Preformulation Studies of a Liposomal Formulation Containing Sirolimus for the Treatment of Dry Eye Disease

Mónica Anayántzin Linares-Alba,¹ Magda Berenice Gómez-Guajardo,² Joice Furtado Fonzar,³ Dennis E. Brooks,⁴ Gustavo Adolfo García-Sánchez,² and Maria Josefa Bernad-Bernad¹

Abstract

Purpose: The aim of this study was to develop and characterize a liposomal product containing sirolimus to be administered subconjunctivally for the treatment of nonresponsive keratoconjunctivitis sicca (KCS) or dry eye.

Methods: Formulations were prepared using an ethanol injection method and an adaptation of the heating method in pursuance of the most suitable methodology for future industrial production. Liposomes were loaded with either a high dose of 1 mg/mL of sirolimus or a less toxic dose of 0.4 mg/mL. The effects of critical process and formulation parameters were investigated. Liposomes were characterized in terms of size, zeta potential, polydispersity, differential scanning calorimetry, morphology, entrapment efficiency, phospholipid content, thermal stability, and sterility. The formulation was evaluated clinically in dogs with spontaneous KCS.

Results: Sterile liposomal dispersions with sizes ranging from 140 to 211 nm, were successfully obtained. High entrapment efficiency of 93%–98% was achieved. The heating method allowed an easier production of liposomes with high entrapment efficiency, to significantly shorten production time and the elimination of the use of alcohol. The poor stability of the obtained liposomes in aqueous dispersion made the inclusion of a lyophilization step necessary to the manufacturing process. *In vivo* testing of the liposomal sirolimus formulations in the spontaneous KCS dog model have produced promising results, particularly with a sirolimus dose of 1 mg/mL, indicating the need for further development and study of proposed formulations in the treatment of canine KCS. Clinical improvement in tear production in dogs with spontaneous KCS treated with the 1 mg/mL dose product was observed.

Conclusions: The heating method allowed easier production of high entrapment efficiency liposomes to significantly shorten production time and the elimination of the use of alcohol. Tear production was increased in dogs administered with the formulation.

Introduction

SJÖGREN SYNDROME (SS) is a chronic inflammatory and lymphoproliferative exocrinopathy in which the glands that produce tears and saliva are destroyed.^{1–3} The diminished gland secretion results in keratoconjunctivitis sicca (KCS) and xerostomia, which in turn cause discomfort, visual disturbance, and tear film instability.⁴ According to the Sjögren's Syndrome Foundation, the disease has a 0.5% of population prevalence, mostly affects the middle-aged, and shows female preponderance (9:1).³

KCS is defined as a tear deficiency or an excessive tear evaporation that causes irritation and damage to the cornea

and conjunctiva.⁵ Current treatment consist of tear substitutes, and tear production stimulants such as cyclosporine A and tacrolimus.^{5–8} Disadvantages of these treatments include irritation caused by topical administration, systemic absorption of the drugs, and failure to return tear production to normal levels.⁹

Companion animals, particularly dogs with spontaneous KCS, are being increasingly appreciated as useful animal models for research, due to the resemblance between human and canine tear film diseases.^{10,11} Some of the advantages include: the size of the canine globe provides benefits for diagnostic tests, the animal eye size compared to the human eye allows an easier scaling of results, canine KCS also

¹Faculty of Chemistry, National Autonomous University of Mexico, Mexico City, Mexico.

²Veterinary Specialty Hospital "Oftalvet," Mexico City, Mexico.

³Faculty of Veterinary Medicine, Sao Paulo State University, Botucatu, SP, Brazil.

⁴College of Veterinary Medicine, University of Florida, Gainesville, Florida.

shows increased prevalence of affected females, and the fact that the dogs share the same environments.^{11,12}

The most common cause of canine KCS is a defect in autoimmunity causing an insufficient production of the aqueous component by the lacrimal and accessory lacrimal glands (Schirmer tear test of <10 mm/min) due to a lymphocytic attack on these tear glands.^{6,7,13–15} In most cases (80%), the histopathologic evidence of lacrimal gland mononuclear cell infiltration and acinar atrophy, presence of circulating autoantibodies, and concurrent immune disease suggest an autoimmune etiology to canine KCS.^{13,14,16}

Cyclosporine A interferes with the proliferation and activity of T-lymphocytes and the macrolide calcineurin inhibitor tacrolimus also reduces lymphocyte activity. Both drugs interfere with calcium independent events of interleukin-2 transcription such that both can be used topically to treat canine KCS. In dog cases with an immune pathogenesis of KCS, ~20%–25%, however, still do not respond to topical treatment with these immune modulating drugs.¹⁷

Sirolimus, also called rapamycin, is a macrolide antifungal, antineoplastic, and immunosuppressive agent discovered in 1975.¹⁸ Since 1999, it has been approved by the United States Food and Drug Administration for the prophylaxis of organ rejection in human patients older than 13 years.¹⁹ Advantages of sirolimus as an immunosuppressant derives from its unique mechanism of action, its improved side-effect profile, and its ability to synergize with other immunosuppressive agents (calcineurin inhibitors) due to the shared metabolism by CYP 3A.²⁰

Sirolimus, although similar in structure to tacrolimus, is not a calcineurin inhibitor. Sirolimus binds to the FK506-binding protein to create an immunosuppressive complex that prevents the activation of the mammalian target of rapamycin (mTOR), which prevents the cell cycle progress from G1 to the S phase. This leads to the suppression of T-lymphocyte activation, proliferation, and antibody production.^{14,21} As a result of its particular mechanism of action, sirolimus safety profiles show decreased nephrotoxicity, neurotoxicity, and hypertension compared with cyclosporine A or tacrolimus.^{22,23}

Specific ocular toxicity studies by Douglas et al.²⁴ demonstrated that intravitreal and subconjunctival injection of 5 and 10 mg of sirolimus did not cause signs of acute toxicity in normal adult horse eyes.²⁴ Fonzar et al.'s study in rabbits and dogs found no evidence of irritation or toxicity caused by multiple subconjunctival injections of 0.4 and 1 mg/mL of sirolimus in a 4-month-long evaluation.²⁵

Despite the mentioned pharmacological benefits, the potential therapeutic use of sirolimus is challenged by its physicochemical characteristics.¹⁹ Sirolimus is practically insoluble in water (2.6 µg/mL), yields high liposolubility (log $P_{O/W}$ 5.77), and contains no ionizable groups.^{26,27} It is reported to be very unstable in ionic mediums and has demonstrated a high rate of degradation under daylight.^{28,29} As reported by Buech et al., the drug has low corneal permeation, and is only promising for ocular surface disorders.^{28,30}

Pharmaceutical nanocarriers, such as liposomes, micelles, nanoemulsions, polymeric nanoparticles, and many others demonstrate a broad variety of useful properties such as longevity in the blood, accumulation in pathological areas with compromised vasculature, specific targeting, enhanced intracellular penetration, and stimuli sensitivity allowing for drug release from the carriers under certain physiological condi-

tions.³¹ Such delivery systems with their individual functions acting in a coordinated way should allow the controlled delivery of pharmaceutical agents with a specific release profile.

The development of such delivery systems for the treatment of dry eye has been reported. For example, Chang et al. indicated that the use of microspheres with encapsulated doxycycline are useful and safe for the treatment of dry eye.³² Likewise, various systems have been developed specifically for the release of sirolimus, including intraocular implants, micelles, nanoparticles, and microparticles.^{33–36} However, such systems present diverse disadvantages requiring expensive materials for their production, the need of surgical placement, use of nonbiodegradable polymers, poor stability, and methodological difficulties for sterilization.

Liposomes are microscopic vesicles composed of lipid bilayers surrounding an aqueous core.³⁷ They can enhance the solubilization and absorption of the encapsulated drug while protecting it from degradation.^{38–41} Since liposomes have a similar composition as cell membranes, it is expected that they are biocompatible and biodegradable preparations.³⁹ Ideal drug candidates for liposomal delivery are those with high lipid or water solubility and potent pharmacological activity. A lipophilic drug is usually bound to the bilayer and is more likely to remain encapsulated during storage due to its partition coefficient.²⁷

Some sirolimus liposomal formulations have been previously reported.^{28,42–45} Some of the major problems limiting the use of liposomes are their stability, poor batch-to-batch reproducibility, difficulties in sterilization, and low drug loading.⁴⁶ There are reported liposomal formulations that allow high entrapment of the drug and good reproducibility.^{25,28,42,46} As for stability, it has been reported that it is possible to significantly increase the shelf life of liposomal formulations by lyophilization of the product.⁴⁷ However, the traditional preparation methods have serious limitations for large-scale production, often involving difficult removal of large amounts of solvents or the use of noninjectable tensioactives.

The heating method is an alternative manufacturing process characterized by the absence of organic solvent or tensioactives for the solubilization of lipids, representing an advantage in terms of toxicity and fabrication time.⁴⁸ Also, it does not subject the active substance to high shear force treatments.⁴⁹ Given those characteristics, it seems more likely to scale this heating process to industrial production. Taking into account the low corneal permeability of the drug and the higher permeability of the conjunctiva (along with main loss by drug clearance), we propose a subconjunctival administration route.⁵⁰

The purpose of this study was to develop, characterize, and evaluate soybean lecithin–cholesterol-based liposomes loaded with sirolimus by an adaptation of the heating method.⁴⁸ The physicochemical characteristics of scalable manufactured liposomes were also compared with liposomes prepared by ethanol injection by Fonzar et al. that were tested to be effective *in vivo* in dogs with KCS.²⁵

Methods

Materials

For liposome preparation, sirolimus was kindly provided by Laboratorio Santgar, purchased from Henan Legend Wealth Trading Co. Sodium phosphate monobasic monohydrate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$), sodium hydroxide (NaOH) and

glycerol anhydrous were obtained from J. T. Baker. Sodium chloride (NaCl), ethanol absolute, and high-performance liquid chromatography (HPLC)-grade methanol were obtained from J. T. Baker. Soybean lecithin and cholesterol were purchased from Sigma-Aldrich. Nitrogen, high purity gas (N_2), was obtained from the Infra Group.

For analytical evaluations, ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) and ammonium thiocyanate ($NH_4 \cdot SCN$) were obtained from Merck Chemicals. Chloroform was purchased from J. T. Baker. All chemicals were used as received. Distilled water was purchased from CONQUIMEX. Ultrapure water of quality 18.2 M Ω was produced from distilled water with a Barnstead Nanopure Diamond system.

Liposome preparation

Phosphate buffered saline preparation. Phosphate buffered saline (PBS) was prepared by dissolving the necessary amount of $NaH_2PO_4 \cdot H_2O$ and NaOH in distilled and deionized water to obtain a 0.1 M solution. If necessary, pH was adjusted to 7.45 by the addition of NaOH saturated solution. NaCl needed to reach isotonicity was added.

Ethanol injection liposome (IEL) preparation. Liposomes composed of lecithin and cholesterol (Table 1) were prepared by ethanol injection.²⁵ Cholesterol and lecithin were dissolved in the minimum required amount of ethanol anhydrous with constant stirring (450–500 rpm) for 40 min. For loaded formulations, sirolimus was dissolved in the minimum required amount of ethanol anhydrous by manual agitation. Subsequently, alcoholic solutions were mixed together for 20 min at 400 rpm.

Later, the required amount of PBS was heated to 45°C. Then, with constant stirring at 400 rpm, the alcoholic mixture was dripped on PBS at a rate of 1 drop every 10 s. The dispersion was stirred at 350 rpm until complete ethanol evaporation. Finally, the dispersion was sterilized by filtration through a polyvinylidene difluoride (PVDF) hydrophilic membrane of 0.22 μ m pore size (Durapore; Millipore Co.) with a N_2 pressure inlet of 2 bars, under aseptic conditions. The sterile dispersion was collected in amber glass sterile vials and kept in refrigeration until use.

Heating method liposome preparation. Liposomes with the same proportion were prepared by an adaptation of the heating method (see Table 1 for composition).⁴⁸ Cholesterol and lecithin were hydrated with PBS for 1 h under N_2 . The lipid dispersions were then mixed together in the presence of glycerol (glycerol was in a 3% v/v concentration) and the

volume made up to the total volume of the desired batch. The mixture was heated to 70°C while stirring at 750 rpm for 30 min. The resultant dispersion was left at room temperature under N_2 for 30 min. For loaded formulations, the required sirolimus amount was added to the mixture and dispersed before making up the total volume.

The dispersion was extruded with a Stainless Steel Pressure Filter Holder (Millipore Co.) through PVDF hydrophilic membrane of 0.45 μ m pore size (Durapore HVLP; Millipore Co.) with a N_2 pressure inlet of 2 bars. Finally, the dispersion was sterilized by filtration through a PVDF hydrophilic membrane of 0.22 μ m pore size (Durapore; Millipore Co.) with a N_2 pressure inlet of 2 bars, under aseptic conditions. The sterile dispersion was collected in amber glass sterile vials and kept in refrigeration until use.

Physicochemical characterization of liposomal formulations

Particle size and particle size distribution. The mean particle size and polydispersity index (PDI) for each liposomal formulation were determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS analyzer ZEN 3600 (Malvern Instruments Ltd.). PDI was used as indicative of particle size distribution of the liposomal vesicles population. Analysis was performed at 25°C using clear folded capillary cells with 173° backscatter as angle of detection, recording 3 measurements per data point.

Zeta potential (ζ). Zeta potential of liposomal vesicles was measured by electrophoretic light scattering using a Zetasizer Nano ZS analyzer. Zeta potential was determined as an indicator of dispersion stability. Analysis was performed at 25°C using clear folded capillary cells. Each reported value is the average of 3 measurements.

Thermal analysis

The differential scanning calorimetry (DSC) of formulations was carried between –5°C and 100°C under an inert atmosphere, with a heating rate of 5°C/min. Analysis was performed using the differential scanner calorimeter DSC821e (METTLER TOLEDO).

Transmission electron microscopy

Transmission electron microscopy (TEM) images for morphological characterization were obtained using the JEM-2010 microscope (JEOL). A sample of each formulation was centrifuged at 14,000g using Amicon Ultra 0.5 mL Centrifugal Filters with a 10 K molecular weight cut off (Millipore Co.).

Sample preparation. Concentrated liposomes were washed with ultrapure water twice, centrifuged, and finally reconstituted with ultrapure water. For sample observation, a drop of liposomal suspension was placed on the surface of a 3 mm carbon grid and was allowed to air dry.

Sirolimus determination

Ultraviolet (UV) analysis of sirolimus was performed on a S2000 spectrometer using a DT1000 deuterium light source, a SAD500 serial port interface (Ocean Optics, Inc.), and a 10 mm path length quartz cuvette (Prolab).

TABLE 1. LIPOSOMAL FORMULATIONS

Formulation code	Composition	Preparation technique	Sirolimus dose
HMV	Lecithin	Heating method	0 mg/mL
HM04	Cholesterol (6:1 molar ratio)		0.4 mg/mL
HM1	Glycerol (3% v/v)	Ethanol injection	1 mg/mL
IEV	Lecithin		0 mg/mL
IE04	Cholesterol (6:1 molar ratio)		0.4 mg/mL
IE1			1 mg/mL

Sirolimus calibration curve. Stock solution of sirolimus (100 µg/mL) was prepared using a mix of ethanol absolute and PBS (2:8) as solvent. Aliquots of standard stock solution were pipetted out and suitably diluted with solvent mix to get the concentration ranging from 0.5 to 20 µg/mL.

Standard solutions were scanned over the range of 200–400 nm against solvent mix blank. Wavelength of maximum absorbance (λ_{max}) was 281.52 nm. The calibration curves, made in triplicate, were constructed by plotting concentration against absorbance of standard solutions at λ_{max} .

Method validation. UV spectrophotometric quantification method for sirolimus was validated by means of linearity, precision, and accuracy according to Del Rivero et al.⁵¹

Determination of entrapment efficiency (EE%)

The EE% was determined to evaluate the effect of changing liposomal composition and preparation technique on the properties of liposomes.

Sample preparation. Liposomal dispersions were centrifuged at 14,000g using Amicon Ultra 0.5 mL Centrifugal Filters with a 10 K molecular weight cut off (Millipore Co.) to remove the nonencapsulated drug. A volume of 400 µL of the supernatant were mixed with 400 µL of ethanol and 800 µL of PBS. Samples were vortexed for 1 min in snap-cap microtubes. Dilution factor was considered for later calculations. Concentration of sirolimus in the supernatant was determined by interpolation in the average calibration curve. EE% was calculated as follows:

$$EE\% = \frac{C_{\text{total}} - C_{\text{free}}}{C_{\text{total}}} * 100$$

Phospholipid determination

Visible spectrophotometric analysis of phospholipids was performed by the quantification of a colored complex formed between phospholipids and ammonium ferrothiocyanate. Analysis was performed on a S2000 spectrometer using a DT-1000CE-BT tungsten light source, a SAD500 serial port interface (Ocean Optics, Inc.) and a 10 mm path length glass cell (Perkin Elmer). Measurements were conducted at the λ_{max} of the complex, 492.23 nm.

Ammonium ferrothiocyanate reagent preparation. Standard solution of ammonium ferrothiocyanate was prepared by dissolving 27.03 g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and 30.4 g of $\text{NH}_4 \cdot \text{SCN}$ in 1 L deionized distilled water.⁵²

Lecithin calibration curve. Stock solution of lecithin (0.1 mg/mL) was prepared using chloroform as solvent. Aliquots of stock solution were pipetted out in a stoppered test tube to prepare standard solutions in a concentration range from 0.01 to 0.1 mg/mL, and enough chloroform was added to make the final organic phase volume of 2.0 mL. Subsequently, 2.0 mL of ammonium ferrothiocyanate reagent (AFR) were added. The biphasic system was then vortexed for 2 min.

Chloroform phase was scanned over the range of 400–700 nm against chloroform blank, maximum 10 min after having formed the colored complex. Wavelength of maxi-

mum absorbance was determined. The calibration curves, made in triplicate, were constructed by plotting concentration against absorbance of standard solutions at 492.23 nm.

Sample preparation. A sample of each formulation [400 µL of heating method liposomes (HMLs), 40 µL of IEV and IE04 and 20 µL IE1] was mixed with 2 mL of AFR with manual agitation. Then, 2 mL of chloroform were added and the biphasic system was vortexed for 2 min in stoppered test tubes. Following, samples were centrifuged at 500 rpm for 3 min. Absorbance of organic phase at λ_{max} of the colored complex was measured, maximum 10 min after having formed it. Phospholipid concentration in samples was determined by interpolation in the average calibration curve.

Thermal shock test

All formulations were subjected to extreme temperature conditions to obtain information about the physicochemical stability of the product in the sterile primary container (amber glass vial and rubber stopper with aluminum seal). Samples were stored at 5°C ±1°C and 40°C ±1°C for 10 days protected from light. Particle size, ζ and EE% were measured on a daily basis.

Thermal degradation of sirolimus

Because sirolimus can potentially degrade when exposed to 70°C in liposome preparation, thermal degradation studies were accomplished and analyzed by using a HPLC. Sirolimus and the excipients required to prepare HMLs were mixed in HPLC-grade methanol at room temperature and diluted to a 30 µg/mL sirolimus concentration. One of the samples was exposed to 70°C for 1 h while stirring at 750 rpm to make evident the thermal degradation of the drug at those conditions.

Quantification of sirolimus was performed in a 1260 Infinity Quaternary HPLC System (Agilent Technologies) equipped with a C18 (4.6 × 150 mm, particle size 5 µm) analytical column (Thermo Scientific). Analysis was performed using a gradient elution profile with methanol and water. Methanol content was increased during the analysis from 30% to 100%. The mobile phase flow rate was set to 0.5 mL/min at 50°C ±1°C, 10 µL injection volume, and analysis time of 10 min. The chromatograms were registered using a DAD detector at 278 nm. Data were obtained and automatically integrated through the OpenLAB Chromatography Data System EZChrom Edition (Agilent Technologies).

Biological evaluation

Sterility test. Sterility test was performed in accordance with the Mexican Pharmacopeia (FEUM) standards. Tests were performed on samples stored in their primary container, incubated at 25°C, 60% relative humidity (RH), and 40°C, 60% RH for 3 months with monthly analysis.

Preliminary in vivo testing

Roughly, dogs previously diagnosed with KCS at the Oftalvet Veterinary Specialty Hospital that did not respond to traditional topical treatment (with cyclosporine A or tacrolimus) were included. Dogs were subconjunctivally administered with liposomes loaded with a 1 mg/mL sirolimus dose.

TABLE 2. PHYSICAL CHARACTERIZATION OF LIPOSOMAL FORMULATIONS

Formulation	Before filtration			After filtration		
	Size (nm)	PDI	ζ (mV)	Size (nm)	PDI	ζ (mV)
HMV	428.0 $\pm$ 9.3	0.563 $\pm$ 0.01	-20.9 $\pm$ 1.07	168.6 $\pm$ 0.8	0.077 $\pm$ 0.01	-21.1 $\pm$ 0.78
IEV	388.0 $\pm$ 10.6	0.639 $\pm$ 0.03	-11.6 $\pm$ 1.82	178.9 $\pm$ 3.4	0.190 $\pm$ 0.03	-22.3 $\pm$ 1.18
HM04	229.1 $\pm$ 6.7	0.559 $\pm$ 0.05	-13.6 $\pm$ 0.66	144.6 $\pm$ 0.6	0.228 $\pm$ 0.01	-15.3 $\pm$ 1.12
IE04	251.9 $\pm$ 2.0	0.221 $\pm$ 0.02	-12.4 $\pm$ 0.60	143.4 $\pm$ 1.5	0.123 $\pm$ 0.02	-13.9 $\pm$ 0.47
HM1	559.7 $\pm$ 6.1	0.616 $\pm$ 0.02	-13.5 $\pm$ 1.42	211.6 $\pm$ 0.5	0.264 $\pm$ 0.01	-16.1 $\pm$ 1.04
IE1	1,894.5 $\pm$ 26.2	1 $\pm$ 0.00	-2.2 $\pm$ 0.10	140.9 $\pm$ 2.1	0.112 $\pm$ 0.02	-15.2 $\pm$ 0.71

Values are mean diameter $\pm$ standard deviation. Sample size (n) was 3 for all formulations. PDI, polydispersity index.

Treated dogs were tested by means of Schirmer's test 1 and 2 (STT1 and STT2) to quantify lacrimal production (normal and with local anesthesia, respectively) as well as stability of the tear film was evaluated by the measurement of the tear film break up time. Furthermore, potential signs of irritation were monitored by the clinical evaluation of veterinary ophthalmologists after each application (following the McDonald-Shadduck score system). The study was conducted in accordance with the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research.

Statistical analyses

All data were normally distributed; therefore, in instances of mean comparisons, F -test for equality of variances followed by t -test for independent samples was used to assess significance. Alpha levels were set at 0.05 for all analyses. The statistical package StatPlus®: Mac (AnalystSoft, Inc.) was used for all data analysis.

Results

Physical characterization

Particle size, particle size distribution, and ζ . Table 2 shows the effect of preparation method on mean diameter size, PDI, and ζ . Generally, all liposomal preparations showed a particle size around 200 nm after filtration and PDI <0.3. Ethanol injection method produced generally bigger liposomes with higher polydispersity before filtration. Anyhow, after filtration, size and polydispersity were similar for all formulations. Zeta potential was in all cases negative, but not lower than -23 mV in any case.

Figure 1 presents the physical changes of the formulations over time at room temperature. The liposome size was increasing with time, most significantly in those prepared by ethanol injection. The PDI also increased in most cases, although never reaching values >0.3. For its part, zeta potential remained negative in all cases, and in some cases an increase in the value of the zeta potential was observed.

FIG. 1. Physical changes of formulations over time at room conditions for each formulation stored in its primary container. (A) Particle size, (B) polydispersity index, and (C) zeta potential. Data are mean values. $n=3$.

TABLE 3. TRANSITION TEMPERATURES OF LIPOSOMAL FORMULATIONS ($N=1$)

Formulation	Temperature ($^{\circ}\text{C}$)
HMV	45.9
HM04	71.0
HM1	63.8
IEV	38.7
IE04	56.6
IE1	53.1

Differential scanning calorimetry

Table 3 shows the observed transition temperatures of liposomal formulations from -5°C to 100°C . We observed endothermic transitions between 38°C and 71°C . Interestingly, results suggest that the addition of a small amount of sirolimus causes a displacement of the transition to higher temperatures. However, if the amount of sirolimus keeps increasing, the transition temperature decreases.

Transmission electron microscopy

Figure 2 shows TEM micrographs where spherical structures are observed. Their size is in general about 250 nm, similar to the information obtained from DLS.

Sirolimus determination

All specifications for method validation were met for sirolimus concentrations between 0.5 and $20\mu\text{g/mL}$ (data not shown). The equation of the average calibration curve was $A=0.0551C-0.007$ with an R^2 of 0.99978.

Determination of entrapment efficiency

In Fig. 3 is observed that the entrapment efficiency (EE%) was high in all formulations. Also, it can be noticed that the heating method produced a higher encapsulation percentage compared with its ethanol injection counterpart at the same sirolimus dose.

Phospholipid determination

The average calibration curve of soybean lecithin was $A=6.2317C-0.0978$ with a R^2 of 0.99396. Table 4 shows the phospholipid concentration of each formulation, lower for HMLs and higher for IELs as expected. It is noteworthy that in HML the amount of lipids was kept constant, taking into account the reported functional lipid doses. For IELs, the amount of lipids was increased in the same proportion as sirolimus for each formulation (13 times higher lipid dose than HML).

Thermal shock test

From Fig. 4 we can say that independently from temperature conditions, the amount of free sirolimus increases with time, even though the release rate seems to be faster at a higher temperature. About physical changes (Fig. 5), size and PDI does not seem to depend on temperature, and generally behave similarly to room conditions, increasing with time. Moreover, zeta potential values were also increasing with time for most formulations, but for IE1 the increment was abrupt.

Thermal degradation of sirolimus

Three peaks (0.8, 3.84, and 4.18 min) were observed in the chromatogram of sirolimus samples (Fig. 6). The first

FIG. 2. Transmission electron microscopy—micrographs of formulations. (A) IEV, (B) IE04, (C) IE1, (D) HMV, (E) HM04, and (F) HM1. $n=1$.

FIG. 3. Entrapment efficiency percentage of liposomal formulations. Data are mean percent values. Bars are standard deviation. $n=3$.

peak was attributed to the solvent front. The chromatogram of the heated solution showed the same peaks, but with different area percent ratio, the 4.18 min peak was increased in a 4% amount (Table 5).

Sterility test

The prepared liposomal formulations complied with the sterility test in each period of analysis for the 2 incubation conditions tested.

In vivo preliminary results

Table 6 summarizes the increment in quantity and quality of lacrimal production of 4 dogs (7 eyes) treated with liposomes loaded with 1 mg/mL of sirolimus for a 1.5-month period.

Discussion

In general, the preparation of liposomes by the heating method was technically easier and faster in comparison with the ethanol injection method. It also permitted the elimination of the use of ethanol and its subsequent evaporation, which was the most time-consuming step of the process. Also, the manufacture, by heating, allowed the production of larger batches without increasing the time of production. The dispersions prepared by heating had an opalescent appearance, whereas those prepared by ethanol injection had a translucent white appearance.

TABLE 4. FINAL PHOSPHOLIPID CONTENT OF EACH FORMULATION

Formulation	Phospholipid concentration (mg/mL)
HMV	0.48 ± 0.01
HM04	0.44 ± 0.005
HM1	0.42 ± 0.02
IEV	2.70 ± 0.20
IE04	2.84 ± 0.06
IE1	5.37 ± 0.11

Values are mean concentration $\pm$ standard deviation; $n=3$.

FIG. 4. Free drug percentage of liposomal formulations under thermal shock conditions for 10 days. (A) 5°C and (B) 40°C. Data are mean percent values. $n=3$.

The size of liposomes before filtration was lower for those prepared by heating due to the extrusion step (Table 2). Particularly in the case of IE1, the size of the liposomes obtained in the manufacture was significantly greater, which resulted in the saturation of the membrane during filtration with a substantial loss of material. Consequently, the sterilization by filtration process was slower (up to 20 h for a 10 mL batch) for formulations prepared by ethanol injection due to saturation of the membrane, compared to the heating method (15 min for a 10 mL batch).

After filtration, the average size of liposomes loaded with 0.4 mg/mL of sirolimus was equal. For empty liposomes and those with 1 mg/mL of sirolimus, the size was statistically different ($P<0.05$). This is probably related to the population of vesicles with the size required to pass the sterilization step that was generated in the manufacturing of the product.

Both methods have been reported to produce liposomes of the desired size (<200 nm). We attribute the detected differences between manufacturing methods to the existence of an extrusion step in the heating method technique. Aside from the manufacturing process, we can say that small amounts of sirolimus favor the packing of the lipids with the consequent decrease in size. Yet, an increment in the

FIG. 5. Physical changes of liposomal formulations under thermal shock conditions for 10 days. (□) HMV, (■) IEV, (△) HM04, (▲) IE04, (○) HM1, and (●) IE1. Data are mean percent values. $n=3$. (A) Size at 5°C, (B) PDI at 5°C, (C) Zeta potential at 5°C, (D) Size at 40°C, (E) PDI at 40°C and (F) Zeta potential at 40°C.

amount of sirolimus (above a certain quantity) induces bilayer disorder, associated with the large size of the drug molecule, resulting in increased size of the liposomes.⁵³

In all cases the PDI results were below 0.26, which indicates low polydispersity of the systems.⁵⁴ The zeta potential of formulations was equal between the liposomes with the same dose of drug, regardless of the method of manufacture. This is because the outer surface of the membrane was equal in all cases and it has negatively charged deprotonated phosphate groups. Unfortunately, the values obtained were not sufficiently low (< -30 mV) to be considered as stable formulations.⁵⁵

The above was evidenced by monitoring physical changes at room temperature (Fig. 1), where the progressive en-

largement of the vesicles over time was observed. A similar trend was observed in the behavior of zeta potential, which has been increasing over time to a greater extent in the formulations prepared by ethanol injection. The value of PDI generally showed upward fluctuations, but remained low (< 0.3).

The observed behavior of zeta potential is interesting given the fact that all prepared liposomes are constituted of virtually the same components, and dispersed in the same medium. Also, it is linked to a maximization of particle size and an increment of free sirolimus. An explanation to such observations might be that free sirolimus being extremely hydrophobic, once out of the liposome, remains close to the bilayer and interacts with the outside surface of the membrane, thanks to its polar surface area ($195 \text{ \AA}^2$),^{56,57} causing the mentioned physical changes.

The increase in the value of the zeta potential indicates a decrease in the electrostatic repulsive force between the vesicles, promoting their agglomeration, and thus results also in the increase of the particle size and polydispersity of the formulation.⁵⁴ For the formulation IE1, the increase in size and zeta potential was particularly dramatic, probably because of the greater amount of lipids in dispersion. Even though, during the 4-month follow-up of the formulations,

FIG. 6. Chromatograms of sirolimus standard (dotted line) and sample heated to 70°C (solid line).

TABLE 5. PEAK AREA AND AREA PERCENTAGE OF SIROLIMUS STANDARD AND HEATED SIROLIMUS SAMPLE ($N=1$)

Sample	Retention time (min)	Peak area	Peak area (%)
Sirolimus standard	3.840	389,604,883	94
	4.187	22,581,880	5
Sirolimus exposed to 70°C for 1 h	3.840	344,380,473	91
	4.187	36,015,841	9

TABLE 6. *IN VIVO* PRELIMINARY RESULTS

<i>Test</i>	<i>Initial</i>	<i>Third application</i>
STT1 (mm/min)	5.8 ± 5.6	10.2 ± 5.7
STT2 (mm/min)	0 ± 0	2.5 ± 3.9
TBUT (s)	0 ± 0	2.5 ± 2.6

Values are mean ± standard deviation; $n=7$.

STT1, Schirmer's test 1; STT2, Schirmer's test 2; TBUT, tear film break up time.

there was no sign of phase separation, making evident the usefulness of the liposomal system to prevent precipitation of the drug in the aqueous medium.³⁸

DSC thermograms of formulations showed endothermal transitions between 30°C and 80°C (Table 3). The observed transitions were blunted in the thermogram, which is not surprising taking into account that the phospholipids that were used as raw material were not purified, but an extract from soybean. Also, it has been reported that the presence of cholesterol in the bilayer significantly decreases the enthalpy of transition of lecithin at the proportion used in our formulations.^{58,59}

The obtained results show that the transition temperature is increased with the addition of drug to the formulation, but not in a linear relationship with concentration of sirolimus. With a 1 mg/mL dose of drug, the transition decreases again. Given the fact that sirolimus is a highly lipophilic molecule, it is most likely that it gets immersed in the lipid bilayer, which may lead to physical changes of the membrane.⁶⁰

Apparently in our systems, the addition of a low quantity of sirolimus promotes a more ordered structure of the bilayer. However, an increase of the drug concentration results in the disruption of the structure of phospholipids, and probably through steric hindrance, sirolimus molecules may be able to reduce the forces between the ordered chains, and the intercalated drug molecules increase the distance between phospholipid molecules and consequently the freedom degree of the hydrocarbon chain.⁶¹ This is also consistent with the previous size observations.

TEM confirmed the formation of liposomes. The scanning of the grids revealed the presence of vesicles with predominant spherical shape in empty as well as drug-loaded liposomal dispersions (Fig. 2). Some of the images showed amorphous structures and disrupted bilayers suggesting destruction of the vesicles through sample preparation and observation. Therefore, it might be convenient to retry the observation using a technique that allows preserving the sample before observation, for example, cryo-TEM.

The EE%, described as the percentage of the added amount of drug that has been encapsulated in the liposome, was found to be between 93% and 98% (Fig. 3). These results were satisfactory, in the sense that only 2%–7% of the added drug was not encapsulated. For both doses used, a statistically greater ($P<0.05$) percentage of encapsulation was observed in the formulations prepared by heating. These high percentages of encapsulation are expected because sirolimus is structurally an extremely hydrophobic molecule, and thus has a high affinity for the interior of the lipid bilayer.

Phospholipid contents of the final dispersions are listed in Table 4. The calculated values represent approximately the 10% of the added quantity for IEL and the 17% for HML, which means that a large amount of phospholipids was lost through the filtration process. Foregoing is consistent with

the particle size before filtration results that indicate that most of the vesicles obtained before filtration were too big to be recovered after filtration. Our results imply that it may be fundamental to find ways to decrease the initial size of the vesicles to avoid loss of raw material.

Using ethanol injection it would be necessary to reduce the injected volume and decrease the lipid:ethanol ratio in the alcoholic mixture^{62,63}; with the consequent increase of fabrication time and/or reduction of batch size. For the heating method, it could be possible to obtain smaller liposomes by conducting the extrusion step at a temperature above the phase transition temperature of the phospholipids and if required, increase the number of extrusion cycles. Since the stage of extrusion was very fast, the limitation would be the capacity of the extrusion device used. Thus, the batch size and number of cycles should be adjusted so that the occlusion of the membrane is prevented.

The thermal shock results (Fig. 4) exhibit a leakage of the drug from liposomes and enlargement of the vesicles over time (Fig. 5), irrespective of temperature and being more evident for IE1. Such results indicate the necessity to maintain liposomes in a dry state during storage to prevent the leak of sirolimus. Also, it would be useful to increase the stiffness of the lipid bilayer; results suggest that the insertion of the macrolide into the lipid bilayer at a sufficient concentration (HM1) avoids the leakage of the drug, probably by limiting the movement of the phospholipid. The addition of a lyophilization step turns to be crucial to enhance product stability and homogenize the free trapped drug ratio at the administration moment.

Taking into account the chemical stability of the drug molecule, the formation process using heat could have an impact on the molecule and consequently alter the formulation immunosuppressive capability. Figure 6 presents the chromatogram of a sirolimus sample before and after being exposed to 70°C. The main difference observed is the increase in the 4.18 min peak that indicates that this peak corresponds to the degradation product. However, according to previously reported heat stress studies carried by Nukala et al., heat exposure up to 150°C does not produce toxic degradation products, but it could lead to isomeric transformation.⁶⁴

Analysis by HPLC (Table 5) showed an increase of the peak area percent of the 4.18 min peak from 5% to 9% in the heated sample. In accordance with the quality certificate provided by the sirolimus manufacturer, raw material was mainly isomer B. However, 5% of isomer C could be present in the bulk powder. This is consistent with the observed peak area percentage of the sirolimus standard. Thus, it seems that heating at 70°C for 1 h leads to isomer C transformation. As previously reported, the immunosuppressant activity is maintained as long as the macrocycle structure is preserved.⁶⁵ Since both isomers present a macrocyclic structure, probably there is no significant loss in the immunosuppressant activity due to the heating step.⁶⁶ Actually, a commercially available oral formulation (Rapamune®) for the prophylaxis of organ rejection, reports the assay test as a sum of both isomers.⁶⁷

Sterility tests proved that both manufacturing methods in conjunction with the selected primary container permitted the production of sterile dispersions. In all cases, the product preserved their sterility regardless of storage temperature, RH, and time. However, the possibility of the addition of an antimicrobial agent to the formulation remains to be explored if a multidose presentation seems practical.

FIG. 7. Images of the clinical ocular improvement of a female Yorkshire Terrier dog with keratoconjunctivitis sicca that was treated with liposomes loaded with 1 mg/mL of sirolimus. (A) Pre-injection and (B) after third application.

Finally, preliminary *in vivo* results (Table 6) are remarkable. After 3 applications of liposomes loaded with 1 mg/mL of sirolimus, the normal lacrimal production was duplicated. The basal lacrimal production that was inexistent at the beginning of the treatment also was increased. Likewise, the tear film appeared to be more stable. Additionally, the clinical appearance (Fig. 7) of the eyes was substantially improved after the third application. The conjunctival discharge was diminished as well as congestion and vascularization. Also, it is important to mention that the corneal pigment was significantly decreased in most cases.

Conclusion

Sterile liposomal dispersions with sizes ranging from 140 to 211 nm were successfully obtained. High entrapment efficiency of 93%–98% was achieved. The heating method allowed an easier production of liposomes with high entrapment efficiency, to significantly shorten production time and the elimination of the use of alcohol. The observed stability of our liposomes in aqueous dispersion suggests the necessity of a lyophilization step in the manufacturing process. Also, results indicated the indispensability of optimization of formulation and manufacturing process, particularly to decrease size of the vesicles after formation and preventing loss of material in the filtration process. *In vivo* testing of the liposomal sirolimus formulations in the spontaneous KCS dog model have produced promising results, particularly with a sirolimus dose of 1 mg/mL, indicating the need for further development and study of proposed formulations in the treatment of canine KCS. Usefulness as a treatment of human SS remains to be studied.

Acknowledgments

The present work benefited from the valuable input of materials, equipment, and funds of the Oftalvet Veterinary Specialty Hospital, Laboratorio Santgar, and the National

Autonomous University of Mexico. The authors thank Dr. Jesús Gracia for his excellent technical assistance with computational resources.

Author Disclosure Statement

Linares, BSc., reports nonfinancial support from Laboratorio Santgar during the conduct of the study; personal fees from Laboratorio Santgar, outside the submitted work. Dr. Bernad reports nonfinancial support from Laboratorio Santgar, during the conduct of the study. In addition, Dr. Bernad has a patent planned. Dr. Gomez has nothing to disclose. Dr. Fonzar reports nonfinancial support from Laboratorio Santgar, during the conduct of the study. Finally, Dr. García reports that a patent has been submitted.

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Received: April 8, 2015

Accepted: September 15, 2015

Address correspondence to:
 Dr. Maria Josefa Bernad-Bernad
 Facultad de Química
 National Autonomous University of Mexico
 Edificio F1, (Oficina 001)
 Av. Universidad 3000
 Ciudad Universitaria
 Coyoacán CP 04510
 Mexico City
 Mexico

E-mail: bernadf@comunidad.unam.mx