J. Pharm. Pharmacol. 1996, 48: 801–805
Received October 2, 1995
Accepted March 3, 1996

The Preparation of Norfloxacin-loaded Liposomes and their In-vitro Evaluation in Pig's Eye

HUNG-HONG LIN, SHIU-MAN KO, LI-REN HSU* AND YI-HUNG TSAI

*School of Pharmacy, Kaohsiung Medical College, Taiwan, and *Department of Pharmacy, Chia-Nan College of Pharmacy, Taiwan, Republic of China*

Abstract

The effectiveness of norfloxacin as an antibacterial agent in ophthalmology is limited by poor drug delivery and limited ocular bioavailability. Liposomes containing norfloxacin have been prepared from different phospholipids using a novel technique with an encapsulation efficiency sixteen times greater than that of a conventional film method. The in-vitro release of the norfloxacin and the transcorneal characteristics of the liposomes have been evaluated. Differential scanning calorimetry was used to determine the interaction occurring between liposomes and cornea.

The release of liposome-entrapped norfloxacin was affected by the pH of the environment. In the in-vitro corneal perfusion studies, norfloxacin-loaded liposome was transferred through the cornea at a slower rate than was the free drug. Norfloxacin-loaded liposomes were accumulated primarily in the cornea. The drug corneal retention of the lipids increased in the order dimyristoyl-L-α-phosphatidylcholine < dipalmitoyl-L-α-phosphatidylcholine < distearoyl-L-α-phosphatidylcholine. In the corneal drug-elimination study, liposomal norfloxacin increased the loading of the drug in cornea; the maximum value of the loading occurred 5 h after dosing. The drainage of liposomes from the cornea was somewhat slower than the solution form.

Accumulation of norfloxacin in the cornea was greater for the liposome-entrapped drug. The results suggest that norfloxacin-loaded liposomes are absorbed by the cornea via endocytosis.

Norfloxacin is a 4-quinolone antibacterial agent with widespread action against Gram-negative microorganisms. In ophthalmology it is used to treat pyogenic inflammations of the eye (e.g. conjunctivitis, keratitis, blepharitis and postoperative infections; Holmes et al 1985). A major problem in ophthalmic drug delivery is poor ocular bioavailability (< 10% of the applied dose; Tang-Liu et al 1987) and the limited rate of corneal absorption. Because the cornea is a membrane containing both lipophilic and hydrophilic layers, it can be effectively permeated by drugs such as norfloxacin with both lipophilic and hydrophilic characteristics. The pH of the instilled solution is known to affect the ocular absorption of weak basic drugs (Sieg & Robinson 1977; Kyyronen & Urtti 1990). It is advantageous for corneal permeation to adjust the pH of the solution to increase the proportion of un-ionized drug in the instilled dose. The aqueous solubility of un-ionized norfloxacin is, however, low and when its solubility was increased by conversion to the ionized form, it would not readily permeate the cornea.

The process of ocular drug uptake may be modified by the physical properties of the vehicle in which the drug is placed. Because liposomes are vesicle-like structures with a concentric series of alternating compartments of aqueous regions and phospholipid bilayers, they can entrap both lipophilic and hydrophilic compounds. One recent application of liposomes is as drug carriers in ophthalmology (Meisner & Mezei 1995). Administration of the liposome-encapsulated compounds proved to be more effective than the same therapeutic regimen of drug solution (Dharma et al 1986). For this reason, liposomes have gained considerable attention for topical ocular drug delivery.

Correspondence: Yi-Hung Tsai, School of Pharmacy, Kaohsiung Medical College, Kaohsiung, Taiwan, Republic of China.

Liposomes can be prepared by several different methods (Babgham et al 1965; Szoka et al 1980; Talsma et al 1994); each provides vehicles with special characteristics and different encapsulation efficiencies; the percentage encapsulation is extremely low if the drug is entrapped as a solution in the aqueous phase. In this study, we have attempted to develop a method which combines the ethanol injection method (Pons et al 1993) with freeze-drying to increase liposome encapsulation efficiency.

Materials and Methods

Materials

Norfloxacin, dimyristoyl-L-α-phosphatidylcholine (DMPC), dipalmitoyl-L-α-phosphatidylcholine (DPPC) and distearoyl-L-α-phosphatidylcholine (DSPC) were purchased from Sigma Chemical Co. (St Louis, USA). All other reagents and solvents were of analytical or equivalent grade.

Preparation of liposomes

Two different procedures were used to prepare the liposomes.

Method A. Liposomes were prepared by the orthodox 'film method'. The desired amount of lipid in absolute ethanol was applied to the wall of a round-bottomed flask as a thin film by removing the ethanol by rotary evaporation. The lipid film was hydrated with a solution of norfloxacin in pH 5·0 phosphate-buffered saline (PBS). The mixture was gently shaken for 30 min and was then sonicated in an ultrasonic bath (Elma, Germany) above the phase-transition temperature of the lipid materials for 10 min under a nitrogen atmosphere.

Method B. Liposomes were prepared by a modification of the ethanol injection technique. Norfloxacin was added directly to

an ethanolic solution of the lipid and the solution was then rapidly injected into a fivefold greater volume of magnetically stirred phosphate-buffered saline. The liposomal dispersions were frozen in a dry ice-acetone bath and were dried by means of a freeze dryer (Labconco, Kansas, USA). The lyophilized powder was then rehydrated with double-distilled water. After mild vortex mixing the flask was left at room temperature for 30 min before use. The total lipid concentration was adjusted to 9·2 mM.

Assay of entrapped norfloxacin

The norfloxacin-containing liposomes were separated from untrapped norfloxacin by filtering the liposome dispersion under vacuum (5 mmHg) through a 0·025 μm filter (Millipore, MA, USA); the liposomes were then washed with phosphate-buffered saline to complete the removal of the free drug.

The amount of entrapped norfloxacin was determined by lysis of the liposomes with absolute ethanol. The liposome dispersions were mixed with an equal volume of absolute ethanol to give a clear solution and the norfloxacin concentrations were estimated by HPLC. The amount of norfloxacin entrapped in the liposomes was expressed as encapsulation efficiency in μmol norfloxacin per μmol phospholipid.

Size distribution determination

Light-scattering measurements were performed with a Coulter submicron particle-size analyser (Model N4MD). The liposome preparations were diluted 1 : 20 with filtered PBS (pH 5·0). The instrument settings were: temperature, 25°C; viscosity, 0·01 P; refractive index, 1·333; scattering angle, 90°; run time, 200 s; range, approximately 0–3000 nm.

Release from liposomes

After the separation of the free drug, the liposomes were diluted 1 : 100 with PBS (pH 5·0 or pH 7·6). The liposome dispersion was transferred to test tubes with ground-glass stoppers which were then immersed in a thermostatted (37°C) water bath with mild mechanical shaking. At timed intervals samples were removed from the thermostatted bath. Each sample was immediately extruded through filters under vacuum. The concentration of norfloxacin was estimated spectrophotometrically at 275 nm. Each experiment was performed three times. The release of norfloxacin from liposomes was recorded continuously for 48 h.

In-vitro perfusion studies

Horizontal-type diffusion cells were similar to the apparatus of the Franz diffusion assembly (Chien et al 1983). The corneas used as the membrane for penetration experiments were excised from pigs (L.Y.D., hybrid), 10–12 months. Within 1 h of the death of the animal, the eyeballs were excised at the slaughterhouse and transported to the laboratory in ice-cooled GBR buffer, pH 7·65 (O'Brien & Edelhauser 1977). The solution was used throughout the penetration studies and prepared in two parts. The first part contained sodium chloride (12·4 g L^{-1}), potassium chloride (0·716 g L^{-1}), monobasic sodium phosphate monohydrate (0·206 g L^{-1}) and sodium bicarbonate (4·908 g L^{-1}); the second part contained calcium chloride dihydrate (0·23 g L^{-1}), magnesium chloride hexahydrate (0·318 g L^{-1}), glucose (1·8 g L^{-1}), and oxidized glutathione (0·184 g L^{-1}). Equal volumes of both solutions were mixed before use.

The cornea was mounted between two half-diffusion cells. The area available for diffusion was 0·785 cm^2 and the volume of liposomal solution in the donor cell was 6·5 mL. The receiver compartment was filled with GBR buffer and agitated by magnetic stirring at 700 rev min^{-1}. The system was oxygenated with carbogen (95% O_2, 5% CO_2) throughout the experiment. The apparatus was maintained at 37 ± 0·5°C with a water jacket. Samples (0·4 mL) were withdrawn from the receiver compartment at appropriate times and the concentration of norfloxacin was determined by HPLC. After each sampling, the same volume of fresh phosphate buffer was added to the receiver compartment to keep the volume constant.

Measurement of the residual amount of norfloxacin within the cornea

The amount of norfloxacin remaining in the cornea after the application or the removal of liposomal preparations was determined after careful removal of the corneas from the diffusion cells and rapid washing several times with distilled water. The cornea samples were weighed and homogenized in phosphate buffer solution (pH 3·0) by means of a tissue homogenizer. The homogenate (total volume 5·0 mL) was centrifuged and the supernatant was filtered through a 0·45 μm pore-size nylon membrane (Msi. Micro Separation Inc.) and analysed by HPLC.

High-performance liquid chromatography

The amount of norfloxacin in each sample was determined by high-performance liquid chromatography with pipemidic acid as internal standard. The conditions were: pump, model L-6000 (Hitachi); column, 4·6 × 250 mm Spherisorb C_{18} (Phase Separations Ltd, UK); mobile phase, 0·015 M tetrabutylammonium iodide solution-acetonitrile (95 : 5, v/v); UV detector, model L-4000 (Hitachi); wavelength, 275 nm; flow rate, 1 mL min^{-1}. Peak areas were determined by means of a model D-2500 Chromato-integrator (Hitachi).

Differential scanning calorimetry (DSC)

Differential scanning calorimetry was used to establish structure-property relationships for the cornea at the molecular level. DSC thermograms were obtained with a Perkin-Elmer DSC7 differential scanning calorimeter. Cornea sample sizes were in the range 6–9 mg and were sealed in volatile type aluminium pans. Thermograms were recorded from 20 to 120°C at a scan rate of 5°C min^{-1}.

Results and Discussion

Some amphiphilic drugs, e.g. the quinolones, can be entrapped in liposomes with high encapsulation efficiencies by the ethanol injection method. We chose the route which combined this method and freeze drying. It produces liposomes free from ethanol residues and is easily subjected to sterilization and industrial scale-up. The liposomes prepared by freeze drying had, furthermore, higher encapsulating capacity than those prepared by the ordinary film method (Ohsawa et al 1984). Particle size and encapsulation efficiency for methods A and B are compared in Table 1 for the DMPC bilayer matrix. The

Table 1. Particle-size distribution and encapsulation of norfloxacin in liposomes of different lipid composition prepared by different procedures.

Lipid composition*	Particle size (nm)	Encapsulation efficiency† (μmol norfloxacin/μmol lipid)
DMPC‡	250	0·010 ± 0·002
DMPC§	1090	0·159 ± 0·007
DPPC§	1410	0·149 ± 0·008
DSPC§	2230	0·139 ± 0·005

*DMPC, dimyristoyl-L-α-phosphatidylcholine; DPPC, dipalmitoyl-L-α-phosphatidylcholine; DSPC, distearoyl-L-α-phosphatidylcholine. †Each value is the mean ± s.d., n = 3. ‡Liposomes prepared by method A. § Liposomes prepared by method B. Both methods were performed at room temperature with an initial drug : lipid ratio of 0·2.

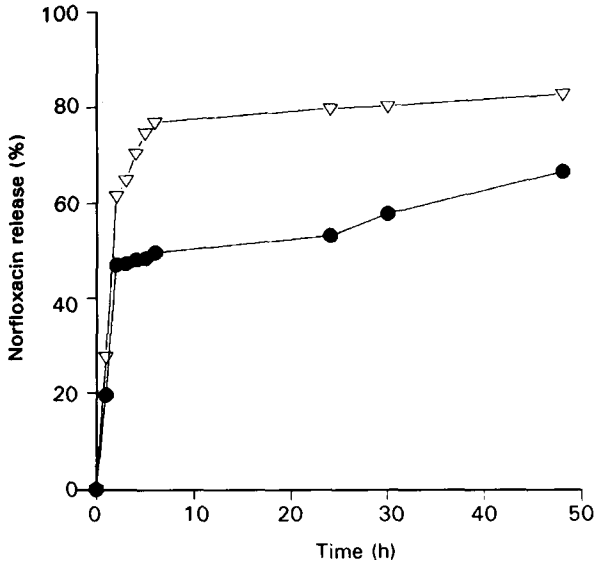

FIG. 1. In-vitro release profiles of norfloxacin from DMPC liposome prepared using method B: ▽ pH 7·6, ● pH 5·0 (n = 3).

'film' method (method A) is by far the simplest method for preparing liposomes. Entrapment of norfloxacin in the resulting liposomes is relatively low (10% or less). A drug : DMPC molar ratio of 1 : 5 yielded a mean particle size ranging from 160 to 340 nm. Greater encapsulation efficiency was obtained by method B; it is apparent that the liposomes prepared by method B have a larger mean diameter. The encapsulation efficiency of the method is, moreover, 16-fold that of method A. Norfloxacin is an amphiphilic drug. Under pK_{a1} it will dissolve in water and be encapsulated within the aqueous compartment of liposomes prepared by the film method. In contrast, the drug is usually bound to the lipid bilayer, i.e. 'dissolved' in the lipid phase, in liposomes prepared by the ethanol injection-freeze drying method (Meisner & Mezei 1995). This might be the reason for the increased efficiency of encapsulation of method B. When norfloxacin was entrapped in liposomes prepared from DPPC or DSPC, the increased chain length of the lipids resulted in a significant increase in particle size but the efficiency of encapsulation was reduced.

The influence of environment pH on norfloxacin release was determined by diluting the liposomes with a large volume of

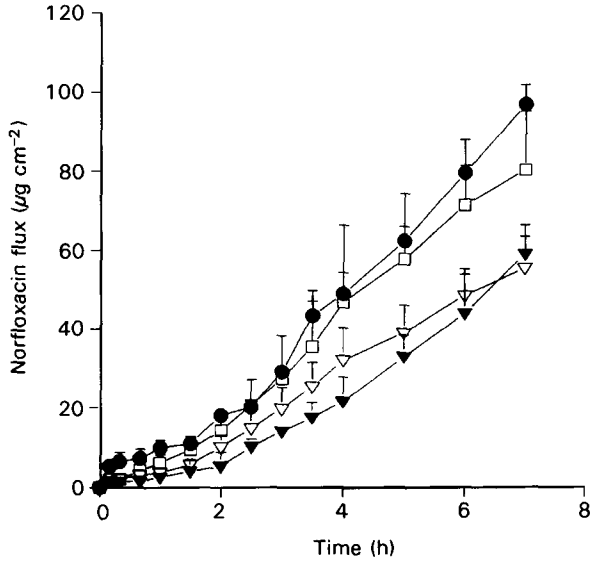

FIG. 2. Effect of liposome lipid composition on the diffusion of norfloxacin through isolated pig cornea: □ DMPC, ▽ DPPC, ▼ DSPC; ● norfloxacin buffer. Points and vertical bars represent means and s.d., respectively (n = 3).

pH 7·6 or pH 5·0 isotonic buffer solution. The results show that the rate of drug release at pH 7·6 is greater than at pH 5·0 (Fig. 1). The pK_{a1} and pK_{a2} values of norfloxacin are 6·30 and 8·55, respectively (Jaw 1991) and so this phenomenon could be attributed to the greater hydrophobic character of the norfloxacin at pH 7·6, which makes the permeation through the liposome membrane easier (Puglisi et al 1995). The release profiles of liposomes prepared from DPPC and DSPC were similar to those for DMPC (data not reported).

Although, because of easy handing and lower cost, laboratory rodents are more convenient than the pig for transcorneal studies, we selected the pig's eye as the animal model for in-vitro screening of liposomal ophthalmic formulations because the pig's eye appears to be a good animal model for the eye in man (Thijssen et al 1985). Fig. 2 shows the concentration of drug diffusing through the cornea at different time intervals after the use of a norfloxacin-loaded liposome dispersion or a solution of the free drug in buffer solution. The straight line of the curve was defined as the steady-state penetration. Linear regression analysis was used to determine the flux of the drug for each sample. The fluxes calculated from the penetration profiles, and the amount of norfloxacin remaining within the cornea are listed in Table 2 It can be seen that the liposome prepared from DMPC resulted in a higher corneal penetration rate of norfloxacin than those prepared from the other two lipids. The transcorneal flux of the norfloxacin-loaded liposome preparation was, however, significantly ($P < 0·05$) less than that of the free drug solution. Thus although liposome-entrapped norfloxacin was transferred through the cornea more slowly than the free drug, accumulation in the cornea was greater for the liposome-entrapped drug. For the three lipids investigated drug corneal retention decreased in the order DSPC > DPPC > DMPC. It should, however, be emphasized that the entrapped drugs are used solely for treatment of local infection, inflammation and similar diseases and that the entrapped drugs would induce side-effects on entering the

Table 2. Effect of lipid composition on corneal permeability and amount of norfloxacin remaining for 7 h.

Treatment*	Flux ($\mu g\ mL^{-1}\ cm^{-2}\ h^{-1}$)	Amount remaining ($\mu g\ g^{-1}$)
Aqueous solution	16·2 ± 1·6	5·1 ± 0·3
DMPC	13·8 ± 1·8	27·0 ± 3·9†
DPPC	10·5 ± 1·4	28·7 ± 3·3†
DSPC	9·3 ± 0·8†	39·9 ± 2·2†

Each value is the mean ± s.d., n = 3. *DMPC, dimyristoyl-L-α-phosphatidylcholine; DPPC, dipalmitoyl-L-α-phosphatidylcholine; DSPC, distearoyl-L-α-phosphatidylcholine. †Significantly different at an alpha level of 0·05 from that of aqueous solution.

anterior chamber. Ophthalmic liposome could, therefore, bring about preferential delivery of the entrapped drug to the cornea for maximum pharmacological action.

Because use of DSPC resulted in the most pronounced corneal retention of norfloxacin, it was selected for further study of the effect of phospholipid on norfloxacin remaining in the pig's cornea. Fig. 3 shows the time-course of changes in the amount of drug remaining in the cornea on application of norfloxacin ophthalmic solution with or without liposome encapsulation. It can be seen that the amount of norfloxacin accumulated in the cornea reaches steady values of approximately 5 $\mu g\ g^{-1}$ after dosing with 0·05% (w/v) free drug solution for 0·5 h. Norfloxacin levels were significantly higher in corneas treated with liposome-encapsulated drug; the maximum value occurred at 5 h then reached the steady state. The cornea was pretreated with liposomal and solution products for 12 h, then transferred into GBR buffer. The corneal elimination profiles obtained are depicted in Fig. 4. The results indicated that liposomal application increased the loading of the drug in the cornea and that drainage of the liposome-containing norfloxacin from the cornea was somewhat slower than for

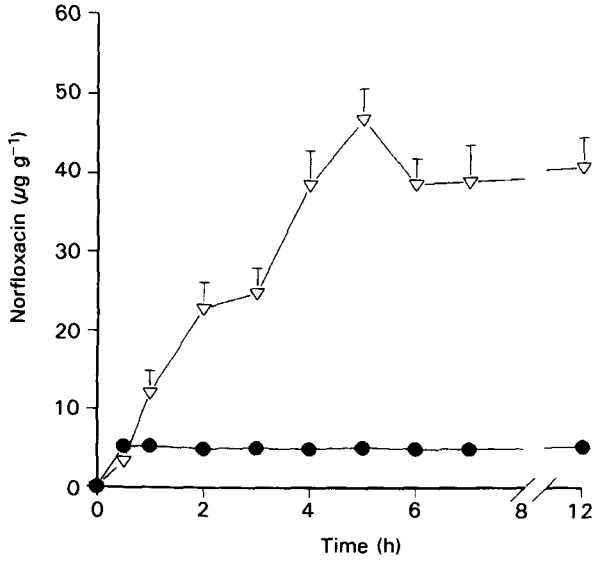

FIG. 3. Norfloxacin distribution in isolated pig cornea dosed with DSPC liposomal norfloxacin (▽) and aqueous norfloxacin solution (●). Points and vertical bars represent means and s.d., respectively (n = 3).

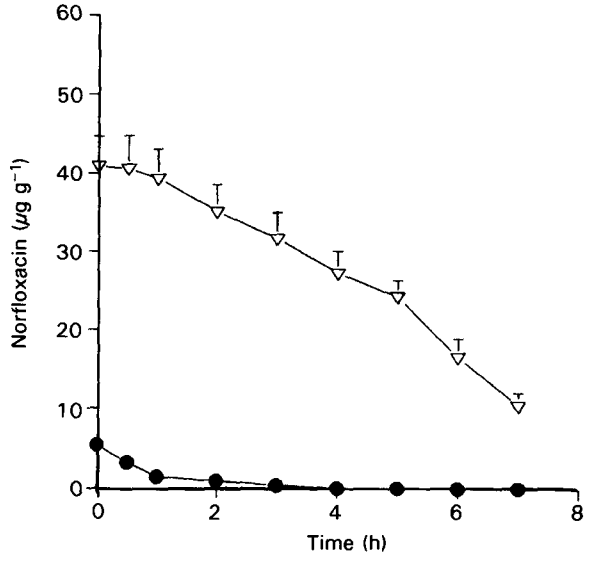

FIG. 4. Residual corneal levels of norfloxacin after pretreatment for 12 h with DSPC liposomal norfloxacin (▽) and aqueous norfloxacin solution (●), then removal. Points and vertical bars represent means and s.d., respectively (n = 3).

the solution form. Consequently, transfer of the entrapped drug to the cornea and the maintenance of pharmacological activity can be enhanced by incorporation into liposomes.

To elucidate the influence of the liposome on corneal structure, differential thermal analysis (DSC) was used to establish structure-property relationships at the molecular and macromolecular level. The lowest trace in Fig. 5 was obtained by treating GBR buffer as a control. One major transition was observed at 56·9°C. Although the presence or absence of norfloxacin had no noticeable effect on the ΔH values of the two corneas, the T_m values of corneas treated with free or liposome-entrapped norfloxacin shifted markedly towards higher temperatures in comparison with the control. According to previous studies (Pagans & Weinstein 1978) the mechanisms of in-vitro liposome-cell interaction include stable absorption, lipid exchange or transfer, fusion and endocytosis. Treatment with liposomes of different phospholipid composi-

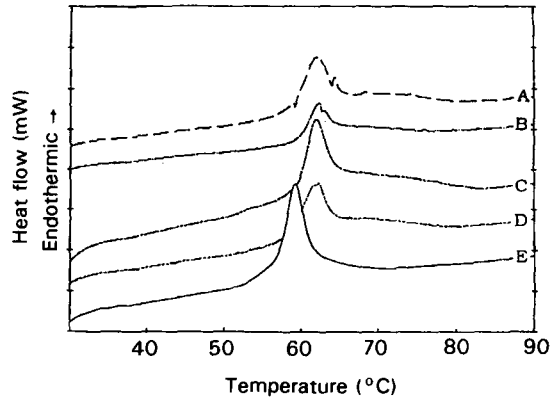

FIG. 5. DSC thermograms of pig cornea after treatment with norfloxacin in A. DSPC liposome, B. DPPC liposome, C. DMPC liposome, D. GBR buffer solution and E. without norfloxacin (as control).

tion caused much alteration in the ΔH values of the cornea and in the shapes of the transition peaks. The liposomes must be transferred into the cornea to alter the constitution of the cells, thereby increasing retention and sustaining the release of drug. Norfloxacin-loaded liposomes are, therefore, probably be taken up by the cornea via endocytosis.

Acknowledgement

The work was partly supported by a grant from Cheng's Pharmaceutical Sciences Foundation, Taipei, Taiwan, Republic of China.

References

Babgham, A. D., Standish, M. M., Watkins, J. C. (1965) Diffusion of univalent ions across the lamellae of swollen phospholipids. J. Mol. Biol. 13: 238–252

Chien, Y. W., Keshary, P. R., Huang, Y. C., Sarpotdar, P. P. (1983) Comparative controlled skin permeation of nitroglycerine from marketed transdermal delivery system. J. Pharm. Sci. 72: 968–970

Dharma, S. K., Fishman, P. H., Peyman, G. A. (1986) A preliminary study of corneal penetration of ^{125}I-labelled idoxuridine liposome. Acta Ophthalmol. 64: 298–301

Holmes, B., Brogden, R. N., Richard, D. M. (1985) Norfloxacin: a review of its antibacterial activity, pharmacokinetic properties and therapeutic use. Drugs 30: 482–513

Jaw, Y. I. (1991) Transdermal delivery of the quinolone antibiotics by ointment dosage forms. Master Thesis; Kaohsiung Medical College

Kyyronen, K., Urtti, A. (1990) Effects of epinephrine pretreatment and solution pH on ocular and systemic absorption of ocularly applied timolol in rabbits. J. Pharm. Sci. 79: 688–691

Meisner, D., Mezei, M. (1995) Liposome ocular delivery systems. Adv. Drug Delivery Rev. 16: 75–93

O'Brien, W. J., Edelhauser, H. F. (1977) The corneal penetration of trifluorothymidine, adenosine, arabinoside and idoxuridine: a comparative study. Invest. Ophthalmol. 16: 1093–1103

Ohsawa, T., Miura, H., Harada, K. (1984) A novel method for preparing liposome with a high capacity to encapsulate proteinous drugs: freeze-drying method. Chem. Pharm. Bull. 32: 2442–2445

Pagans, R. E., Weinstein, J. N. (1978) Interaction of liposomes with mammalian cells. Ann. Rev. Biophys. Bioeng. 7: 435–468

Pons, M., Foradada, M., Estelrich, J. (1993) Liposomes obtained by the ethanol injection method. Int. J. Pharm. 95: 51–56

Puglisi, G., Fresta, M., Mazzone, P. M. (1995) Formulation parameters of fluoroquinolone-loaded liposomes and in vitro antimicrobial activity. Int. J. Pharm. 118: 65–76

Sieg, J. W., Robinson, J. R. (1977) Vehicle effects on ocular drug bioavailability II: evaluation of pilocarpine. J. Pharm. Sci. 66: 1222–1228

Szoka, F., Olson, F., Heath, T. (1980) Preparation of unilamellar liposomes of intermediate size (0·1–0·2 μm) by a combination of reverse phase evaporation and extrusion through polycarbonate membrane. Biochim. Biophys. Acta. 601: 559–571

Talsma, H., Steenbergen, M. J., Borchert, J. C. H., Crommelin, D. J. A. (1994) A novel technique for the one-step preparation of liposomes and nonionic surfactant vesicles without the use of organic solvents. Liposome formation in a continuous gas stream: the 'bubble' method. J. Pharm. Sci. 83: 276–280

Tang-Liu, D. D.-S., Liu, S., Neff, J., Sandri, R. (1987) Disposition of levobunolol after an ophthalmic dose to rabbits. J. Pharm. Sci. 76: 780–783

Thijssen, J. M., Mol, H. J., Timmer, M. R. (1985) Acoustic parameters of ocular tissues. Ultrasound Med. Biol. 11: 157–161