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Original Research

Evaluation of cyclosporine A eye absorption after administration of liposomal or conventional forms in animal model

Sara Nikoofal-Sahlabadi[1], Seyed Ahmad Mohajeri[2], Touka Banaee[3], Ehsan Abedini[4], Bizhan Malaekeh-Nikouei[5*]

[1]School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran
[2]Pharmaceutical Research Center, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran
[3]Eye Research Center, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
[4]Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
[5]Nanotechnology Research Center, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran

Abstract

Objective(s): A lot of researches have investigated the effects of topical cyclosporine A on the eye surface layers' diseases. By now the main limitation in cyclosporine application is the low permeation of the drug into the posterior segments of the eye. The aim of present study was to formulate high permeable dosage form can be beneficial in the topical treatment of the uveitis.
Materials and Methods: To reach higher corneal drug absorption and drug concentration in the posterior segments of the eye, 3 nanoliposomal formulations containing 0.5 mg/ml cyclosporine A were prepared. Liposomal formulations and the commercial product (Restasis®) were instilled in the right and left eyes of the rabbits, respectively. The rabbits were killed in the 3, 7, 14 and 28 days of study and the aqueous humor and vitreous were extracted.
Results: Mean size of liposomal formulation number 1, number 2 and number 3 were 107.2 ± 0.7, 129.3±0.9 and 144.8±1.8 nm and their zeta potential were -5.0±1.7, -5.5±2.3 and 44.6±6.2 mV, respectively. Results of ocular analysis showed that the liposomal formulations could increase the concentration of the drug in the aqueous and vitreous like Restasis®. But, in contrast with what has been expected the findings of this study implicate nanoliposomal formulations prepared could not make a significant difference in concentration of the drug in aqueous and vitreous humor compared to Restasis® (anionic microemulsion).
Conclusion: In conclusion, we can state that liposomes with the same composition as our formulations are not more efficient than microemulsion for cyclosporine as ophthalmic drug delivery.

Keywords: Cyclosporine A, Posterior segment, Nanoliposome, Restasis®

*__Corresponding author:__ *Bizhan Malaekeh-Nikouei, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran.*
Tel: +985118823255, Email: malaekehb@mums.ac.ir

Introduction

Cyclosporine A (CyA), a poorly water soluble drug, is an anti-inflammatory and im-munomodulatory drug which is used to prevent transplanted organs from rejection and is also administered to treat autoimmune diseases such as uveitis and rheumatoid arthritis (1-3). Its mechanism of action is inhibition of interleukin-2 production or action. So that, T-cell proliferation is strongly inhibited (4). The most common and serious side effect of CyA is nephrotoxicity. Hepatotoxicity and hypertention are other adverse effects (5).

Many reports support immunosuppressive effect of CyA and thus this drug is suitable for treatment of corneal transplantation rejection, autoimmune uveitis and dry eye syndrome (6). This drug has a narrow therapeutic window (7, 8). Moreover, systemic administration of CyA increases adverse effects' risk and in this case the drug does not enter the eye unless there is an inflammation such as severe uveitis (9). The adverse effect and low ocular drug delivery following systemic administration have led to lots of efforts for preparing an ophthalmic CyA delivery system which raises the efficacy of drug in various ocular diseases (10-12). Restasis® is the topical commercial product of CyA and is approved by FDA for the treatment of dry eye syndrome (kerato-counjunctivitis sica) (13). Topical CyA has good effects on different eye diseases. Therefore, preparing an efficient locally used dosage form can be beneficial. Ointment formulation is uncomfortable for the patients because the ointment base can lead to blurred vision. So, its application has clinically got limited. According to all these limitations, a lot of efforts must be made in order to prepare a topical formulation which can penetrate the eye better, increase the contact time with the eye surface and also reduce the usage frequency (14). By such formulation we can treat ophthalmic diseases more efficiently.

Liposomes are microscopic vesicles composed of phospholipid bilayers in which aqueous compartments can be enclosed. Liposomes can encapsulate both lipophilic and hydrophilic drugs and boost their efficacy and specificity (15). Some other merits of liposomes are their biodegradability, biocompatibility, reduced toxicity and lack of antigenicity (4, 16). In the present study we prepared three nanoliposomal formulationscontainingCyA. These formu-lations were compared to Restasis® in their ability to pass cornea and increase the drug concentration in aqueous and vitreous humor.

Materials and Methods

Materials

Dipalmitoylphosphatidylcholine (DPPC), dio leoylphosphatidylethanolamine (DOPE) and egg phos phatidylcholine (EPC) were purchased from Lipoid (Germany). Cholesterol (Chol) and stearylamine (SA) were bought from Sigma (St. Louis, MO, USA). CyA was obtained from LC laboratories (Woburn, MA, USA). Chloroform was from Merck (Darmstadt, Germany). Acetonitrile and methanol (HPLC grade) were obtained from Scharlau (Spain).

Nanoliposomes preparation and charac-terization

Three nanoliposomal formulations containing CyA were prepared by solvent evaporation method. The molar ratios of each formulation composition are shown in Table 1.

In summary, the lipid phase and CyA were dissolved in the organic solvent containing chloroform: methanol (2:1, v/v) in a round-bottom flask. The organic solvent was vapo-rized by rotary evaporator (Heidolph, Germ-any) to form a thin lipid film on the wall of the flask. Then the lipid film got hydrated with isotonic phosphate buffer (0.1 mM, pH 7.2) and the film was dispersed by vortexing and bath sonicating. These processes were carried out at a particular temperature depending on the lipid composition of each

Table 1. Molar ratio of components for each liposomal formulation.

Formulation number	DPPC	DOPE	Chol	EPC	SA	CyA
1	1	0.5	0.5	-	-	0.1
2	-	0.5	0.5	1	-	0.1
3	-	-	0.5	1	0.5	0.1

formulation. The resulting liposomes were maintained at room temperature for 1 hour and then were stored in the refrigerator at 4 °C. Different liposomal formulations were extruded repeatedly through 1000, 400, 200 and 100 nm polycarbonate membranes. Formulations were passed 11 times through the polycarbonate filters to make nanoliposomes having uniformed size. The average size, size distribution and zeta potential of nanoliposomes were determined by using Zetasizer (3000HSA, Malvern, UK) after suitable dilution. Then, in order to compare nanoliposomal formulations with Restasis®, the liposomal concentration of CyA was evaluated to get equal with Restasis® if needed.

To determine the concentration of CyA in liposomes, 100 µl liposome and 900 µl methanol were mixed and resulting solution was injected to High pressure liquid chromatography (HPLC). HPLC of CyA (in both liposomes and ocular samples was performed with a Young Lin (South Korea) Acme9000 system, consisting of an SP930D solvent-delivery module, an SDV50A solvent-mixing vacuum degasser, aCTS30 column oven, a UV730 dual-wavelength UV–visible detector, and an ODSA C18 (4.6×150 mm, 5µm) column. Data were analyzed by use of Autochro-3000 software provided by Young Lin. The injection volume was 20µL, the flow rate was 1.5 ml/min, and the column temperature was fixed at 70 ºC. The wavelength of UV detector was set to 205 nm. For chromatographic analysis of CyA, an isocratic method was used. The mobile phase consists of acetonitrile (65%), methanol (20%), and water (15%).

Animal studies

In this study, 36 (3 used for calibration curve + 33 for drug application) New Zealand albino rabbits (72 eyes), weighing 1.8–2.0 kg, were included. Rabbits were divided into three groups: A, B and C. Group A (9 rabbits) received formulation number 1, group B (12 rabbits) was administered by preparation number 2 and finally group C (12 rabbits) received formulation number 3. In all groups, the right eyes were treated with liposomal formulations and commercial formulation (Restasis®) was instilled into the left eyes.

Protocol of examination

Rabbit eyes were examined at 3, 7, 14 and 28 days after ocular preparation administration (duration of examination in group A was 14 days because of fewer number of rabbits in this group). In each mentioned days, 3 rabbits were killed and their aqueous humor and vitreous were extracted. The ocular samples were stored in -20 °C for later analysis with HPLC.

Evaluation of CyA concentration in ocular samples

Standard solutions (1-50 µg/ml) in mobile phase were prepared from stoke solution (1 mg/ml) and were used for spiking calibration samples. For making calibration samples, 25 µl standard solutions were added to 225 µl aqueous/vitreous humor mixture to obtain 0.1-5 µg/ml calibration standards. Then these calibration samples were injected to HPLC, and the Area Under the Curve (AUC) for each concentration was determined and the calibration line was drawn through plotting area under UV

absorption peak for spiked samples against corresponding CyA concen-tration. The ophthalmic samples were stored at freezer (-20 °C). Determination of CyA in ocular samples was performed by using calibration line equation (y=34.685x +4.5338, R^2=0.9952).

Sample preparation for HPLC analysis

To precipitate proteins, 1 ml acetonitrile was added to 250 µl ocular sample in a 1.5 ml microtube. Then the mixture was centrifuged at 11337 g. 0.5 ml supernatant was taken by a sampler (1000 µl) and was transferred to a test tube and was dried under nitrogen stream and got reconstituted in 100 µl mobile phase. 20 µl of this solution was injected to the HPLC loop for analysis.

Statistical approach

SPSS 11.5 for windows was used for statistical data analysis. P plot curves were drawn, for data obtained after each formulation application, to determine if the data had normal distribution. Since the data did not have normal distribution, nonparametric test (wilcoxon test) was used to make comparison between data obtained from ocular samples treated with each liposomal formulation and ophthalmic samples which were under treatment with Restasis®.

Results

Characterization of liposomes

Mean size and zeta potential of the first, second and the third formulations are shown in the Table 2. All three formulations are under 150 nm in size. F1 and F2 are neutral in charge while F3 is positively charged.

Table 2. Mean size (nm) and zeta potential (mV) of three nanoliposomal formulations (Mean±SD, n=3).

Formulation number	Mean size (nm)	Zeta potential (mV)
1	107.2 ± 0.7	-5.0± 1.7
2	129.3 ± 0.9	-5.5±2.3
3	144.8 ± 1 .8	44.6±6.2

Samples analysis with HPLC

The mean concentration of CyA in each eye was calculated for all three formulations and Restasis® (n=3).

The average of concentrations and the standard deviations are shown in Tables 3, 4 and 5.

CyA concentration in the right eyes (aqueous humor and vitreous) of the rabbits after application of all three nanoliposomal formulations did not differ significantly with CyA concentration in the left eye (aqueous humor and vitreous) after Restasis® application ($P > 0.05$).

Table 3. The average of CyA concentration in the aqueous and vitreous of rabbits' eyes after topical application of formulation number 1 (DPPC: DOPE) in the right eye and instillation of Restasis® in the left eye at days 3, 7 and 14 (Mean± SD, n=3).AH: aqueous humor, VIT: vitreous.

Sampling time	AH of the right eye (µg/ml)	AH of the left eye (µg/ml)	VIT of the right eye (µg/ml)	VIT of the left eye (µg/ml)
after 3 days	0.65±0.35	1.21±0.02	0.67±0.1	0.18±0.11
after 7 days	0.47±0.17	0.34±0.08	0.37±0.15	0.49±0.18
after 14 days	0.23±0.07	0.17±0.04	0.44±0.15	0.54±0.14

Discussion

Results of our study indicated that all three prepared liposomal formulations could increase the concentration of the drug in the aqueous and vitreous like Restasis®. But, in contrast with what has been expected the findings of this study implicate nanoliposomal formulations prepared could not make a significant difference in concentration of the drug in aqueous and vitreous humor compared to Restasis®.

Cortesi *et al* utilized cationic liposomes

Table 4. The average of CyA concentration in the aqueous and vitreous of rabbits' eyes after topical application of formulation number 2 in the right eye and instillation of Restasis® in the left eye at days 3,7, 14 and 28 (Mean± SD, n=3). AH: aqueous humor, VIT: vitreous.

Sampling time	AH of the right eye (µg/ml)	AH of the left eye (µg/ml)	VIT of the right eye (µg/ml)	VIT of the left eye (µg/ml)
after 3 days	0.55±0.09	1.54±0.28	0.76±0.007	0.64±0.25
after 7 days	0.47±0.17	1.18±0.007	0.34±0.02	0.29±0.03
after 14 days	0.49±0.07	1.29±0.14	0.7±0.05	0.74±0.12
after 28 days	1.13±0.23	0.37±0.007	0.51±0.05	0.36±0.07

Table 5. The average of CyA concentration in the aqueous and vitreous of rabbits' eyes after topical application of formulation number 3 in the right eye and instillation of Restasis® in the left eye at days 3, 7, 14 and 28 (Mean± SD, n=3). AH: aqueous humor, VIT: vitreous.

Sampling time	AH of the right eye (µg/ml)	AH of the left eye (µg/ml)	VIT of the right eye (µg/ml)	VIT of the left eye (µg/ml)
after 3 days	0.47±0.28	1.22±0.17	2.04±0.07	1.99±0.36
after 7 days	1.77±0.55	2.14±0.05	2.4±0.14	1.41±023
after 14 days	2.10±0.28	0.60±0.41	0.36±0.04	1.29±0.24
after 28 days	0.51±0.02	1.29±0.38	0.55±0.14	2.61±0.26

containing polylysine rich peptide named DTK and HSV-1 glycoprotein B (gB1s) as a topical ophthalmic vaccine. This vaccine was efficient in protecting animals against herpes simplex virus type 1 (HSV1) ocular troubles (17). In another study, Sahoo *et al* demonstrated that administration of liposomes containing phosphodiester oligon ucleotides leads to a slower release into the vitreous and retina. Additionally, this formulation reduces drug distribution into the sclera and lenses (18). By contrast, straford *et al* observed a reduction in epinephrine and inulin absorption in aqueous humor after their encapsulation in liposomes. After corneal analysis, they found that the drug molecules were accumulated there; in the corneal (19). According to these studies liposomes' application for increasing drug concentration in aqueous and vitreous humor is controversial and depends on the drug properties. Since CyA is a high molecular weight peptide, in this study we decided to apply liposomes for drug delivery to posterior segments of the eye. In one of our previous studies the safety of liposomes containing CyA was investigated. In that study, fusogenic liposomes showed no toxicity but the cationic ones had some toxicity in the rabbits' eyes (14). The nanoliposomal formulations prepared in this study did not increase the CyA concentration in aqueous and vitreous humor of the rabbits' eyes as much as we expected. However, these liposomal formulations and microemulsion of CyA (Restasis®) did not show any significant differences in their ability to increase the CyA concentration in the posterior segments of the eye. One of the reasons for such result may be the instability of liposomes on the corneal surface. Due to this lack of stability, some of these liposomes may release their content before passing the cornea. As a result, a few numbers of liposomes may get the chance to penetrate the cornea layer. On the other hand, the most common way of particles' internalization into the cells is endocytosis. We believe that the lower concentration of CyA, than we expected, in the aqueous and vitreous may be the consequence of entering the endosomes and escaping from it and releasing into the cells' cytosole of cornea and lense; or the liposomes may last to lysosomes after entering the endosomes, so that the drug

gets destructed. According to these explanations a small amount of liposomes play the role as carriers for CyA.

Conclusion

In the present study, three nanoliposomal formulations containing CyA were prepared with the same concentration as Restasis®. Our study indicated that nanoliposomes act equally as Restasis®, but despite our expectation none of the nanoliposome formulations are able to increase the CyA concentration in the aqueous and vitreous humor of the rabbits' eyes more than the commercial micro emulsion formulation. To sum up, nanoliposomes as ophthalmic drops are not able to increase the drug concentration in the posterior segments of the eye compared to commercial form.

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