's rigidity increases steadily, resulting in a higher stability and reduced permeability of the liposomal membrane,[18] and hence greater drug retention.[15]

By further inspection of Table 1 it is obvious that the entrapment efficiencies had higher values in case of MLVs liposomes than in REVs liposomes of the same composition

Table 1. Encapsulation Efficiency and T_{8h} of Acetazolamide Reverse-phase Evaporation and Multilamellar Liposomes*

		Reverse-phase Evaporation Liposomes		Multilamellar Liposomes	
Liposomal Formulation Composition (molar ratio)	Liposomal Formulation Charge	Encapsulation Efficiency† (% ± SD)	T_{8h}‡	Encapsulation Efficiency† (% ± SD)	T_{8h}‡
PC:CH (7:2)	Neutral	11.66 ± 0.57	_	12.23 ± 0.38	_
PC:CH (7:4)	Neutral	15.49 ± 1.30	59.05 ± 0.48	23.59 ± 1.56	44.12 ± 1.30
PC:CH:SA (7:4:1)	Positive	25.84 ± 0.43	40.30 ± 1.06	34.29 ± 2.10	42.59 ± 0.74
PC:CH:DP (7:4:1)	Negative	10.11 ± 0.20	62.24 ± 2.47	10.54 ± 0.81	62.29 ± 1.29
PC:CH (7:6)	Neutral	27.88 ± 1.52	54.63 ± 2.40	30.78 ± 0.94	40.69 ± 0.99
PC:CH:SA (7:6:1)	Positive	35.79 ± 1.50	37.37 ± 1.33	36.70 ± 0.54	36.93 ± 1.25
PC:CH: DP (7:6:1)	Negative	11.4 ± 1.28	61.46 ± 0.48	12.28 ± 0.94	55.12 ± 1.74
PC:CH (7:7)	Neutral	35.61 ± 0.61	38.28 ± 2.00	39.73 ± 0.44	36.14 ± 1.98
PC:CH:SA (7:7:1)	Positive	44.48 ± 1.78	35.19 ± 1.36	48.27 ± 1.01	32.09 ± 1.43
PC:CH:DP (7:7:1)	Negative	22.48 ± 1.20	61.44 ± 0.82	25.55 ± 1.50	54.30 ± 3.74

*PC indicates phosphatidylcholine; CH, cholesterol; DP, dicetyl phosphate; and SA, stearylamine.

†Each value is an average of 3 determinations.

‡T_{8h} indicates the percentage acetazolamide released after 8 hours.

and molar ratio. This finding may be because multilamellar vesicles contain multiple lamellae capable of loading a higher mass of hydrophobic drug than reverse-phase evaporation vesicles.[19] Liposomes prepared with PC:CH (7:2) molar ratio, showing the lowest encapsulation efficiency, were excluded from further studies.

Concerning the effect of charge-inducing agents on the encapsulation efficiency of acetazolamide in the prepared REVs and MLVs liposomes, results showed that positively charged liposomes exhibited the highest encapsulation efficiency, followed by neutral ones, and then by negatively charged liposomes, using the same PC:CH molar ratio. This order of entrapment efficiency would result because acetazolamide is a weak acid, so an electrostatic attraction would occur between drug anion and the positively charged stearylamine. This attraction would account for the higher encapsulation efficiency when compared with the negatively charged liposomes, where such interaction would not be possible. On the other hand, in case of negatively charged liposomes, it is likely that charge repulsion may occur between the drug molecules and the negatively charged DP, thus suppressing the loading efficiency.[20] The highest encapsulation efficiency (48.27%) was observed with the positively charged multilamellar liposomes composed of PC:CH:SA (7:7:1) molar ratio (ie, the positive liposomes with the highest CH content), while negatively charged liposomes with the lowest CH content PC:CH:DP (7:4:1) molar ratio exhibited the lowest encapsulation efficiency (10.54%). Similar results were also obtained during the incorporation of charge-inducing agents into azathioprine liposomes[14] and indomethacin liposomes.[21]

Characterization of Acetazolamide Liposomes

Photomicroscopic Analysis

The photomicrograph of acetazolamide reverse-phase evaporation liposomes is shown in Figure 1. It reveals the presence of homogenous population of unilamellar vesicles with one phospholipid bilayer and oligolamellar vesicles consisting of a few concentric bilayers. The prepared liposomes are well-identified spheres that have a large internal aqueous space relative to the sphere diameter. In contrast, the photomicrograph of acetazolamide multilamellar liposomes shown in Figure 1 reveals also the presence of well-identified spheres of multilamellar vesicles that consist of many concentric phospholipid bilayers. It is to be noted that MLVs are larger in size than REVs liposomes.

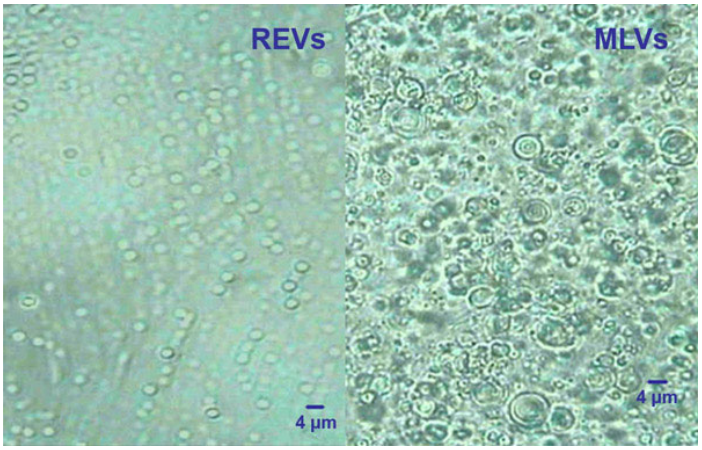

Figure 1. Photomicrograph of acetazolamide reverse-phase evaporation and multilamellar liposomes composed of PC:CH (7:4) molar ratio (original magnification ×400).

Particle Size Measurement

The results of particle size measurement for freshly prepared neutral, positively charged and negatively charged REVs and MLVs liposomal dispersion with lipid components in the molar ratio of 7:4 are presented in Table 2. The particle size distribution of all the tested REVs and MLVs liposomal formulations showed unimodal normal symmetrical frequency distribution patterns.

The mean particle diameter was estimated to be 5.81 μm for neutral reverse-phase evaporation acetazolamide liposomes. Negatively charged REVs liposomal formulation of the same molar ratio showed a mean particle diameter of 5.86 μm, which is slightly higher than that of neutral liposomes. Positively charged liposomes showed the highest mean particle diameter, which accounts for 7.66 μm. Similar results were obtained for multilamellar liposomes, where the mean diameters of the neutral, negatively charged, and positively charged MLVs vesicles with lipid content PC:CH (7:4) molar ratio were 6.97, 7.94, and 9.34 μm respectively. These results can be attributed to the inclusion of a charge

Table 2. Particle Size of Acetazolamide Reverse-phase Evaporation and Multilamellar Liposomes*

Liposomal Formulation Composition (Molar Ratio)	Liposomal Formulation Charge	Mean Diameter (μm)	
		Reverse-phase Evaporation Liposomes	Multilamellar Liposomes
PC:CH (7:4)	Neutral	5.81	6.97
PC:CH:SA (7:4:1)	Positive	7.66	9.34
PC:CH:DP (7:4:1)	Negative	5.86	7.94

*PC indicates phosphatidylcholine; CH, cholesterol; DP, dicetyl phosphate; and SA, stearylamine.

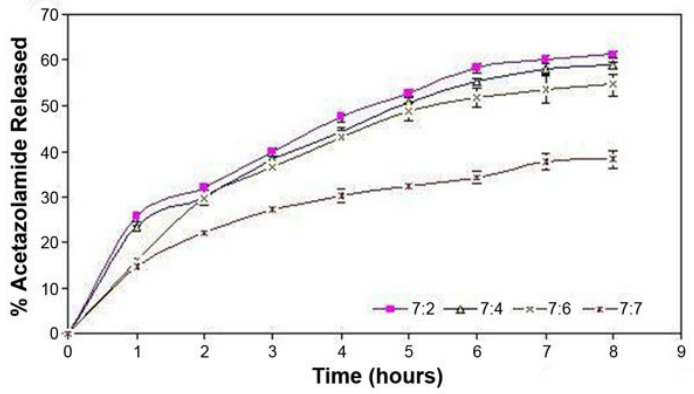

Figure 2. Effect of cholesterol concentration on the release of acetazolamide from reverse-phase evaporation liposomes.

inducer in liposomes, which increased the spacing between the adjacent bilayers,[22] resulting in the formation of liposomes larger in size compared with the neutral ones. Furthermore, the positively charged lipid electrostatically attracts the drug anion, which may be expected to push phospholipids head groups apart, hence increasing the particle diameter.[23] This increase in particle size would account, as mentioned previously, for the higher encapsulation efficiency of the positive liposomes compared with the neutral and negative ones.

By comparing the mean particle diameter of REVs and MLVs liposomal formulations shown in Table 2, it could be detected that the multilamellar liposomes are larger in size than REVs liposomes prepared with the same lipid molar ratio, which would account for their higher entrapment efficiency values as previously described.

In Vitro Drug Release Studies

The effect of cholesterol content on acetazolamide release from neutral REVs and MLVs liposomal formulations could be depicted from Figures 2 and 3, respectively. From

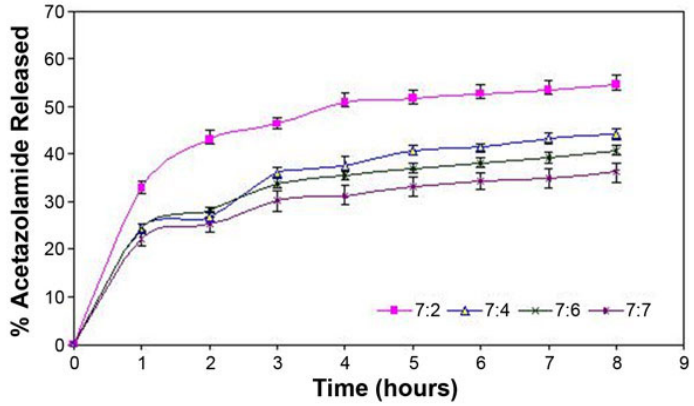

Figure 3. Effect of cholesterol concentration on the release of acetazolamide from multilamellar liposomes.

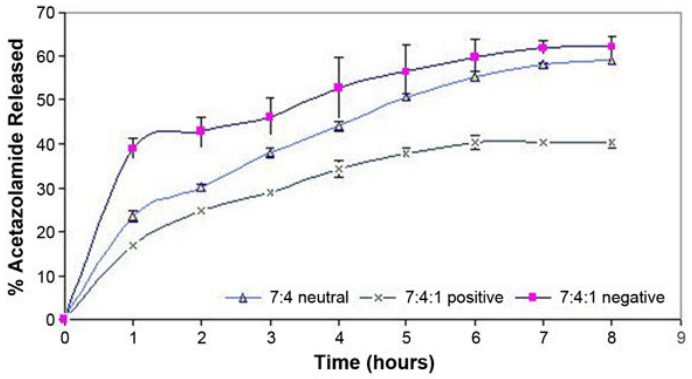

Figure 4. Effect of charge on the release of acetazolamide from reverse-phase evaporation liposomes, 7:4 molar ratio, in phosphate-buffered saline.

the release profiles, it is obvious that the increase of cholesterol molar ratio in the prepared liposomal formulations progressively decreased the release of acetazolamide from the vesicles. The percentages of drug released from liposomal formulations after 8 hours of the experiment (T_{8h}) are presented in Table 1. On the basis of the molar ratio of the lipid content (PC:CH), T_{8h} for the neutral liposomal preparations can be arranged in the following decreasing order: 7:4 > 7:6 > 7:7 corresponding to 59.05%, 54.63%, and 38.28% for REVs liposomal formulations and 44.12%, 40.69%, and 36.14% for MLVs liposomal formulations. Differences were significant at $P < .05$. The results can be explained by the presence of cholesterol in the bilayers above the phospholipid T_c ,which modulates membrane fluidity by restricting the movement of the relatively mobile hydrocarbon chains, reducing bilayer permeability,[24] and decreases the efflux of the encapsulated drug, resulting in prolonged drug retention.[25]

By comparing the release data of acetazolamide REVs liposomes with that of multilamellar liposomal formulations, it is obvious that the release of acetazolamide is slower from

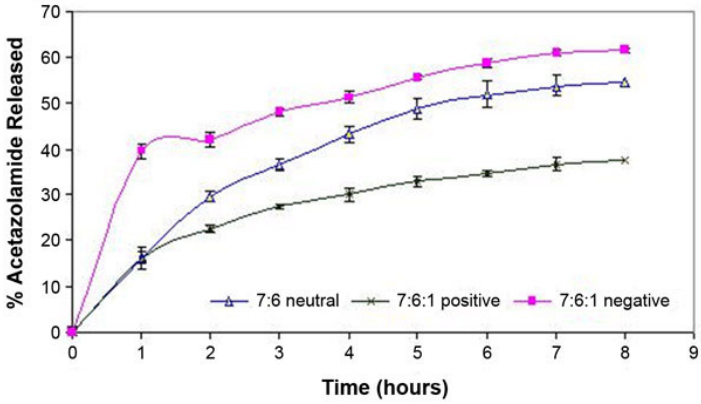

Figure 5. Effect of charge on the release of acetazolamide from reverse-phase evaporation liposomes, 7:6 molar ratio, in phosphate-buffered saline.

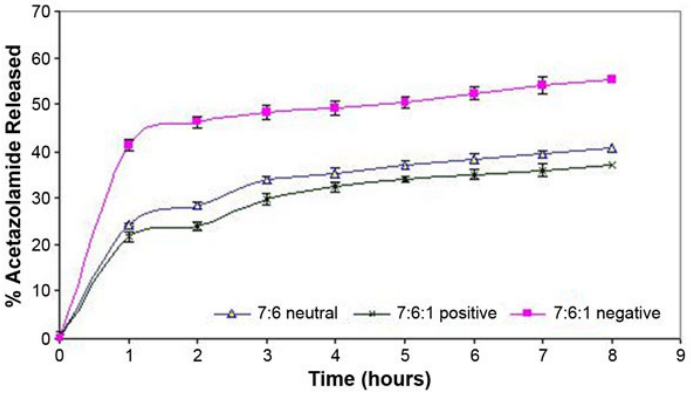

Figure 6. Effect of charge on the release of acetazolamide from multilamellar liposomes, 7:6 molar ratio, in phosphate-buffered saline.

multilamellar liposomes than from REVs liposomes of the same lipid molar ratio. The multilamellar vesicles consist of several concentric spheres of lipid bilayers separated by aqueous compartments. Therefore MLVs would play a role as a lipid reservoir. Because acetazolamide is embedded in the hydrophobic regions of the multilamellar vesicles, its release rate from the multilamellar vesicles would be expected to occur over a prolonged period of time.[26]

Concerning the effect of charge on the release of drug from liposomal formulations, Figures 4 and 5 illustrate acetazolamide release profiles from neutral and charged REVs liposomes with lipid content PC:CH in the molar ratio of 7:4 and 7:6, respectively. Also, Figure 6 illustrates the release profiles of acetazolamide from neutral, positively and negatively charged multilamellar liposomal formulations with lipid content PC:CH in the molar ratio of 7:6. From all the release profiles, it is obvious that the negatively charged liposomes showed the highest rate and extent of drug release, followed by neutral and positive ones. Similar patterns were exhibited by the neutral and charged REVs liposomes with lipid content PC:CH in the molar ratio of 7:7 and multilamellar liposomes with lipid content PC:CH in the molar ratios of 7:4 and 7:7 (figures not shown).

By reviewing data in Table 1, comparing the percentage of drug released from neutral and charged REVs and MLVs liposomes after 8 hours, it is obvious that positive liposomes were always the ones giving the lowest percentage drug release followed by the neutral liposomes then the negative ones. Once more, this order may be due to the electrostatic attraction forces that may exist between the acid moiety of the drug and the amine moiety of the positive lipid; in addition, the charged lipids serve to tighten the molecular packaging of the vesicle bilayer.[27] Meanwhile, electrostatic repulsion may occur between the drug and negatively charged liposomes resulting in a higher percentage of drug release. One-way ANOVA revealed that differences are significant at $P < .05$ except between the neutral and the positive multilamellar liposomes prepared with PC:CH:SA in the molar ratio of 7:4:1.

By further inspection of Figures 4-6, it is obvious that an initial phase of rapid drug release is apparent during the

Table 3. Diffusional Order of Release of Acetazolamide From Different Liposomal Formulations Using the Correlation Coefficient Parameter (r)*

Liposomal Formulation Composition (molar ratio)	Type of Liposomes	Liposomal Formulation Charge	r		
			Zero Order	First Order	Diffusion
PC:CH (7:4)	REVs	Neutral	0.979	0.989	0.994
PC:CH:SA (7:4:1)	REVs	Positive	0.937	0.947	0.974
PC:CH:DP (7:4:1)	REVs	Negative	0.979	0.985	0.988
PC:CH (7:6)	REVs	Neutral	0.948	0.969	0.982
PC:CH:SA (7:6:1)	REVs	Positive	0.961	0.970	0.999
PC:CH:DP (7:6:1)	REVs	Negative	0.983	0.981	0.992
PC:CH (7:7)	REVs	Neutral	0.940	0.954	0.971
PC:CH:SA (7:7:1)	REVs	Positive	0.963	0.973	0.990
PC:CH:DP (7:7:1)	REVs	Negative	0.974	0.974	0.975
PC:CH (7:4)	MLVs	Neutral	0.940	0.952	0.970
PC:CH:SA (7:4:1)	MLVs	Positive	0.959	0.965	0.983
PC:CH:DP (7:4:1)	MLVs	Negative	0.978	0.983	0.990
PC:CH (7:6)	MLVs	Neutral	0.948	0.958	0.980
PC:CH:SA (7:6:1)	MLVs	Positive	0.948	0.956	0.977
PC:CH:DP (7:6:1)	MLVs	Negative	0.972	0.980	0.990
PC:CH (7:7)	MLVs	Neutral	0.951	0.959	0.982
PC:CH:SA (7:7:1)	MLVs	Positive	0.917	0.927	0.963
PC:CH:DP (7:7:1)	MLVs	Negative	0.968	0.977	0.992

*PC indicates phosphatidylcholine; CH, cholesterol; DP, dicetyl phosphate; and SA, stearylamine.

first hour. This finding could be because of acetazolamide partitioning out of the charged bilayers or to the result of desorption of the drug bound to the charged liposome surface. A phase of rapid release has previously been described for hydrophilic drug from charged liposomes,[28] for hydrophobic drugs from charged liposomes,[29] and for hydrophobic materials from uncharged liposomes.[12] Hence this effect may be the result of an inherent property of the bilayer or liposome structure or reflect loss of surface-associated material.

It is to be noted that the in vitro release results are consistent with those of the encapsulation efficiency, as the positively charged multilamellar liposomes with the highest cholesterol content PC:CH:SA (7:7:1) molar ratio and the highest encapsulation efficiency (ie, low leakage ability) showed the lowest drug release percentage.

The release data were kinetically treated and the results are tabulated in Table 3. The in vitro release results showed that the release of acetazolamide fitted Higuchi release kinetics, suggesting that the drug transport occurred mainly by diffusion-controlled mechanism. Our results are in good agreement with the studies of many research coworkers who found that many drugs were released from liposomes by the same mechanism.[30-32]

DSC Measurements

DSC thermograms of acetazolamide, empty and drug-loaded multilamellar liposomes composed of PC:CH (7:4 or 7:7) molar ratio or PC:CH:SA (7:4:1) molar ratio are illustrated in Figure 7.

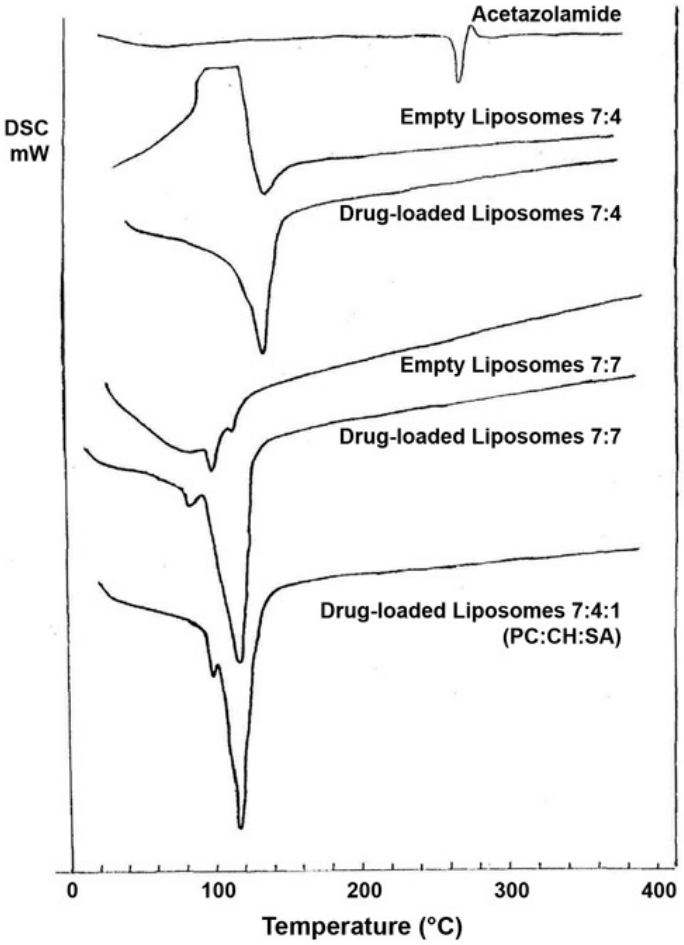

Figure 7. Differential scanning calorimetry (DSC) thermograms of acetazolamide, empty and drug-loaded multilamellar liposomal formulations. PC, phosphatidylcholine; CH, cholesterol; and SA, stearylamine.

DSC thermogram of acetazolamide showed endotherm at 273.7°C. DSC thermogram of empty liposomal dispersion containing PC and cholesterol in the molar ratio 7:4 showed broad endotherm at 100°C followed by major endotherm at 130°C corresponding to, phosphatidylcholine and cholesterol, the lipid bilayer components.[33] The melting endotherm of cholesterol was found to be shifted from 147.4°C to 130°C, signifying that all the lipid components interact with each other to a great extent while forming the lipid bilayer. DSC thermogram of acetazolamide-loaded liposomes composed of PC:CH (7:4) molar ratio interestingly showed disappearance of the melting endotherm of acetazolamide and the major endotherm was shifted from 130°C to 110.7°C. The incorporated acetazolamide associated with lipid bilayers and interacted to a large extent with them. Absence of the melting endotherm of acetazolamide and shifting of the lipid bilayer components endotherm suggested significant interaction of acetazolamide with bilayers.[33] The same results were observed with empty and drug-loaded liposomes composed of PC and cholesterol (7:7) molar ratio. The DSC thermogram of empty 7:7 liposomes showed very small endotherm at 100.5°C, where the intensity of the endotherm reduced markedly because of increased cholesterol contents in comparison to empty liposomes 7:4 molar ratio. DSC thermogram of acetazolamide-loaded liposomes composed of PC and cholesterol (7:7) molar ratio showed disappearance of the melting endotherm of acetazolamide and appearance of 2 distinct endotherms at 90.7°C and 113.9°C, indicating the interaction of acetazolamide with bilayers leading to enhanced entrapment of the drug and decreased rate of release. DSC thermogram of positively charged acetazolamide-loaded liposomes composed of PC:CH:SA (7:7:1) molar ratio also showed disappearance of the melting endotherm of acetazolamide, the intensity of the endotherms markedly increased, and the major endotherms shifted from 90.7°C to 100.2°C and from 113.9°C to 114.5°C in comparison to 7:4 drug-loaded liposomes, indicating good interaction of all components. The DSC results of 7:7:1 liposomes suggest enhanced entrapment efficiency of acetazolamide in the lipid bilayer in the presence of positive-charge inducer together with increased molar ratio of cholesterol in the prepared liposomes.

Stability Study

Physical stability study of acetazolamide REVs and MLVs liposomes was conducted at 4°C for a period of 3 months. Drug leakage, from the liposomal formulations with lipid components in the molar ratio of 7:4, was evaluated at definite time intervals, and the results are demonstrated in Table 4 in terms of percentage acetazolamide retained in the liposomes.

After 90 days, the percentages of acetazolamide retained in the liposomal formulations were 68.51%, 87.40%, and 75.13% for neutral, positively charged, and negatively charged reverse-phase evaporation liposomes respectively. Similarly, after the same period of time, the percentages of acetazolamide retained in the liposomal formulations were 69.40%, 89.43%, and 77.11% for multilamellar liposomes. It can be noted that there is no obvious difference in physical stability results between reverse-phase evaporation and multilamellar liposomes prepared with the same lipid molar ratio when stored in the refrigerator. Student *t* test shows that there is no significant difference between them at $\alpha = 0.05$.

It is also obvious that positively charged liposomes show the highest stability manifested by the highest drug retention, followed by the negatively charged liposomes, then neutral liposomes. Surface charge is one of the important factors that improve the stability by reducing the rate of aggregation and fusion of liposomes during storage.[34]

It is obvious from the results that despite the partial hydrolysis that would occur for the phosphatidylcholine, the liposomes made from them are sufficiently stable under refrigerator storage, and the advantages of the lipid membrane were retained.[35]

In Vivo Studies

Neutral and charged REVs and MLVs liposomal formulations prepared with PC:CH (7:4) molar ratio were selected for in vivo studies as they exhibited higher T_{8h} than their corresponding liposomes prepared with PC:CH in the molar ratios of either 7:6 or 7:7. Also, multilamellar liposomal formulation composed of PC:CH:SA (7:7:1) molar ratio was involved in the study. This formulation showed the highest encapsulation efficiency percentage among all the prepared liposomal formulations. The values of the reduction in IOP in mmHg produced by a single dose of each of the selected preparations are shown in Tables 5 and 6.

Acetazolamide liposomal preparations produced a significant lowering in IOP compared with the solution of free drug. Plain liposomes showed no effect on ocular hypotensive activity. By comparing the IOP lowering activity after 3 hours of topical administration, the tested liposomal preparations and drug solution can be arranged in the following descending order: positive MLVs (PC:CH:SA, 7:4:1) > neutral MLVs (PC:CH, 7:4) > positive REVs (PC:CH:SA, 7:4:1) > positive MLVs (PC:CH:SA, 7:7:1) > neutral REVs (PC:CH, 7:4) > negative MLVs (PC:CH:DP, 7:4:1) > negative REVs (PC:CH:DP, 7:4:1), and reference solution > plain liposomes corresponding to the values of −7.8, −5.5, −4.65, −4.3, −4.2, −3.7, −0.67, −0.67, and 0. One-way ANOVA revealed significant differences between all pairs at $P < .01$.

The maximum IOP reduction was observed among the rabbits of group VIII receiving the positively charged multilamellar liposomes prepared with PC:CH:SA (7:4:1) molar ratio. The IOP reduction reached a value of −7.8 mmHg after 3 hours of topical administration and was sustained during the time of the experiment (8 hours). The superiority of the positive liposomes with the molar ratio 7:4:1 could be explained on the basis of their high binding affinity to the negatively charged mucin of the corneal epithelium, thus enhancing contact time with the cornea by charged mediated adhesion or electrostatic interaction.[36] Moreover, the positive vesicles are expected to slow down drug elimination by the lachrymal flow, both by increasing solution viscosity and by interacting with the negative charges of the mucus.[1]

Next, the neutral multilamellar liposomes showed promising results as they lowered the IOP to −5.5 mmHg after

Table 4. Physical Stability of Acetazolamide Liposomal Formulations Stored at 4°C*

	Acetazolamide Retained in Liposomal Formulations (% ± SD)					
	Reverse-phase Evaporation Liposomes			Multilamellar Liposomes		
Time (days)	Neutral Liposomes PC:CH (7:4)	Positive Liposomes PC:CH:SA (7:4:1)	Negative Liposomes PC:CH:DP (7:4:1)	Neutral Liposomes PC:CH (7:4)	Positive Liposomes PC:CH:SA (7:4:1)	Negative Liposomes PC:CH:DP (7:4:1)
0	100	100	100	100	100	100
15	89.15 ± 0.62	96.45 ± 0.16	92.54 ± 0.10	89.12 ± 0.06	97.11 ± 0.07	92.30 ± 0.27
30	80.41 ± 0.17	93.63 ± 0.90	88.12 ± 1.50	81.33 ± 0.26	94.76 ± 2.80	88.17 ± 0.49
45	73.85 ± 2.40	91.76 ± 0.43	82.19 ± 1.67	75.16 ± 0.16	93.35 ± 0.40	83.67 ± 0.50
60	70.00 ± 3.20	90.80 ± 1.40	77.88 ± 1.4	71.90 ± 0.28	91.44 ± 0.78	78.01 ± 0.43
90	68.51 ± 0.13	87.40 ± 1.20	75.13 ± 1.65	69.40 ± 0.39	89.43 ± 0.09	77.11 ± 0.06

Table 5. Effect of Topically Administered Acetazolamide Reverse-phase Evaporation Liposomes on the Change in Intraocular Pressure in Normotensive Rabbits*

Time (hours)	Δ IOP† ± SD After Topical Administration of Drug Treatment (mmHg)‡				
	Plain Liposomes	Acetazolamide Solution	Neutral Liposomes PC:CH (7:4)	Negative Liposomes PC:CH:DP (7:4:1)	Positive Liposomes PC:CH:SA (7:4:1)
0	0	0	0	0	0
0.5	0	–2.50 ± 0.75	–4.55 ± 0.98	–4.25 ± 0.81	–4.00 ± 1.65
1	–0.45 ± 0.41	–3.70 ± 0.98	–5.35 ± 1.16	–4.20 ± 1.56	–4.20 ± 1.15
2	–0.27 ± 0.46	–3.13 ± 0.98	–5.20 ± 1.47	–3.13 ± 2.16	–5.35 ± 2.01
3	0	–0.67 ± 1.15	–4.20 ± 1.65	–0.67 ± 0	–4.65 ± 1.47
4	0	0	–4.20 ± 1.34	0	–5.33 ± 1.73
5	0	0	–3.13 ± 2.42	0	–5.33 ± 2.43
6	0	0	–2.11 ± 2.18	0	–4.25 ± 2.42
7	0	–0.67 ± 1.15	–2.11 ± 1.56	0	–3.95 ± 2.18
8	0	0	–2.11 ± 1.65	0	–4.10 ± 2.41

*IOP indicates intraocular pressure; PC, phosphatidylcholine; CH, cholesterol; DP, dicetyl phosphate; and SA, stearylamine.
†Average difference in intraocular pressure between the treated and control eye of the same rabbit.
‡All treatments were equivalent to 1% acetazolamide.

3 hours of drug administration, and the effect was sustained for eight hours.

The fourth rank of the (7:7:1) positive multilamellar liposomes despite their high drug loading could be attributed to their tightened structure and decreased permeability of the lipid bilayer, a result of its high cholesterol content, manifested by a low rate of drug release and consequently lower bioavailability than 7:4:1 positively charged multilamellar liposomes.

Comparing the results of both REVs and MLVs liposomal formulations of the same composition and molar ratio, it is obvious that multilamellar liposomes produced a more significant lowering in IOP compared with REVs liposomes and showed a more sustained action towing to the presence of several lipid bilayers, which release the drug slowly over a prolonged period of time.

In an attempt to confirm the sustained effect of the encapsulated acetazolamide in liposomes, the selected acetazolamide vesicular preparations were compared with acetazolamide solution. The results reveal that the IOP-lowering activity of the drug solution observed among the rabbits of group II reached values of −2.50, −3.70, −3.13, and −0.67 mmHg after 0.5, 1, 2, and 3 hours of drug administration, respectively, and the effect was nearly abolished after 3 hours of the experiment; while with the vesicular acetazolamide, the effect was more significant and sustained for a period ranging from 6 to 8 hours with different extents. This result occurs because in the liposomal dosage form, the drug is

Table 6. Effect of Topically Administered Acetazolamide Multilamellar Liposomes on the Change in Intraocular Pressure in Normotensive Rabbits*

Time (hours)	Change in IOP† ± SD After Topical Administration of Drug Treatment (mmHg)‡					
	Plain Liposomes	Acetazolamide Solution	Neutral Liposomes PC:CH (7:4)	Negative Liposomes PC:CH:DP (7:4:1)	Positive Liposomes PC:CH:SA (7:4:1)	Positive Liposomes PC:CH:SA (7:7:1)
0	0	0	0	0	0	0
0.5	0	–2.50 ± 0.75	–3.15 ± 0.98	–4.50 ± 0.81	–3.40 ± 0.81	–1.55 ± 0.77
1	–0.45 ± 0.41	–3.70 ± 0.98	–4.55 ± 0.81	–4.25 ± 1.56	–6.77 ± 0.1.56	–2.57 ± 0.81
2	–0.27 ± 0.46	–3.13 ± 0.98	–5.78 ± 2.16	–3.70 ± 1.04	–6.97 ± 1.15	–5.37 ± 0.58
3	0	–0.67 ± 1.15	–5.50 ± 1.65	–3.70 ± 2.18	–7.80 ± 1.04	–4.30 ± 0.69
4	0	0	–4.25 ± 1.34	–3.13 ± 2.14	–6.77 ± 1.56	–3.50 ± 0.80
5	0	0	–4.20 ± 0.81	–0.67 ± 0.65	–6.43 ± 0.98	–3.27 ± 0.40
6	0	0	–3.91 ± 1.18	–0.67 ± 0.56	–7.47 ± 0.81	–3.03 ± 0.40
7	0	–0.67 ± 1.15	–2.50 ± 1.56	0	–4.53 ± 2.14	–2.57 ± 0.40
8	0	0	–2.11 ± 1.65	0	–3.97 ± 1.15	–2.57 ± 0.40

*IOP indicates intraocular pressure; PC, phosphatidylcholine; CH, cholesterol; DP, dicetyl phosphate; and SA, stearylamine.
†Average difference in intraocular pressure between the treated and control eye of the same rabbit.
‡All treatments were equivalent to 1% acetazolamide.

encapsulated in lipid vesicles that can cross cell membranes. 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, the higher the bioavailability of the drug.[10] Thus, liposomes as drug carriers can change the rate and extent of drug absorption.[16] Hence, a more pronounced sustained reduction in IOP was produced by the encapsulated drug.

It is to be noted that examination of the rabbits' eyes during the study showed no signs of irritation such as lachrymation or increased eye blinking upon instillation of any of acetazolamide preparations.

CONCLUSION

From the comparison study between acetazolamide REVs and MLVs liposomal formulations, it could be concluded that the multilamellar liposomes are larger in size than REVs and exhibited higher values of entrapment efficiencies. The release of acetazolamide is slower from multilamellar liposomes than from REVs liposomes of the same lipid molar ratio. Multilamellar liposomes produced a more significant lowering in IOP and showed a more sustained action than REVs liposomes because of the presence of several lipid bilayers that release the drug slowly over a prolonged period of time. Neutral and positively charged multilamellar liposomes, prepared with PC:CH (7:4) or PC:CH:SA (7:4:1) molar ratio, respectively, would be promising ocular delivery systems for acetazolamide in the treatment of glaucoma.

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