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Original Article

Enhanced Corneal Permeation of Pilocarpine Using Liposome Technology

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ABSTRACT

Background: A novel liposomal pilocarpine formulation as an ophthalmic drug delivery system has been designed to treat patients with glaucoma. The purpose of the present study was to formulate and evaluate liposomes loaded with pilocarpine and to evaluate permeation through rabbit cornea.

Method: Liposomes containing pilocarpine were prepared using thin film method. The quantities of soya lecithin and cholesterol were changed to enhance the encapsulation of the drug. The physicochemical properties of the prepared liposomes were evaluated according to their viscosity, pH, particle size, in vitro drug release, and transcorneal rabbit permeation. Dialysis membrane method was utilized to assess drug release profile.

Results: The results indicated that the mean particle sizes of liposomes were 120.5-212 nm and the pH and viscosity of formulations were in the range of 6.30-6.63 and 43.85-80.1 cps, respectively. According to the release study results, maximumally 60% of the drug released from liposomal formulations after 24 hours of the experiment. Also, the cumulative percentage of the drug permeated through rabbit cornea was differing from 3.86 to 14.9%. Irrespective from the composition and characteristics of the different liposomal formulations, they significantly increased the drug partitioning, permeability coefficient and flux of pilocarpine in rabbit cornea ex vivo model in comparison to control drug solution.

Conclusion: The present study proved that any alteration in composition and nature of pilocarpine liposomal formulations may affect the drug permeability parameters through corneal membrane and also physico-chemical properties. It is probably due to the change in corneal structure in the presence of different liposomes composition.

Keywords: Corneal Permeability, Liposome, Pilocarpine, Rabbit

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Introduction

The human eye is a complex and unique organ with advanced anatomical structure and various physiological barrier against drug permeation for example pre-corneal and corneal barriers, the conjunctival barriers, the blood–aqueous barrier, and the blood-retinal barrier; hence, ocular drug delivery is a major challenging task for scientists and clinicians (1, 2). There are too many administration routes for the treatment of patients with eye problems (2). The common method for anterior segment disease is topical administration, such as ophthalmic ointments and drops. The main eye drops problem is that they rapidly drained away from pre-corneal cavity by tear flow and washout into the nasolacrimal duct due to eye's barriers and the tear film (3). Finally, 1 to 5 percent of the administered drug can be absorbed into the target tissue (7). For these reasons, new drug delivery strategies like nanoparticle-base products have been developed (3).

Pilocarpine hydrochloride is among nonselective muscarinic receptor agonists (4). Pilocarpine eye drops are widely used in the treatment of patient with open-angle glaucoma because of the ability to decrease intraocular pressure by increasing facility of trabecular outflow (5). pilocarpine eye drops has several problems including short half-life (should be taken several time a day), short bioavailability and durability (due to wasting and poor permeation); therefore, high doses of pilocarpine should be administrated for patient to have therapeutic effects and this causes side effects such as ciliary spasm, eye pain, irritation and lacrimation (6).

Liposomes have been used as drug vehicles for many drugs with different applications (7). Liposome is a spherical vesicle formed from phospholipid bilayer membranes with 50-1000 nm diameter and into an enclosed pocket (8-10). It is very versatile and can carry hydrophilic or hydrophobic drugs (11-13). Hydrophobic drug molecules can be entrapped into the lipid bilayers while the inner aqueous core is the main site for encapsulation of hydrophilic drugs. Liposomes are important drug delivery systems because of their biodegradability and biocompatibility(14). Based on many researches, the benefits of using liposomes as drug carriers compared with eye drops, are high penetration efficiency of drug into ocular tissue (15, 16), long duration of action of the drug (17, 18), reduction of side effect and toxicity of drug in frequent administration (19, 20) and improvement of drug bioavailability (21-23). Liposomes are potentially able to make intimate contact with conjunctival and corneal surfaces, therefore increase the probability of ocular drug absorption.

This study was aimed to formulate pilocarpine HCL loaded liposome and evaluate its *in vitro* release and permeation behavior.

Materials and Methods

L-α-Phosphatidylcholine and cholesterol were obtained from Merck (Germany). Pilocarpine hydrochloride was provided from Sina Darou pharmaceutical Co. (Iran). All of the solvents were of the analytical grade. Fresh double distilled water was used in the experiments.

Animals

Male New Zealand white rabbits weighting 2.5 – 3.5 kg were used in the present study which was conducted with the approval of the animal ethics committee, Ahvaz Jundishapur University of Medical Sciences (permit No. 024).

Assay of Pilocarpine Hydrochloride

The amount of Pilocarpine HCl in experiments was determined by UV spectrophotometric method at 216 nm (24).

Liposome preparation

Thin film method was utilized to prepare the liposomes. Cholesterol and soy lecithin were accurately weighed and dissolved in chloroform as solvent, then the mixture was evaporated in a rotary evaporator. The thin film layer forming then was hydrated in phosphate buffer (pH 7.4) and added to the thin film for hydration .The suspension was shaken by vortex and then sonicated for definite times (5 and 15 min)(25, 26).

Particle size determination

The mean diameter and polydispersity index (PDI) of liposomes were measured at room temperature by SCATTER SCOPE 1 QUIDIX , South Korea apparatus.

pH determination

The pH of liposomal formulations was determined using a pH-meter (AG8603, Mettler Toledo, Switzerland). The instrument was calibrated using buffer phosphate pH 7.4 (27).

Viscosity Experiments

The viscosity of liposomes was determined at 25 ± 1 ˚C with a Brookfield viscometer apparatus (DV-11 + pro Brookfield, USA) using spindle No. 34 (28, 29).

Physical Stability studies

The physical stability of each liposomal formulation was assessed by temperature stability. Liposome samples were kept in 4˚C and 25ºC for one month and then evaluated by monitoring any change in their particle sizes.

Entrapment efficiency (EE %)

The encapsulation efficiency of the liposomal samples were determined using an indirect method so that each liposomal formulation was centrifuged for 40 min at 20000 rpm. Then, supernatant was removed and the amount of free drug in the supernatant was measured by spectrophotometer apparatus at 216 nm. The percentage of entrapment efficiency was calculated by the following equation:

$$EE\%\ x = \frac{(\text{W initial drug} - W\ free\ drug)}{(\text{Winitial drug})} \times 100$$

***In vitro* Release of Pilocarpine**

In vitro Pilocarpine release from liposomal formulations was determined using a modified Franz diffusion cells with contact area of 0.348 cm^2. Before the experiment, the cellulose membrane was hydrated in double distilled water at room temperature for 24 hours. Then, it was set between donor and receptor compartments and 0.5 ml of each formulation was put in a donor compartment. The receptor phase was filled with 10 ml of phosphate buffer (pH 7.4) and was continuously stirred by externally driven magnetic bars at 100 rpm and at 37˚C throughout the experiment. An aliquot of 1 ml of sample was withdrawn from the receptor compartment, at definite time intervals (0.5, 1, 2, 3, 4, 5, 6, 7, 8 and 24 h) and replaced immediately with the same amount of fresh phosphate buffer to maintain sink condition. Then, the concentration of the drug was determined and cumulative percentage of released drug was plotted versus time and their behavior was described by fitting to different kinetic models. The maximum r^2 value was considered as the most probable release mechanism (28).

***Ex vivo* Corneal Permeability**

The eye cornea with sclera ring was isolated from a newly sacrificed male New Zealand rabbit. Then, the subcutaneous tissue was completely removed using scalpel and scissors. The cornea was kept in DexSol solution (chondroitin sulfate-based, commercial storage media for preservation of corneal epithelium, Chiron Ophthalmic, Irvine, California)(30, 31).

A modified diffusion cell with effective diffusion area of 0.348 cm^2 was utilized for corneal permeation studies. The extracted rabbit corneas were set between the donor and receptor compartments of the diffusion cell in a manner that sclera ring clamped between two chambers and cornea facing the receptor without any damage due to diffusion cell apparatus. The receptor chamber was filled with 10 ml buffer phosphate (pH 7.4) and the receptor fluid was constantly stirred by externally driven magnetic bars at 100 rpm throughout the experiment and its temperature was maintained at 32±0.5˚C. Pilocarpine HCL liposomal samples (0.5 ml) were accurately weighted and placed onto the corneas. At time intervals (0.5, 1, 2, 3, 4, 5), 1 ml of sample was withdrawn from the receptor for spectrophotometric determination and immediately replaced with the same amount of fresh phosphate buffer to maintain sink condition. The Samples were analyzed spectrophotometrically at 216 nm. The free drug liposomal solution and the pilocarpine solution were used as blank positive and control respectively. The results were plotted as cumulative amount of drug permeated versus time (32).

Data Analysis and Statistics

The cumulative amount of pilocarpine permeated per unit corneal area was measured and plotted versus time. Different corneal permeability parameters including flux (J_{ss}), apparent corneal permeability coefficients (P_{app}), apparent diffusivity coefficient (D_{app}), enhancement ratio flux (ER_{flux}), enhancement ratio diffusion (ER_D) and enhancement ratio permeability (ER_P) were calculated. The corneal permeation rate at steady state (J_{ss}, µg/cm^2h) was determined from the linear section of the slope of the permeation curve. Since the cornea thickness (h) does not show the actual pathway for drug permeation, diffusivity coefficient is defined as apparent D (D_{app}). Apparent permeability coefficient (P_{app}, cm/h) and apparent diffusivity coefficient (D_{app} cm^2/h)

parameters were calculated from the equations 1 and 2,

$P_{app} = J_{ss}/C_0$ (Eq.1)

$D_{app} = h^2/6\ T_{lag}$ (Eq.2)

All the experiments were performed in triplicate and data were expressed as the mean value ± SD. One-way analysis of variance (ANOVA) was applied to identify any significant difference and $P < 0.05$ was the level of significance with 95% confidence intervals.

Results

Eight different liposomes were prepared using full factorial design. The composition of the prepared liposomal formulations are presented in Table 1. The polydispersity index (PI), mean particle sizes, pH and viscosities are shown in Table 2.

Table 1. Composition of Pilocarpine Liposomal formulations

Formulation	Factorial design	Sonication time (min)	Cholesterol (mg)	Lecithin (mg)	Drug (mg)
LP-1	- - -	5	30	70	50
LP-2	+ - -	15	30	70	50
LP-3	- + -	5	10	90	50
LP-4	+ + -	15	10	90	50
LP-5	- - +	5	30	70	100
LP-6	+ - +	15	30	70	100
LP-7	- + +	5	10	90	100
LP-8	+ + +	15	10	90	100

Table 2. pH, Mean particle size, Polydispersity Index and Viscosity of Pilocarpine Liposomes (Mean ± SD, n=3)

Formulation code	pH	Mean particle size(nm)	PDI	Viscosity (cps)
LP-1	6.61±0.014	212±2.8	0.372±0.037	47±3.64
LP-2	6.63±0.028	148±2.8	0.364±0.046	44.1±5.37
LP-3	6.49±0.021	198.5±2.1	0.310±0.00	56.25±3.92
LP-4	6.54±0.007	167±1.4	0.310±0.010	61.2±6.45
LP-5	6.30±0.049	120.5±3.5	0.314±0.006	80.1±7.54
LP-6	6.41±0.028	166±1.1	0.317±0.006	54.6±3.73
LP-7	6.37±0.021	211±3.7	0.317±0.013	43.85±2.31
LP-8	6.35±0.021	141.5±3.6	0.339±0.030	74.15±5.66

The release profile of pilocarpine from LIPs are presented in Figure 1. Drug release profile represented that LP-3 released 66% of the entrapped pilocarpine of the drug in 24 hours of the experiment. Higuchi kinetic was the most probable model for all of the formulations. The percentage of the released drug and release kinetics of the liposomal formulations are summarized in Table 3.

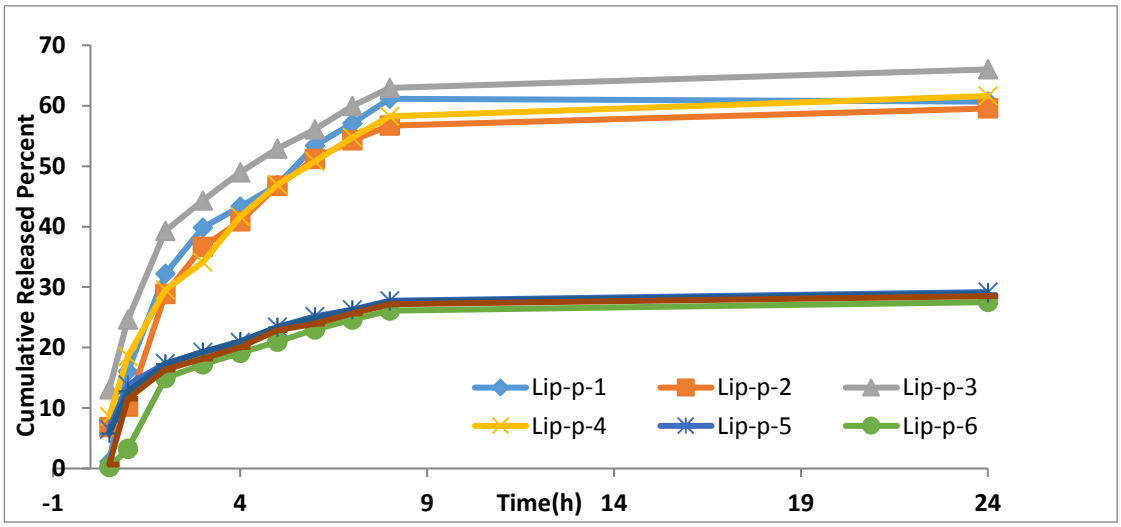

Figure 1. Release profile of Pilocarpine liposomes

Table 3. The Percentage and Kinetics of Release of the Selected Liposomes (Mean ± SD, n=3)

Formulation	%Release, 2h	%Release, 24h	Kinetics of Release	R^2
LP-1	32.19±0.069	60.68±0.090	Higuchi	0.6816
LP-2	28.85±0.122	59.54±0.513	Higuchi	0.7168
LP-3	39.28±0.174	66.00±0.012	Higuchi	0.7239
LP-4	29.54±0.183	61.61±0.052	Higuchi	0.7637
LP-5	17.39±0.055	29.17±0.038	Higuchi	0.7375
LP-6	14.96±0.002	27.51±0.170	Higuchi	0.6893
LP-7	17.25±0.232	28.96±0.053	Higuchi	0.7264
LP-8	16.38±0.123	28.50±0.078	Higuchi	0.6816

The extent of permeated pilocarpine through the rabbit corneal membrane per area of diffusion cells (µg/cm^2) as a function of time (h) was plotted (Figure 2 and Table 4).

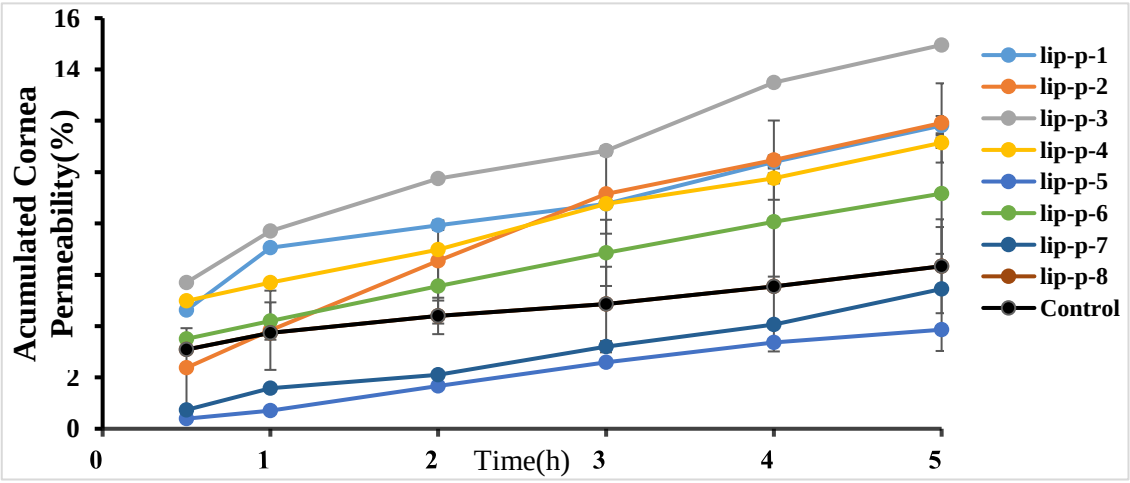

Figure 2. Accumulated Cornea Permeability Percent of pilocarpine for Liposomal Formulations (Mean ± SD, n=3)

Table 4. Pilocarpine Corneal Permeability Parameters from Liposomal Formulations (Mean ± SD, n=3)

formulation	J_{ss}(μg/cm^2.h)	D_{app}(cm^2/h)	P(cm/h)	%P5h	ER_{flux}	ER_D	ER_p
Control	63.73±2.52	0.001±0.00001	0.012±0.005	6.3±0.01	-	-	-
LP-1	65.42±2.114	0.003±0.0007	0.035±0.011	11.82±1.17	1.036±0.393	2.035±0.046	2.776±1.053
LP-2	116.61±3.45	0.001±0.0001	0.062±0.018	11.92±0.457	1.818±0.487	0.893±0.065	4.863±1.303
LP-3	84.368±1.42	0.023±0.02	0.044±0.0007	14.96±0.4	1.324±0.04	14.108±1.2	3.529±0.1
LP-4	81.211±9.88	0.0006±0.00001	0.043±0.005	11.15±0.782	1.278±0.197	0.381±0.007	3.387±0.523
LP-5	119.503±1.2	0.007±0.001	0.027±0.0002	3.860±0.022	1.876±0.057	4.282±0.646	2.124±0.064
LP-6	154.4±7.8	0.002±0.0001	0.035±0.001	9.163±0.032	2.425±0.166	1.339±1.322	2.766±0.190
LP-7	122.46±3.28	0.001±0.00001	0.028±0.0007	5.45±0.006	1.924±0.120	0.975±0.006	2.205±0.138
LP-8	92.186±2.19	0.001±0.00002	0.021±0.0005	6.332±0.040	1.448±0.077	1.000±0.000	1.662±0089

The results showed that drug flux of all liposomal formulations through rabbit cornea increased more than diffusion. All liposomal formulations with different compositions and properties significantly increased partitioning, flux, and permeability coefficient from rabbit cornea.

Discussion

The Liposomal samples displayed the mean range of viscosity from 43.85 to 80.1 cps in 50 rpm, pH range of 6.30 – 6.63, and particle size range of 120.5 – 212 nm. Analysis of variance represented that the correlation between particle size and sonication time were significant. It means that particle size increases with less sonication time. LP-1 and LP-5 showed the highest and lowest particle size respectively. One of the most important characteristics in nano drug delivery systems is particle size. Any reduction in particle size leads to a huge increase in surface area that could improve drug bioavailability. This increase in particle size would account, as mentioned previously by Rania et al. in 2007 (33), for the higher encapsulation efficiency of the positive liposomes compared with the neutral and negative ones. The polydispersity index described the uniformity of the particle sizes. All of the polydispersity indexes were smaller than 0.5; hence, the obtained results display the narrow distribution of particle size in the samples.

The results showed that the sonication time had a significant impact on the viscosity. Increased viscosity may improve the pre-ocular retention time and thus, increase the amount of the permeated drug through cornea. All of the liposomal formulations were more viscose in comparison with aqueous solution. It was shown that all of the formulations had relatively proper homogeneity and one-month duration stability. The comparison of mean particle sizes at the beginning and after one month storage showed no significant difference ($P > 0.05$). Also, polydispersity index did not change significantly during and after one month storage, indicating the stability of the formulations.

A correlation between drug release in 2 hours (R2h) and 24 hours (R24h) was found using analysis of variances, indicating that all of the independent variables significantly affect the drug release ($P<0.05$). It seems that pilocarpine HCl release profile is directly affected by hydrophilic characteristics. Pilocarpine is most likely loaded into the inner aqueous core or attached to the surface of liposomes. High drug release in the first 2 hours may be due to the detachment of surface drug. Furthermore, it can be inferred that the increased viscosity of LIPs may lower drug diffusion retards drug release from the membrane. The results can be explained by the presence of cholesterol in the bilayers above the phospholipid Tc, which adjusts membrane fluidity by restricting the movement of the relatively mobile hydrocarbon chains, reducing bilayer permeability, resulting in prolonged drug retention (34).

The correlation between apparent diffusivity coefficients (Dapp) and sonication time was significant and by lowering the sonication time, Dapp increased. Furthermore, there was no significant correlation between formulation parameters and Papp ($P>0.05$). Dapp parameter of LP-2 and LP-3 formulations were 0.023 and 0.062 cm^2h^{-1}, respectively, which were much higher than control (pilocarpine aqueous solution). Also, drug permeated percentage during 5 hours of experiment (%P5h) and drug/lipid ratio was significant and decreasing drug/lipid ratio caused increase of %P5h.

It has been previously shown that formulation of pilocarpine in liquid crystal nanoparticles had a prolonged effect on decreasing intraocular pressure (35).

Additionally, *in vivo* studies have increased apparent permeability and residence time of pilocarpine in ophthalmic preparations (36). It is reported that colloidal systems such as liposomes maintain the drug acting at the ophthalmic site of action which are suitable for absorption of hydrophobic drugs (37). According to the results of a previous study, formulation of micronized pilocarpine in poly-lactic acid microcapsules prolonged the time of miosis significantly (38).

Conclusions

The present findings revealed that the amount of cholesterol, lecithin and drug besides sonication time in the LIP formulation majorly affects the release and permeability parameters. The kinetics of drug release from all the selected LIPs were roughly described by the Higuchi model and showed a prolonged release compared to the pilocarpine aqueous solution.

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Conflict of interest

Authors declare that there is no conflict of interest.

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