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Preparation and evaluation of liposomal formulations of tropicamide for ocular delivery

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Abstract

Tropicamide, a mydriatic, cycloplegic drug was entrapped in liposomes. Liposomes were investigated by laser counting studies, transmission electron microscopy and differential scanning calorimetry for characterization. The precorneal clearance of liposomes was compared with solution by γ-scintigraphy in the rabbit. The neutral liposomes failed to demonstrate significant enhancement in precorneal retention in comparison with aqueous solution. The potential of liposomes as an ophthalmic drug delivery system was investigated by comparing pupil dilatory effect of tropicamide by topical instillation, in the rabbit eye, of the solution and various drug-loaded liposomal forms, i.e. neutral liposomes, positively charged liposomes and neutral liposomes dispersed in 0.25% (w/v) polycarbophil gel. The positively charged liposomal formulation and liposomes dispersed in polycarbophil gel were found to be more effective than neutral liposomal dispersion when data were statistically treated at the 5% level of significance.

Keywords: Liposomes; Pupil dilatory effect; γ-Scintigraphy; Tropicamide

1. Introduction

One of the major problems of ocular drug delivery is to provide and maintain an adequate concentration of drug in the precorneal area. In solutions many drugs display poor penetration through the corneal barrier. Rapid nasolacrimal drainage of the instilled drug from tear fluid and non-productive absorption through the conjunctiva may lead to a short duration of action. Tear turnover and drug binding to tear fluid proteins are additional precorneal factors that contribute to the poor ocular bioavailability of many drugs, when instilled in the eye in the solution dosage form. The rate of release of drug from the tear fluid to ocular tissues is initially high when instilled as solution, but rapidly declines; this may result in a transient period of overdose and the associated risk of side-effects, followed by an extended period of subtherapeutic levels before the next dose is administered. This indicates the need for an ocular drug delivery system which has the convenience of a drop but will serve as slow release depot.

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Table 1
Effect of time of shaking on encapsulation efficiency

Time (h)	% Entrapped[a]
0	15.35 ± 0.71
3	18.36 ± 1.55
6	21.8 ± 0.72

[a] Each value is mean of three determinations.

Liposomes have shown potential to fulfil these requirements; they have the ability to entrap hydrophilic compounds in the aqueous compartments and to incorporate hydrophobic molecules in the lipid bilayers. The potential of liposomes in ocular delivery has been investigated previously, with conflicting reports (Singh and Mezei, 1983, 1984; Meisner et al., 1989).

In this study, the effect of liposomal encapsulation of tropicamide on precorneal drainage profile and mydriatic activity was studied. The effect of formulating a gel and of incorporating positive charges on liposomes was investigated by evaluating extent and duration of pupil dilatory effect.

2. Materials and methods

2.1. Materials

Tropicamide was generously donated by FDC, India. Phospholipon 90 and Phospholipon 90H were obtained as gift samples from Nattermann Phospholipid (Germany). Stearylamine and cholesterol were purchased from Sigma (USA) and Loba Chemie (India) respectively. Noveon™ AA-1 Polycarbophil was obtained as a gift sample from BFGoodrich (USA). All other reagents were of analytical grade. Distilled water was used throughout this study.

2.2. Methods

Aqueous liposomal formulations were prepared by the conventional lipid film hydration method.

2.2.1. Preparation of empty liposomes

Liposomal dispersion without drug (empty liposomal dispersion) was prepared using a mixture of P-90, P-90H and cholesterol in 3:8:4 molar proportion. A mixture of these lipids in chloroform was taken in a round-bottomed flask with glass beads. A thin film formed on the inner side of the round-bottomed flask and on the surface of the glass beads due to evaporating chloroform under vacuum in: (A) Edward's Modulyo vacuum drier (stationary flask) at room temperature; and (B) a rotary flash evaporator at 40°C. The required amount of isotonic phosphate buffer (pH 7.0) was added above phase transition temperature and the flask was shaken for 6 h with intermittent sonication using a bath sonicator (Imeco Ultrasonics).

2.2.2. Preparation of drug (tropicamide) containing liposomes

Tropicamide was incorporated into liposomes by mixing a solution of drug in chloroform (1%, w/v) to the phospholipid/chloroform mixture prior to evaporation. Preparation of liposome suspension was carried out in similar way as described above, to provide a final lipid concentration of 28.9 mg/ml and drug concentration of 5 mg/ml.

Table 2
Effect of method of preparation on encapsulation efficiency and particle size

Method	Liposomal product	% Entrapped (after 6 h shaking)[a]	Mean particle size (μ)[b]
Keeping round-bottomed flask stationary (A)	Empty	–	2.629 ± 0.145
	Drug loaded	5.48 ± 0.98	2.919 ± 0.036
Using rotary flash evaporator (B)	Empty	–	0.454 ± 0.029
	Drug loaded	21.8 ± 0.72	0.424 ± 0.000

[a] Each value is mean of three determinations.
[b] Each value is mean of two determinations.

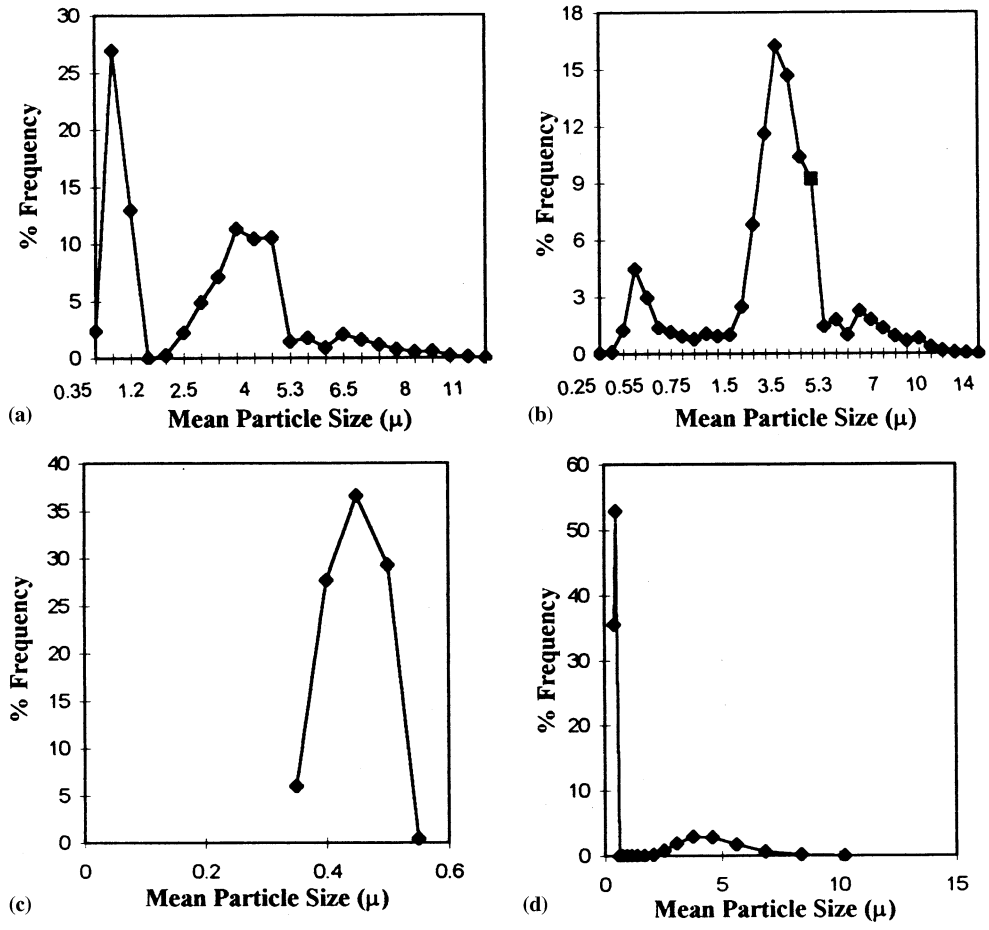

Fig. 1. Particle size distribution of liposomal dispersions: (a) empty liposomes (Method A); (b) drug-loaded liposomes (Method A); (c) empty liposomes (Method B); and (d) drug-loaded liposomes (Method B).

Positively charged liposomes were prepared by incorporating stearylamine in lipid phase.

2.3. Characterization of liposomal dispersion

2.3.1. Determination of encapsulation efficiency

The liposomal suspension was suitably diluted with buffer and centrifuged at 15 000 rpm for 30 min in Remi R-24 research centrifuge. The supernatant was analyzed for drug content at 257 nm on a Shimadzu UV-120 spectrophotometer, and encapsulation efficiency was calculated as a fraction of drug in the liposome pellet expressed as a percentage of total drug content.

Encapsulation efficiency was determined before shaking and after 3 and 6 h of shaking to study the effect of shaking on encapsulation.

2.3.2. Particle size measurement

The mean particle size and size distribution of liposomal dispersions were studied using a Shimadzu SALD-2001 laser counter.

2.3.3. Transmission electron microscopy

A small aliquot of the liposomal dispersion was negatively stained using uranyl acetate. The samples were examined using a $JEOL_{JEM}$ 100S electron microscope employing an accelerating voltage of 80 keV.

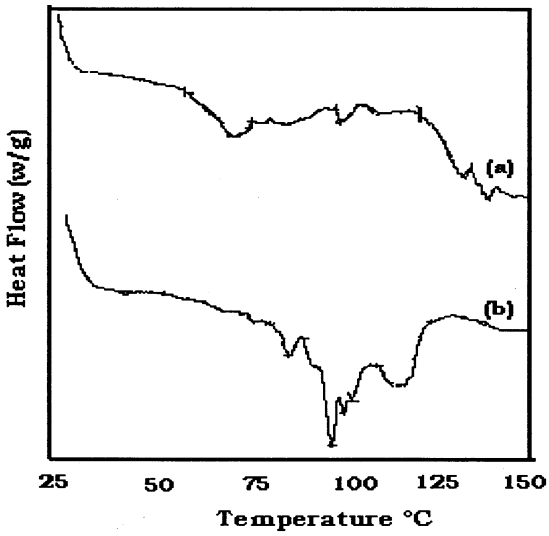

Fig. 2. Differential scanning thermograms of (a) empty liposomal dispersion and (b) drug-loaded liposomal dispersion.

Table 3
Precorneal clearance of tropicamide formulations (mean ± S.E.)

		DTPA solution	Liposomes
Cornea	% Remaining (600 s)	10.17 ± 3.04	11.1 ± 1.47
	AUC relative to solution	1.00 ± 0.26	1.58 ± 0.18
	k_{d1} ($min^{(1)}$	2.07 ± 0.16	2.99 ± 0.86
	$t_{0.5}$ (min)	0.34 ± 0.03	0.22 ± 0.07
	k_{d2} ($min^{(1)}$	0.06 ± .0.01	0.06 ± 0.01
	$t_{0.5}$ (min)	13.2 ± 3.0	11.9 ± 0.01
Inner canthus	AUC relative to solution	1.00 ± 0.79	1.14 ± 0.24

2.3.4. Differential scanning calorimetry (DSC)

DSC was performed by heating the samples in sealed aluminium pans from 30 to 150°C at a rate of 10°C/min.

2.3.5. Stability of the liposomal dispersions

The empty and tropicamide-loaded liposomal dispersions were stored at 4 and 25°C. They were observed visually as well as for microscopic appearance at the end of 15, 30, 45, 60, 90 and 120 days from the date of preparation.

2.3.6. ^{99m}Technetium-liposome radiolabelling

A ^{99m}Tc-liposome radiolabelling technique based on the method of Farr et al. (1985) was used. Briefly, 0.5 ml of the liposomal dispersion (28.9 mg/ml), 70 MBq of [^{99m}Tc]pertechnetate and 200 μl of 1 mg/ml solution of stannous chloride in deoxygenated water were mixed simultaneously and vortexed. No free pertechnetate was found. The mixture was then allowed to stand for 20 min at room temperature and radiochemical purity (RCP) was determined using instantaneous TLC and paper chromatography using acetone and saline, respectively, as solvent systems. RCP was found to be greater than 95% and of suitable stability for more than 2 h.

2.3.7. Assessment of the precorneal clearance of liposomal dispersion

Precorneal drainage of liposomes was assessed by γ-scintigraphy in a group of three male NZW rabbits (3–4 kg) by the method of Davies et al. (1991) using a 20-μl dose of each preparation. The precorneal clearance was measured using a Elscint

Table 4
Pupil dilation study of tropicamide formulations (mean ± S.E.)[a]

	Drug solution	Drug in gel	Liposome	Positively charged liposomes	Liposomes dispersed in gel
IR_t (max)	0.19 ± 0.05	0.19 ± 0.81	0.18 ± 0.03	0.16 ± 0.75	0.18 ± 0.01
IR_t (6 h)	0.01 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.06 ± 0.01	0.03 ± 0.01
AUC (0–6 h)	54.70 ± 14.69	66.34 ± 3.11	47.89 ± 10.61	64.08 ± 3.1	68.77 ± 7.17
t (max)	15	15	20	30	30

[a] IR_t (max), maximum response intensity; IR_t (6 h), mydriatic response intensity at 6 h of instillation; AUC (0–6 h), area under mydriatic response intensity–time graph from 0 to 6 h (min); t (max), time to reach maximum mydriatic response intensity (min).

409A γ-camera attached to a 4-mm pinhole collimator and tuned to detect γ radiation of energy within the range of 140 ± 10% keV. The test animals were held stationary in restraining boxes and positioned such that the eye was 4 cm from the collimator aperture. This allowed for the acquisition of a clear image situated within a predetermined area in which the radiation count rate variation is less than 10% for a uniform distribution of activity. A total of 20 μl of labeled liposomal dispersion was instilled into the lower fornix of the conjunctival sac, and the eye was manually blinked to distribute the solution over the cornea. A dynamic protocol of 3 s/frame was employed, followed by a static image taken after 600 s postinstillation. The images were stored on the dedicated camera computer for further analysis.

For image analysis, three anatomical regions of interest (ROI) were defined: cornea, inner canthus and lacrimal duct, together with a fourth region, which encompassed the total area of observation. To correlate the data, the activity associated with each ROI in each new frame of study was normalized to the activity associated with the new total area of observation.

^{99m}Tc-labelled diethylenetriaminepentaacetic acid ([^{99m}Tc]DTPA) solution was used as a standard for comparison.

2.3.8. Pupil dilation studies

Sterile aqueous solution containing 0.1% (w/v) tropicamide was prepared in isotonic buffer. The neutral liposomal dispersion, freshly prepared, was centrifuged to form lipid plug. The supernatant was completely removed and the lipid plug retained was stored, inverted, at 4°C. The lipid plug was resuspended in sterile buffer or dispersed in sterile Noveon™ AA-1 polycarbophil gel (pH 7) to give a final gel formulation containing 0.1% (w/v) tropicamide entrapped within vesicles and 0.25% (w/v) of Noveon™ AA-1. Positively charged liposomes were centrifuged, treated in a similar way and lipid plug was suspended in sterile buffer. Sterile Noveon™ AA-1 polycarbophil gel (pH 7) containing 0.1% (w/v) tropicamide was also formulated.

Tropicamide produces a rapid mydriatic response and therefore bioavailability was assessed by measuring pupil diameter. All studies were conducted in a crossover manner on three unanesthetized preconditioned male NZW rabbits (3–4 kg), with a rest period of not less than 1 week allowed for each rabbit between successive studies. The test animals were positioned in restraining boxes in the normal upright position in a room with constant light intensity and devoid of distractions. All rabbits were acclimatized to the laboratory testing conditions for 30 min prior to initiating the study.

Baseline pupil diameter measurements were taken every minute for 5 min prior to dosing. A 20-μl dose of each preparation was instilled into the lower fornix of the conjunctival sac and the eye was manually blinked. Experiments were repeated in cases where spillage of solution onto the eyelashes or eyelids was evident. Pupil diameter measurements were made on treated eyes at various time intervals postinstillation. The relative mydriatic response intensities at time t (IR_t) for each formulation were calculated by the equation $IR_t = (I_t - I_o)/I_o$, where I_o is average baseline diameter and I_t the pupil diameter at time t. Activity parameters were calculated as described by Davies et al. (1991) and subjected to statistical analysis employing ANOVA and paired t-test for variability ($P < 0.05$).

3. Results and discussions

The data in Tables 1 and 2 showed the dependence of liposomal tropicamide entrapment on shaking time and method of preparation. Keeping the round-bottomed flask stationary (Method A) might have resulted in relatively thick film which gave liposomes with less entrapment of drug, i.e. 5.48 ± 0.98%, compared to entrapment efficiency of 21.8 ± 0.72% obtained in liposomes prepared using the rotary flash evaporator (Method B). Bangham (1982) has showed that the hydration and entrapping process is most efficient when the film of dry lipid is kept thin.

The entrapment efficiency was increased to 21.8 ± 0.72% after 6 h of shaking, as shown in Table 1. It is reported that the time allowed for hydration and conditions of agitation are critical

in determining the amount of aqueous buffer (or drug solution) entrapped within the internal compartments of MLV (Martin, 1990). The entrapment efficiency for positively charged liposomes was found to be $24.64 \pm 0.52\%$.

The data in Table 2 show the effect of method of lipid film formation on mean particle size and particle size distribution. The thin film formed by Method B showed marked decrease in mean particle size and also gave unimodal particle size distribution for drug-loaded liposomes (Fig. 1d) and bimodal particle size distribution for empty liposomes (Fig. 1c), as compared to trimodal particle size distribution (Fig. 1a,b) for liposomal dispersions prepared by Method A. Incorporation of drug in liposomes did not have significant effect on mean particle size in either of the methods. Positively charged empty and drug-loaded liposomal dispersions showed increase in mean particle size of 2.83 ± 0.12 and 1.99 ± 0.05 μm, respectively. Increase in liposome size and size of the internal aqueous compartments was reported and was ascribed to the electrostatic repulsion of adjacent bilayers (Johnson, 1973). Transmission electron microscopy revealed the presence of spherical multilamellar vesicles in empty as well as drug-loaded liposomal dispersions.

DSC thermograms of empty liposomal dispersions containing phospholipids and cholesterol showed small endotherms from 50 to 100°C, with a major transition at 55°C (Fig. 2a). The melting endotherm of cholesterol was found to be shifted from 151 to 140.79°C, signifying that all lipid components interact with each other while forming the lipid bilayer.

The DSC thermogram of drug-loaded liposomal dispersion (Fig. 2b) interestingly showed transition at 72.5°C, indicating an increase in the phase transition temperature of liposomes upon loading with tropicamide. The tropicamide endotherm shifted slightly from 99.7 to 97.24°C, and cholesterol exhibited a significant shift of endotherm from 151 to 115.09°C, respectively. Positively charged liposomal dispersions showed a broad endotherm (81–129°C) showing good interaction of all components. The phase transition temperature can give good clues about liposomal stability, permeability and whether a drug is entrapped in the bilayer or in the aqueous compartment (Weiner et al., 1989).

Aqueous liposomal dispersions stored at 4 and 25°C showed sedimentation which, on slight shaking, redispersed easily. Reduction in percent encapsulation due to leakage and aggregation was 25% of initial value after storage of 1 month at 25°C. Microscopic observations revealed an increase in particle size. Mean particle size increased from an initial value of 0.424 to 2.668 ± 0.489 μm after storage of 2 months at 4°C. There was no perceptible change in color of dispersions stored at 4 and 25°C.

The precorneal clearance studies demonstrated that liposomal dispersion behaves in a similar manner to the solution, the precorneal drainage being a biphasic phenomenon with an initial rapid phase (30–60 s) followed by a much slower basal drainage phase. Kinetic analysis of the corneal drainage phase gives rise to two drainage rate constants, of which the initial drainage rate constant is two orders of magnitude greater than the basal drainage rate constant (Table 3). However no statistical difference was found in the rate constants of solution and liposomal dispersion (ANOVA, $P < 0.05$). Calculation of the corneal AUC (0–600 s) was taken as a measure of the corneal residence of the instilled solution. Analysis of variance (5% confidence limits) of the AUC demonstrated that the precorneal retention of the liposomes was not significantly different than that of aqueous solution. This indicated that the potential of liposomes in ocular drug delivery is limited by their rapid clearance from the precorneal region.

Fig. 3 compares the pupil dilatory effect of tropicamide from an aqueous solution, gel, drug-loaded liposomes, positively charged drug-loaded liposomes and vesicles dispersed in gel. The mydriatic response intensity curves are of similar shape for all formulations. The main activity parameters are summarized in Table 4. The area under the mydriatic response intensity–time curve (AUC) for each formulation is of particular importance, as it is indicative of the bioavailability of tropicamide from each formulation. The bioavailability of tropicamide from liposomes was equivalent to that of solution

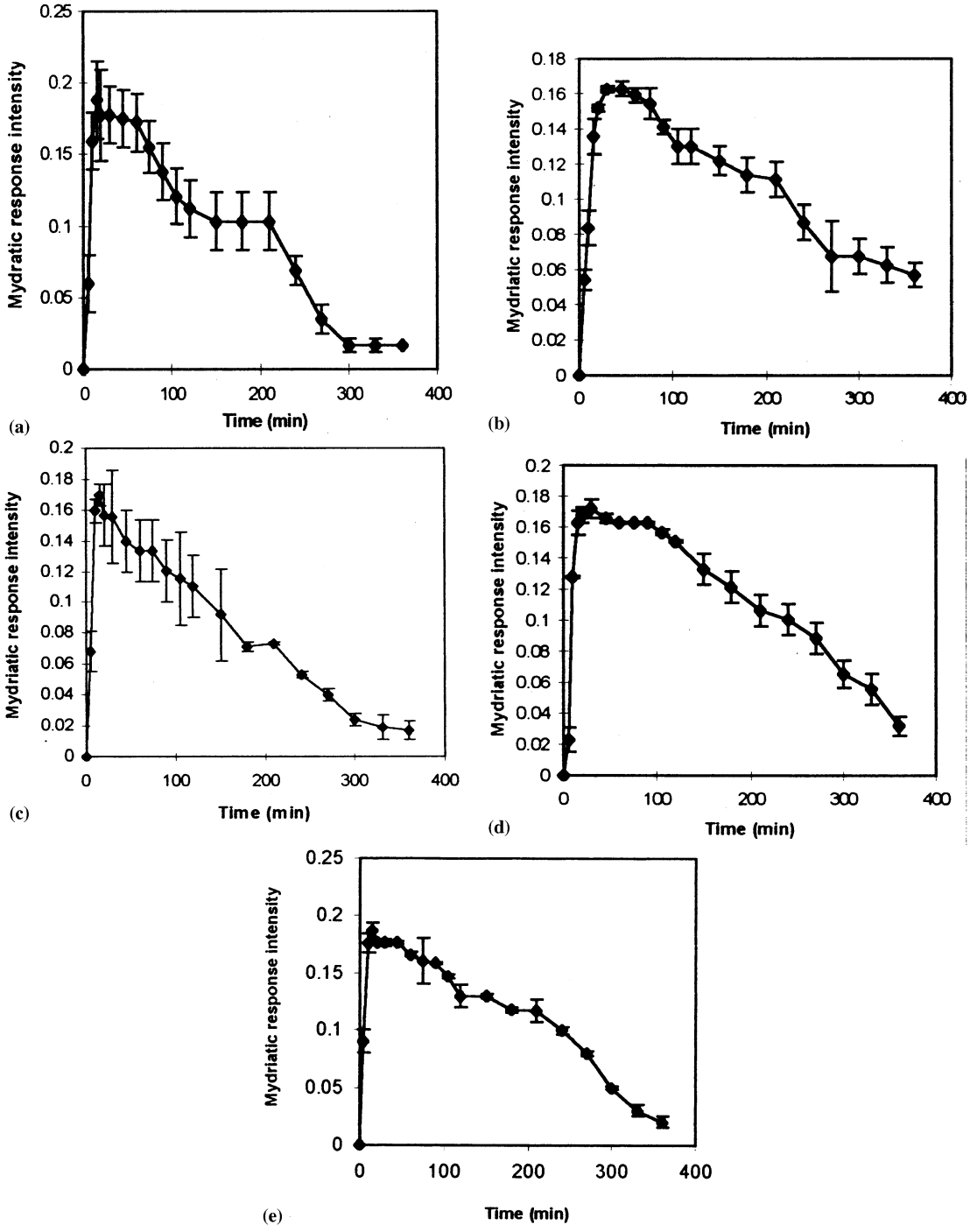

Fig. 3. Mydriatic response intensity elicited by tropicamide formulations (mean ± S.E.): (a) aqueous solution; (b) neutral liposomal dispersion; (c) positively charged liposomal dispersion; (d) liposomes dispersed in gel; and (e) free drug in gel.

which may be due to lack of specificity of liposomes for the cornea.

As liposomes were not retained in the eye for a sufficiently long time to exhibit prolonged drug action, liposomes were formulated into a gel and, as another approach, positive charges were incorporated into liposomes.

Statistical analysis (ANOVA) revealed that AUC for positively charged liposomes and liposomes dispersed in gel were significantly different than drug-loaded liposomes ($P < 0.05$). t_{max} and IR_t (6 h) were found to have increased for both formulations. Interestingly, higher standard deviations in AUC values were noted for drug solution and dispersion containing neutral liposomes. Incorporation of positive charge and increase in viscosity resulted in more consistent and higher values of AUC.

Positively charged vesicles are reported to exhibit a prolonged precorneal retention (Fitzgerald et al., 1987). At physiological pH, the corneal epithelium is negatively charged and thus, in the case of positively charged vesicles, electrostatic attraction may enhance adsorption. Studies have shown that positively charged vesicles can enhance the bioavailability of entrapped drug over neutral or negatively charged vesicles. The literature suggests that ocular delivery via liposomes may benefit lipophilic compounds to a greater extent than hydrophilic compounds (Meisner et al., 1989). The capacity of positively charged vesicles, to exhibit prolonged corneal retention by adsorption onto the corneal surface, would be expected to result in delayed ocular absorption of drug which would exhibit prolonged efflux rates.

It is well documented that the precorneal residence of ophthalmic solutions can be promoted by the inclusion of viscosity-enhancing polymers. This approach may, therefore, prolong the precorneal retention of vesicle formulations. Liposomes containing tropicamide when dispersed in polycarbophil gel were found to be more effective in duration of drug action than drug-loaded neutral liposomes. However, no statistically significant difference was found when AUC of tropicamide gel was compared with that of vesicles dispersed in gel. Increase in viscosity rather than entrapment in vesicles appears to have increased AUC of gel due to longer precorneal residence. Fitzgerald (1985) studied the in vivo precorneal clearance rates of SUVs and MLVs in the presence of 0.45% (w/v) HPMC and 3.0% (w/v) poly(vinyl alcohol) solutions in which vesicles suspended in polymer solutions were retained on the corneal surface for a significantly longer period than those suspended in buffer.

4. Conclusion

The process of 'lipid film hydration' resulted in formation of multilamellar, spherical liposomes of tropicamide. The method of preparation and shaking time resulted in marked difference in percentage encapsulation in spite of identical lipid concentrations and compositions. γ-Scintigraphic studies confirmed that the practical limitation of neutral liposomes of tropicamide in ocular drug delivery is lack of specificity in the cornea. Increase in viscosity by formulating a gel resulted in prolonged drug action. Positively charged liposomal formulation was found to be more effective than drug-loaded neutral liposomes when pupil dilation data was statistically treated at 5% level.

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