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International Journal of Pharmaceutics 198 (2000) 29–38

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

Prolonged effect of liposomes encapsulating pilocarpine HCl in normal and glaucomatous rabbits

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Received 8 February 1999; received in revised form 27 July 1999; accepted 6 October 1999

Abstract

The possibility of using liposomes as an ophthalmic drug delivery carrier for the lipophilic drug, pilocarpine HCl, was investigated on the eyes of normal and glaucomatous pigmented rabbits. The intraocular pressure (IOP) of rabbits was measured, using a Shi∅tz tonometer, as a function of time after topical administration with free drug, neutral and negatively charged multilamellar vesicles (MLVs) encapsulating pilocarpine HCl. The results showed that administration with neutral MLVs displayed the most prolonged effect with respect to negatively charged MLVs and free drug. The efficiency of MLVs encapsulating pilocarpine HCl, measured using spectrophotometric technique, was found to be 96% in our modified preparations. The storage stability of MLVs encapsulating pilocarpine HCl was investigated by measuring phase transition and size distribution using light scattering technique. The results show that liposomes encapsulating pilocarpine HCl have kept their integrity and physicochemical properties for at least 15 months, which makes them suitable for commercial use.

Keywords: Liposome characterization; Pilocarpine hydrochloride; Glaucomatous pigmented rabbits; Intraocular pressure (IOP); Light scattering

1. Introduction

The main objective of ophthalmic therapy is to provide and maintain an adequate concentration of the active ingredient at the site of action. Several free drug eye drops are found to have poor penetration into ocular tissues (Benson, 1974; Bloomfield et al., 1978) and the duration of action of the drug is therefore too short (Hanna et al., 1978; Hanna, 1980). Hence frequent administration is needed which would increase the risk of drug toxicity and side-effects (Patton and Francoeur, 1978; Salminen et al., 1984).

Several experiments have demonstrated that administration of drugs entrapped in liposomes as eye drops improves drug bioavailability (Niesman, 1992; Gregoriadis and Florence, 1993; Meisner and Mezei, 1995; and references there in). The bioavailability of liposomes encapsulating drugs has been found to be highly dependent on liposome surface charge (Shaeffer and Krohn, 1982;

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0378-5173/00/$ - see front matter
PII: S0378-5173(99)00348-8

Fitzgerald et al., 1987; El-Gazayerly and Hikal, 1997; Kompella et al., 1998) and on whether the drug is lipophilic or hydrophilic (Shaeffer and Krohn, 1982; Singh and Mezei, 1983; Benita et al., 1984; Meisner et al., 1989).

Although positively charged liposomes enhance the adsorption of liposomes to mucin layer overlying the corneal epithelium resulting in improved drug transfer, they were found to be toxic because of their stearylamine content (Taniguchi et al., 1988), causing pain and unpleasantness following instillation. On the other hand neutral liposomes were found to be safe for ophthalmic applications (Taniguchi et al., 1988). Positively charged liposomes were therefore not used in this work.

Although encapsulated liposomes have been widely investigated as a carrier for ophthalmic medications, little attention has been given to studying their encapsulation efficiency and storage stability. In this work, we present the results of using pilocarpine HCl encapsulated MLVs to extend the time of reduced intraocular pressure (IOP) of rabbit's eye measured using a Shi∅tz tonometer. The encapsulation efficiency of MLVs was measured using spectrophotometric technique. The storage stability of the two types of MLVs prepared in this work, was tested by measuring the phase transition behavior of MLV suspensions using light scattering technique. The size distribution of MLVs was also measured using low angle scattering data and the theory of Fraunhoffer diffraction.

2. Material and methods

L-α-Dipalmitoyl phosphatidyl choline (DPPC) with a molecular weight of 734 (99% pure), trizma buffer with a molecular weight of 121.1, and dicetyl phosphate (DCP) with a molecular weight 546.9 (99% pure) were purchased from Sigma. Pilocarpine hydrochloride drug with a molecular weight of 244.72, was purchased from Boehringer–Ingelheim, Germany, 0.9% sodium chloride was obtained from ADWIC (Pharmaceuticals Division), and chloroform ('ARISTAR' grade) from BDH. All chemicals were used without further purification.

Female, pigmented rabbits (Breeding Farm, Egyptian Research Institute of Ophthalmology), weighing 2.3–2.6 kg and 6–8 months old were used throughout this study. The rabbits were divided into three groups, each group composed of three rabbits. They were fed on balanced diet pellets.

2.1. Liposome preparation

L-α-Dipalmitoyl phosphatidyl choline (DPPC):pilocarpine HCl molar ratio 7:2 was used to prepare neutral MLVs using the method of Bangham et al. (1974). Briefly, 10 mg DPPC and the drug powder were transferred to a 50-ml round bottom flask. Then 15 ml of chloroform was added, and the flask shaken until lipid was completely dissolved in the chloroform. The solvent was evaporated under vacuum until a thin dry film of lipid was formed. The flask was left under vacuum for 12 h to ensure the evaporation of all traces of chloroform. Then 10 ml of buffer (10 mM Trizma adjusted to pH 7) was added to the flask which was flashed through with nitrogen and immediately stoppered. The flask was mechanically shaken for 1 h at a temperature of 45°C. The suspension was then centrifuged at 8000 rpm and the supernatant was discarded. The liposomes were then resuspended in 10 ml buffer solution. The concentration of pilocarpine/ml of buffer was calculated to be 0.1 mg/ml, equivalent to 0.01% pilocarpine HCl. DPPC:pilocarpine HCl:DCP molar ratio 7:2:1 was used to prepare negatively charged MLVs.

2.2. Encapsulation efficiency measurements

The encapsulation efficiency of the samples was measured using a spectrophotometer (Uvikon 930, UK). The wavelength was adjusted to 215 nm (the resonance absorption of pilocarpine HCl). The absorption of the supernatant of each sample (centrifuged at 8000 rpm for 20 min) was compared to standard curve relating absorption and pilocarpine concentration. Mixing pilocarpine HCl with the lipid powder before dissolving in chloroform was found to increase encapsulation efficiency to 96%; if pilocarpine HCl is dissolved

in buffer and added to the dry film of lipid then a reduced encapsulation efficiency results.

2.3. Sample characterization

The size distribution of the samples was determined from the data of low angular light scattering and the theory of Fraunhoffer diffraction (Bayvel and Jones, 1981). The angular light scattering apparatus allows the measurement of the polarized light scattering in the angle range 3–180° and is described elsewhere (Monem, 1986).

The size distribution of the samples $F(a)$, according to Fraunhoffer diffraction, is given by Bayvel and Jones (1981):

$$F(a) = \frac{\sum_{i=1}^{n} A_i(\alpha\theta)\phi_i(\theta)\Delta\theta}{\sum_{l-1}^{m} a_l \sum_{i=1}^{n} A_i(\alpha\theta)\phi_i(\theta)\Delta\theta\Delta a} \quad (1)$$

where

$$A(\alpha\theta) = (\alpha\theta)J_1(\alpha\theta)Y_1(\alpha\theta) \quad (2)$$

where J_1 $(\alpha\theta)$ and Y_1 $(\alpha\theta)$ are the Bessel functions of the first order and first and second type, respectively. The size parameter (α) is given by $(\alpha = 2\pi a/\lambda)$, where a is the particle radius and λ is the wavelength of the incident light in the medium. The function $\phi(\theta)$ is given by:

$$\phi(\theta) = \frac{\mathrm{d}}{\mathrm{d}(\theta)}[\theta^3(I(\theta)/I_0)] \quad (3)$$

where $(I(\theta)/I_0)$ is the normalized scattered light intensity, at an angle θ, measured at the angle range $(\theta = 2–8°)$ in steps of 0.5° at room temperature 23°C. The parameters $\Delta\theta$ and Δa are taken to be 0.5° and 0.1 μm, respectively. The variable parameter (a_l) changed from 1 to 10 μm in steps of 0.1 μm. The size distribution $F(a)$ was then calculated from Eq. (1) using the computer program 'Math Cad'.

The phase transitions of the samples were measured using angular light scattering apparatus. The scattering angle was fixed at $(\theta = 90°)$ and the temperature of the samples was varied from 20 to 45°C. The scattered light intensity I_{VV} (90°) was measured as a function of temperature using an X–Y chart recorder.

2.4. Bioavailability studies

The rabbits were divided into three groups, each group composed of three rabbits. The initial value of intraocular pressure (zero reading) of both eyes of each rabbit was measured.

Each group was designated to receive one of the drug preparations: neutral liposomes, negatively charged liposomes and free-drug solution. All preparations containing 0.1 mg/ml equivalent to 0.01% by weight pilocarpine HCl. All rabbits received a single 25 μl dose of drug preparation in one eye (right eye) except the glaucomatous rabbits, which received a 50 μl dose in case of free-drug solution. The contralateral eye (left) received no drugs and remained as a control. The left eye was left without additional control, such as empty liposomes mixed with DNA, to study the possible drug transfer from right to left eye. Moreover the addition of empty liposomes and/or buffer solutions have been found to have no effect on drug levels in most ocular tissues (Singh and Mezei, 1984; Meisner et al., 1989).

After the instillation of different drug samples, the ocular bioavailability of pilocarpine HCl was assessed by measuring the intraocular pressure using a standardized Shi∅tz tonometer as described by Shields (1987), in both eyes at 15, 30 and 60 min, and then every hour until the intraocular pressure of the treated eye (right eye) returned to the initial value for each rabbit.

In the glaucomatous rabbits, the glaucoma was produced experimentally by means of subconjunctival injection of 5% solution of phenol in almond oil as sclerosing fluid, causing a rise in intraocular pressure but no apparent macroscopic or microscopic damage to the eye (Maurice and Luntz, 1966).

3. Results and discussion

Fig. 1 shows the measured drop in the IOP of the treated right eye (closed circles) and the untreated left eye (closed squares) of normal rabbit as a function of time after administration with neutral MLVs encapsulating pilocarpine HCl (a), negatively charged MLVs encapsulating the drug

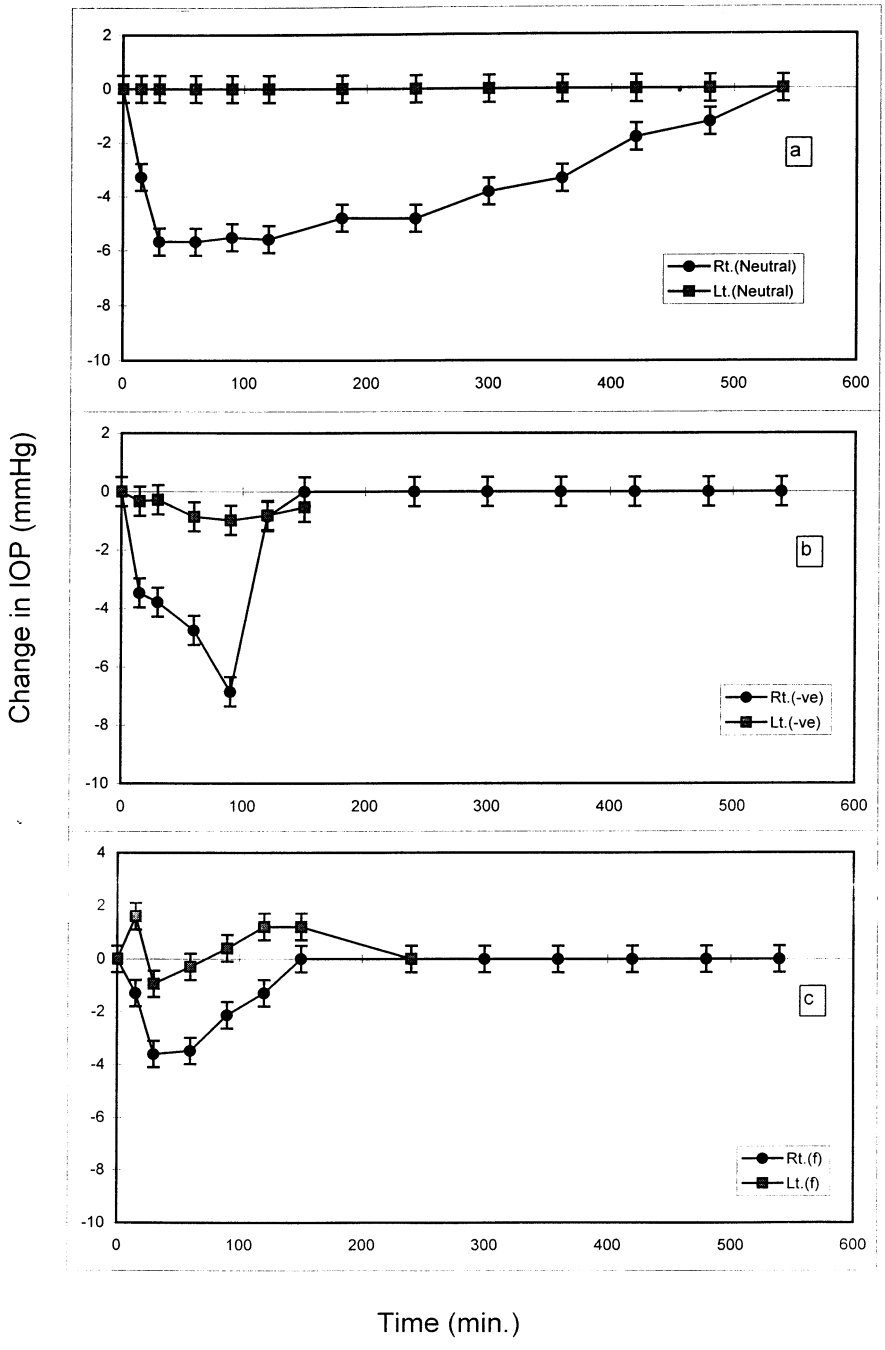

Fig. 1. The change in the intraocular pressure (IOP) as a function of time for normal rabbits. A remarkable drop in IOP of the treated right eye was seen after topical administration of neutral MLVs encapsulating pilocarpine HCl (a), negatively charged MLVs (b) and the free drug (c). The drug dose used was 5 μg. The average values of the initial IOP were 20.7 and 19.5 mmHg for right and left eye, respectively. Error bars represent the percentage of the ratio of the standard deviation to the mean value.

(b) and the free drug (c). Administration with neutral MLVs decreases the IOP from an initial value of 20.7 to ~15 mmHg during a period of 30 min. The lowered IOP remains for ~4–5 h before it rises to its initial value. No changes in the IOP were observed for the untreated eye during the course of measurements.

Administration with negatively charged MLVs and the free drug shows a similar behavior, where the IOP decreases and remains at its lower value for remarkably shorter periods of time (~1 h) compared to those observed for neutral MLVs. The untreated left eye shows a slight decrease in its IOP for rabbits treated with negatively charged MLVs while the change was irregular for rabbits treated with the free drug alone.

The same protocol was used for three sets of glaucomatous rabbits. The average values of the initial IOP of the right and left eye were 26.5 and 24.5 mmHg, respectively. Fig. 2 shows the drop in the IOP of the treated right eye (closed circles) and the contralateral untreated left eye (closed squares). Administration with neutral MLVs encapsulating the drug shows a remarkable drop of the IOP from 26.5 to ~15 mmHg in a period of 60 min (Fig. 2a). The lowered IOP remains below 20 mmHg for a period of ~7 h. The IOP of the untreated left eye shows a drop to ~18 mmHg by 100 min and then fluctuates around 22 mmHg throughout the rest of the measuring course (9 h).

Fig. 2b and c shows the drop in the IOP of the treated right eye and the untreated left eye for glaucomatous rabbits treated with negatively charged MLVs encapsulating the drug and the free drug, respectively. The lowered IOP of the treated right eye remains for only 2 h for both treatments. A remarkable drop in the IOP of the untreated left eye was observed for rabbits treated with the free drug alone (Fig. 2c). The characteristic curves for IOP of both right and left eye seem to coincide for glaucomatous rabbits treated with free drug alone. A possible free drug transfer from the treated right eye to the untreated left eye may occur. The former observation was not observed for glaucomatous rabbits treated with both neutral and negatively charged MLVs.

The observed drop in the IOP of the untreated left eye of glaucomatous pigmented rabbits (Fig. 2c) was rather ambiguous for two reasons: first, it was not observed for normal rabbits and second, the concentration of postulated drug transferred to the left eye is supposed to be too low to cause such an observed drop in its IOP. Further investigation should be conducted to elucidate the different mechanisms of drug transfer from one eye to the other, especially in glaucomatous rabbits.

The results presented in this work asses the advantage of using neutral MLVs as an ocular drug carrier system: this could be due to the effective adsorption of ocular tissues to neutral MLVs. For instance the corneal epithelium is coated with negatively charged mucin (Shek and Barber, 1987) which provides stable adsorption surface to neutral MLVs as well as positively charged MLVs. In this work positively charged MLVs were avoided because of their toxicity and irritability (Taniguchi et al., 1988).

Fig. 3 shows the scattered light intensity I_{VV} (90°) from DPPC-MLVs (a), neutral MLVs encapsulating HCl measured directly after preparation (b), and 15 months after preparation (c). The lower and main transitions of DPPC-MLVs were found to occur at 35 and 41°C (Fig. 3a), in agreement with the reported results (Chen et al., 1980). The incorporation of pilocarpine HCl in the lipid bilayers of DPPC-MLVs (molar ratio 7:2) had changed the phase transition properties of such lipid (Fig. 3b). A continuous drop in the scattered light intensity starts at ~27 and ends at ~43°C. The disappearance of the lower transition and main transition broadening usually accompany the incorporation of lipophilic molecules into lipid bilayers, such as cholesterol (Ladbrooke et al., 1968). Fig. 3c shows the scattered light intensity from neutral DPPC- pilocarpine HCl MLVs as a function of temperature measured 15 months after preparation. The transition starts at ~28 and ends at ~38°C. Although a small shift in the transition temperature (~3°C) was observed for stored samples, their phase transition characteristics were similar to those observed for samples measured directly after preparation.

Fig. 4a and b shows the scattered light intensity I_{VV} (90°) as a function of temperature for negatively charged MLVs encapsulating pilocarpine HCl measured directly after preparation (a) and

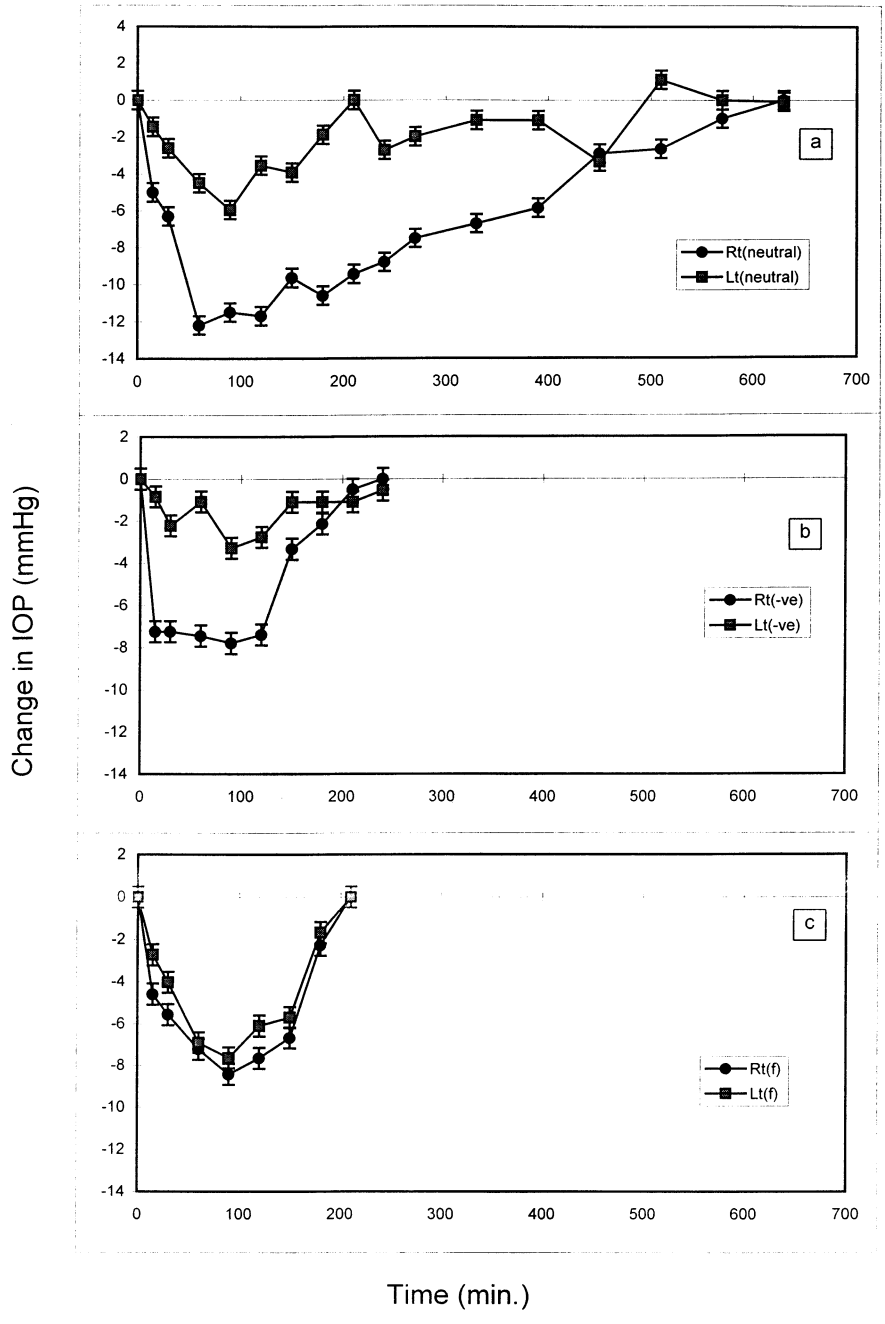

Fig. 2. The change in IOP as a function of time for glaucomatous pigmented rabbits. Topical administration of neutral MLVs (a), negatively charged MLVs (b) and free drug (c). The drug dose was ~5 μg. The average values of the initial IOP were 26.5 and 24.5 mmHg for right and left eye, respectively.

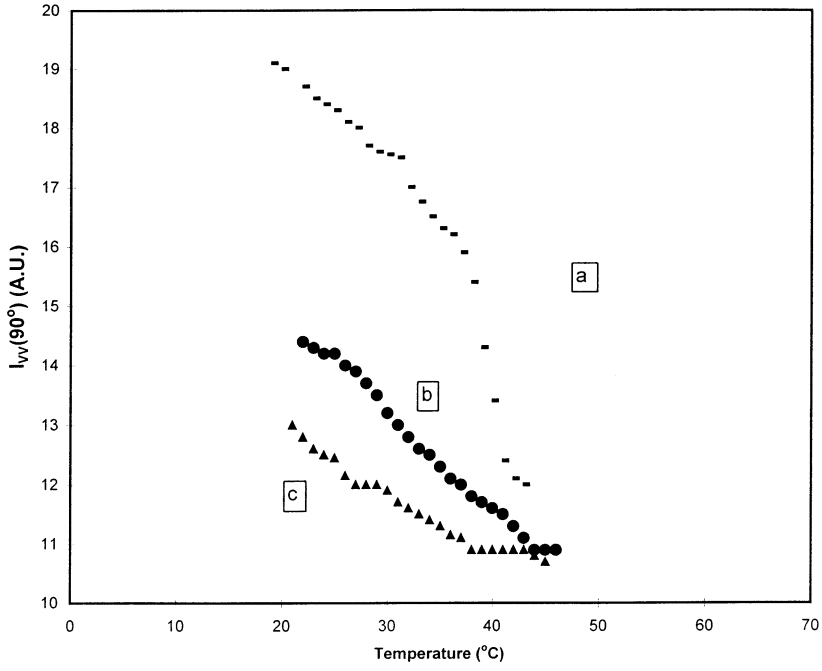

Fig. 3. The change in the scattered light intensity I_{vv} (90°), measured in arbitrary units (A.U.) as a function of temperature for pure DPPC-MLVs (a), neutral MLVs encapsulating pilocarpine HCl measured directly after preparation (b) and 15 months after preparation (c). Temperature scan rate was $\sim$0.5°C/min. Lipid concentration was 0.5 mg/ml. Samples were gently stirred during measurements.

10 months after preparation (b). The lower transition of samples measured directly after preparation starts at $\sim$30°C and ends at 36°C while the main transition occurs at $\sim$41°C. A broadening of the lower transition and diminishing of the amplitude of the main transition may characterize a bilayer composed of DPPC:pilocarpine HCl:DCP molar ratio 7:2:1. The phase transition characteristics for samples measured 10 months after preparation were found to be similar to those observed for samples measured directly after preparation with only a 1°C shift to lower temperature. The samples were kept under nitrogen at 4–8°C.

Fig. 5 shows the size distribution of neutral MLVs measured using the low angular data and Eq. (1). It is clear that the size distribution shows no changes between samples measured directly (a) and 15 months after preparation (b). The measurement of the size distribution of MLVs is another biophysical technique to ensure that samples kept under nitrogen for 15 months suffer no aggregation, fusion and/or dissociation. A similar size distribution was obtained for negatively charged MLVs measured directly and 10 months after preparation (data not shown).

Fig. 6 shows the absorption spectra of pilocarpine HCl. The maximum absorption was found to occur at 215 nm (Fig. 6a). The standard curve was then measured relating absorption, at 215 nm, and pilocarpine concentration (Fig. 6b). Measurements were taken in the concentration range 0.015–0.11 mg/ml. Concentrations of the drug below 0.01 mg/ml cannot be detected using the spectrophotometer used in this work. The absorption of the supernatant of samples measured directly after preparation was used to evaluate the encapsulation efficiency of stored MLV samples. The absorption of supernatant of samples measured 10 months and 15 months after

preparation shows no traces of pilocarpine HCl indicating that MLVs encapsulating the drug are still stable. These results lend confidence to the results of phase transition and size distribution (Figs. 3–5), respectively.

4. Conclusion

We may conclude that neutral MLVs encapsulating pilocarpine HCl exhibit the most prolonged drug action in reducing intraocular pressure (IOP) when used as topical eye drops. Negatively charged MLVs encapsulating pilocarpine HCl and free pilocarpine HCl exhibit shorter drug action (nearly one third that of neutral MLVs). Although only the right eyes of glaucomatous rabbits were treated with the different types of drugs, the left eyes show a reduction in the IOP indicating possible direct drug transfer from right to left eye.

Three different parameters namely, MLV phase transition, MLV size distribution and drug release to the suspending medium, had to be measured to evaluate encapsulation efficiency and storage stability of MLVs encapsulating pilocarpine HCl. The phases and phase transitions of MLV bilayer are very sensitive to impurities and drug incorporation within the lipid bilayer. The shape of MLV phase transition was found to be sensitive to lipid type and molar ratio of incorporated molecules in the bilayer as well as lipid degradation. The stability of MLVs could then be tested by measuring its phase transition under certain storage conditions. The size distribution of MLVs also plays an important role in testing their aggregation and/or fusion. The third parameter is to measure release of the fused drug from the bilayer to the suspending medium using spectrophotometric techniques.

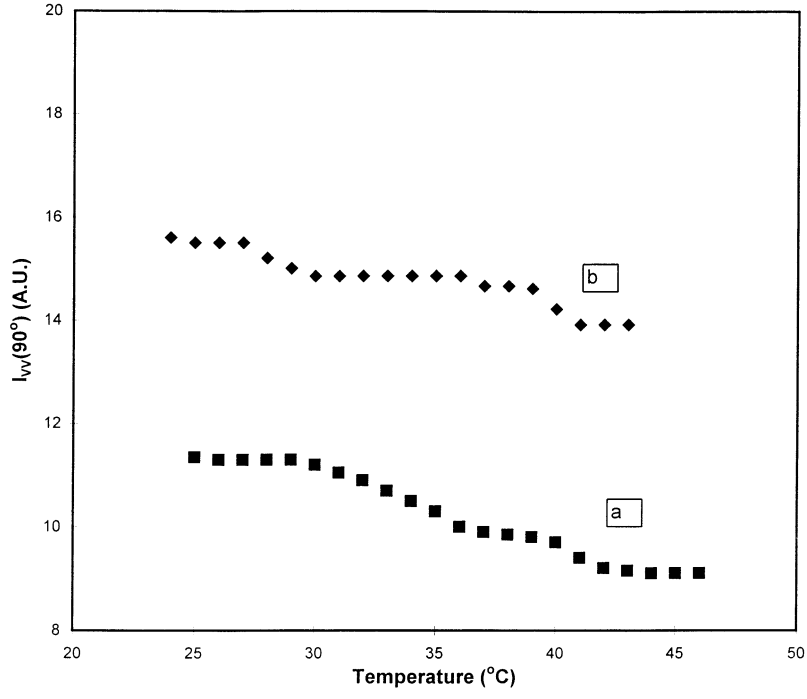

Fig. 4. The change in the scattered light intensity I_{VV} (90°) as a function of temperature for negatively charged MLVs measured directly after preparation (a) and 10 months after preparation (b). Temperature scan rate was 0.5°C/min and lipid concentration was 0.5 mg/ml.

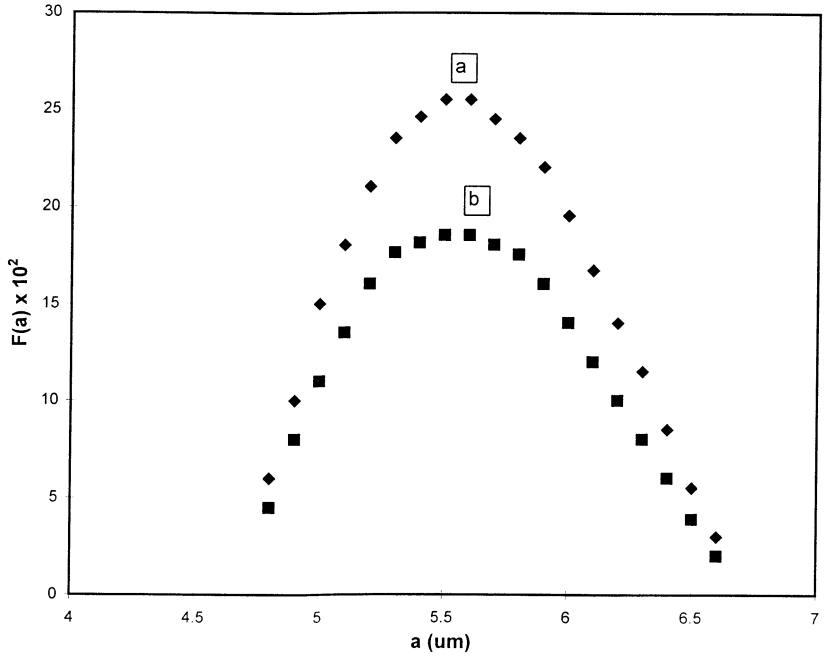

Fig. 5. The size distribution of MLV $F(a)$, calculated using Eq. (1), for neutral MLVs measured directly after preparation (a) and 15 months after preparation (b). Concentration of MLVs was ~ 0.1 mg/ml to avoid multiple scattering.

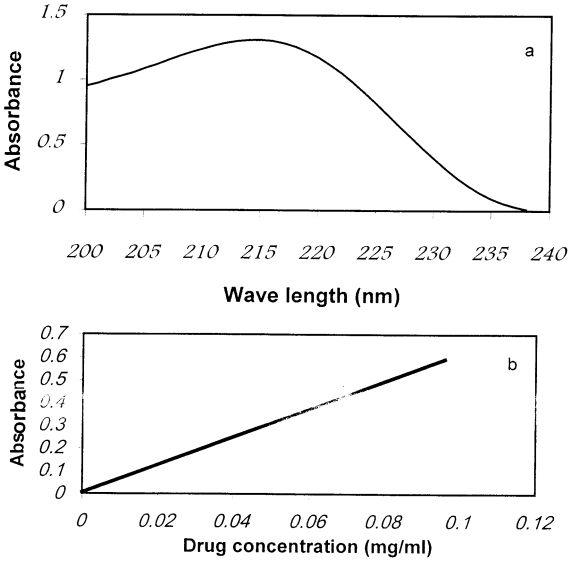

Fig. 6. The absorption spectra of pilocarpine HCl (a) and the standard curve relating absorption and pilocarpine HCl concentration measured at 215 nm (b).

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