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Cyclosporine A loaded SLNs: Evaluation of cellular uptake and corneal cytotoxicity

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ABSTRACT

Cyclosporine A (CsA) loaded solid lipid nanoparticles (SLNs) for topical ophthalmic applications were prepared by high shear homogenization and ultrasound method using Compritol 888 ATO, Poloxamer 188 and Tween 80, to investigate the cellular uptake of rabbit corneal epithelial cells (RCE) and to evaluate the cytotoxicity. The size of the optimized formulation was 225.9 ± 5.5 nm with a polydispersity index of 0.253 ± 0.05 . The zeta potential and entrapment efficiency was detected as -16.9 ± 0.7 mV and 95.6%, respectively. The CsA release was found to be enzyme (lipase/co-lipase complex) dependent. SLNs were sterilized at 110 and 121 °C. The cytotoxicity was evaluated in vitro by means of RCE cells and was higher at 121 °C sterilization temperature, probably due to a supposed leakage of Tween 80 following lipid re-crystallization. The permeation and penetration of CsA across/into the corneal cells were evaluated using in vitro and ex vivo experiments. The cellular uptake was investigated by replacing CsA with the fluorescent dye Rhodamine B. The penetration enhancement properties were supported by confocal laser scanning microscopy analysis. The internalization of SLNs in cornea and in RCE cell lines was confirmed, pointing out the possibility of CsA targeting to the cornea.

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1. Introduction

Cyclosporin A (CsA) is a neutral, lipophilic, cyclic endecapeptide used as an immuno-suppressive agent in the treatment of severe inflammatory and immune-related ocular diseases (Kuwano et al., 2002). Systemic administration of CsA has been found to be effective in treating many ocular disorders and preventing corneal graft rejection. However, most of these diseases require life-long treatment at relatively high doses to achieve the therapeutic concentration of CsA in the eye (50–300 ng/g ocular tissue), which can lead to severe adverse effects such as nephrotoxicity, hepatotoxicity and cardiotoxicity (Italia et al., 2006). Thus, it has been suggested that topical ophthalmic application of CsA could provide a good alternative to the systemic drug delivery. However, low solubility of CsA in water (0.04 mg/ml), makes the drug difficult to formulate topically (Guo et al., 2005).

In ophthalmology, it has been shown that nanoparticles are promising systems to enhance the bioavailability of drugs applied topically (Qaddoumi et al., 2004). The results of the different

studies show the potential of nanoparticles as ocular drug delivery systems for either hydrophobic or hydrophilic drugs (Bourlais et al., 1998; Ding, 1998). These systems are able to encapsulate and protect the drug against degradation, improve tolerance, increase corneal uptake and intraocular half-lives (Lallemant et al., 2003).

Solid lipid nanoparticles (SLNs) were derived from o/w emulsions by replacing the liquid lipid (oil) by a solid lipid at room temperature. SLNs have many advantages over polymeric nanoparticles: SLNs are based on more biocompatible materials than polymeric nanoparticles; they can be prepared using techniques which are easy to scale up; they can be obtained at higher concentrations (>2%) and they are stable during storage (Mehnert and Mäder, 2001; Müller et al., 2000). Moreover, due to the high stability as aqueous dispersion, SLNs can be formulated as nano dispersions in liquid dosage forms. Therefore, they can be administered as eye-drops based on colloidal suspension. Such a formulation avoids blurred vision and is comfortable due to the small SLN size. Many hydrophobic drugs have been successfully incorporated into SLNs like tobramycin, pilocarpine and vitamin A (Cavalli et al., 2002, 1995; Jenning et al., 2000a). Moreover, topical ocular administration of SLN dispersions was shown to improve the passage of the drug into the aqueous humour (Manjunath et al., 2005).

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The lipid matrix employed in SLN is usually a physiological lipid with low acute and chronic toxicity (Müller et al., 2000). However, the potential ophthalmic tolerability of the SLNs must be assessed via toxicity studies because of the presence of the surfactants. Cell cultures appear as the most promising alternatives to evaluate the corneal toxicity and the rabbit corneal epithelial cell line has been used as a model for physiology, immunology and toxicology studies (Burgalassi et al., 2004, 2001). The ex vivo studies were performed in porcine cornea, as they are easily obtained from the slaughterhouse, and it has been proposed as a useful model for ophthalmic permeation studies (Reichl and Muller-Goymann, 2003).

The first aim of our study was to prepare sterile, CsA loaded SLN that can be used topically for ophthalmic applications. Corneal toxicity was evaluated using an in vitro approach (RCE cell lines) by means of neutral red (NR) cytotoxicity assay, in which the cell survival/viability is evidenced with the ability of viable cells to internalize and bind NR (Babich and Borenfreund, 1990). The permeation and penetration of the drug CsA across/into the corneal cells was evaluated using an in vitro (RCE cells) and an ex vivo (excised pig cornea) approach. The cellular uptake was confirmed by replacing CsA with a fluorescent dye, Rhodamine B (RhB), so that the penetration enhancement properties could be supported by direct observation of the dye with a confocal laser scanning microscopy analysis (CLSM).

2. Materials and methods

2.1. Materials

Compritol 888 ATO (Glyceryl behenate) was generously supplied by Gattefossée (France). Poloxamer 188 (Pluronic F68) was kindly donated by BASF (Germany). Tween 80 (Polysorbate 80), CsA and RhB were obtained from Sigma Aldrich (Italy). All HPLC reagents and lipase/co-lipase were purchased from Sigma Aldrich (Italy). The other chemicals were obtained from Carlo Erba (Italy). All filters were purchased from Sartorius. The cornea epithelial cells of rabbits (RCE) were obtained from European Cell Culture Collection (No. 95081046, ECACC, Salisbury, United Kingdom).

2.2. Preparation of CsA and RhB loaded SLNs

CsA and RhB loaded SLNs were prepared by high shear homogenization and ultrasound method (Mehnert and Mäder, 2001). The lipid phase – Compritol 888 ATO (C888) and CsA – and the aqueous phase – Poloxamer 188 (P188) and Tween 80 (Tw 80) in bidistilled water – were heated to 85 °C, separately. The aqueous phase was poured into the lipid phase, under the stirring speed of 24,000 rpm with UltraTurrax (T25). The particles were dispersed in bidistilled water and kept at –20 °C, for 10 min. SLNs were ultrasonicated to avoid agglomeration during re-crystallization. They were kept

at 4 °C for 1 day. The CsA amount was kept constant as 0.05% in the whole dispersion. Blank SLNs were prepared in a similar way, without the drug.

Production parameters such as mixing time (15, 10 and 5 min), ultrasonication time (10, 30 min and 1 h), temperature of dilution water (room temperature, ice bath, +4 °C) and total formulation volume (25, 50 and 100 ml) were investigated for preformulation studies (Table 1).

The ratio of ingredients in the formulations was also tested in terms of particle size and polydispersity index (Table 2), after determination of the optimum production procedure.

The amount of RhB, a hydrophobic fluorescent dye, employed for preparation of SLNs in confocal microscopy studies, was kept in the same concentration as CsA in the nano-dispersions and RhB loaded SLNs were prepared in the same way.

The formulations were prepared without adding any buffer. The pH was determined immediately after preparation (by Crison-micro pH 2002) to check the compatibility with an ophthalmic administration.

2.3. Measurement of particle size and polydispersity index

Particle size (PS) and polydispersity index (PI) were measured at 25 °C by Photon Correlation Spectroscopy (Beckman Coulter N5) at an angle of 90° after dilution of formulations with bidistilled and filtered (0.45 µm) water. Each sample was measured in triplicate.

2.4. Measurement of zeta potential

The zeta potential of SLN dispersions was measured at 23 °C, at a diffraction angle of 14°, under an electrical field of 15 V/cm, by Zetasizer (Nicomp 380 ZLS). The measurements were conducted in triplicate.

2.5. Sterilization of SLNs

After the preparation of CsA loaded and blank SLNs, these were sterilized at 121 °C for 15 min and at 110 °C, for 30 min via steam sterilization in a thermo-controlled autoclave (Pbi Alfa Junior). Three different batches of each sample were evaluated for their PS and PI to validate the preparation procedure.

2.6. Solubility studies of CsA with surfactants employed

The effect of surfactants on the solubility of CsA in water was determined by adding an excess amount of drug to the medium. The surfactant and co-surfactant concentrations were kept as 0.2% and 0.1% for P 188 and Tw 80, respectively. The suspensions were stirred for 2 h and the samples were filtered before the measurement through a filter membrane with a 0.45 µm pore size (Millipore).

Table 1

The particle size (PS) and polydispersity index (PI) of SLN formulations prepared under the production conditions of mixing time (MT), dilution temperature (DT), ultrasonication time (UT) and total volume of formulation (VF)

MT (min)	PS ± S.D. (nm)	PI ± S.D.	DT	PB ± S.D. (nm)	PI ± S.D.
15	228.3 ± 13.2	0.321 ± 0.06	25 °C	288.5 ± 12.1	0.333 ± 0.04
10	226.5 ± 11.6	0.316 ± 0.09	Ice bath	263.4 ± 14.6	0.309 ± 0.02
5	222.5 ± 4.2	0.245 ± 0.05	+4 °C	233.2 ^a ± 5.3	0.246 ± 0.01
UT (min)			VF (ml)		
0	268.3 ^b ± 16.6	0.215 ± 0.02	25	225.3 ^c ± 11.3	0.253 ± 0.03
10	224.8 ± 8.50	0.249 ± 0.06	50	248.2 ± 9.7	0.259 ± 0.04
30	228.3 ± 10.8	0.363 ± 0.05	100	264.5 ± 6.4	0.293 ± 0.02
60	223.7 ± 9.1	0.316 ± 0.05			

a,b,c $P < 0.05$.

The results are the means ± S.D. ($n = 3$).

Table 2
The particle size (PS) and polydispersity index (PI) of blank and Cyclosporin A (CsA) loaded SLN formulations with various ratios of Compritol 888 ATO (C 888), Poloxamer 188 (P 188) and Tween 80 (Tw 80)

Concentrations of ingredients% (w/v)						
Code	C 888	P 188	Tw 80	CsA	PS ± S.D. (nm)	PI ± S.D.
F1	1	0.4	–	–	258.3 ± 16.7	0.281 ± 0.01
F2	0.8	0.4	–	–	238.2 ± 12.4	0.228 ± 0.02
F3	0.6	0.4	–	–	246.2 ± 13.4	0.224 ± 0.03
F4	0.4	0.4	–	–	220.4 ± 18.7	0.240 ± 0.04
F5	0.4	0.3	–	–	255.2 ± 14.5	0.293 ± 0.04
F6	0.4	0.2	–	–	206.7 ± 8.70	0.218 ± 0.02
F7	0.4	0.1	–	–	253.1 ± 15.6	0.206 ± 0.03
F8	0.4	0.2	0.2	–	233.7 ± 4.10	0.269 ± 0.05
F9	0.4	0.2	0.1	–	227.2 ± 5.60	0.214 ± 0.05
F10	0.4	0.2	0.05	–	218.1 ± 4.40	0.168 ± 0.04
F11	0.4	0.2	0.2	0.05	214.5 ± 9.50	0.353 ± 0.03
F12	0.4	0.2	0.1	0.05	225.9 ± 5.50	0.253 ± 0.05
F13	0.4	0.2	0.05	0.05	Aggregation	

The results are the means ± S.D. (n=3).

The drug amount was determined by a validated HPLC method (Husek, 1997) using an HPLC apparatus (series 200, PerkinElmer) equipped with a UV detector set at 210 nm, a column oven set at 70 °C, and using C18 column (Partisil ODS-3 5U 250 mm × 4.6 mm; Waters, Alltech). The mobile phase, fluxed at 1.5 ml/min, was a mixture of water/CH₃CN/ter-butylmethyl ether at ratio of 590/340/72, and containing H₃PO₄ 0.02 M and sodium lauryl sulphate 0.01 M, buffered at pH 3.65 with NaOH.

2.7. Drug entrapment efficiency

SLN dispersion was ultracentrifuged for 1.5 h at 55,000 rpm (ALC Centrifuge 4206). The supernatant was used for CsA analysis by HPLC and the quantity of free drug was determined. The encapsulated amount of CsA was calculated by subtracting the free amount of CsA from the total amount in the dispersion. Each batch was evaluated three times. Entrapment efficiency (EE%) was calculated by the following equation where W_i is the amount of initial drug and W_f is the amount of free drug.

$$EE\% = \frac{W_i - W_f}{W_i} \times 100$$

2.8. DSC analysis

Differential scanning calorimetric (DSC) analysis was performed by a Mettler STAR system equipped with a DSC821. The samples were sealed in aluminum pans under nitrogen air atmosphere at a flow rate of 50 ml/min and evaluated in 20–250 °C temperature ranges. CsA, C 888, physical mixture of C 888:CsA (10:1) and SLN formulation were evaluated.

2.9. TEM analysis

The morphological examination of the SLNs was performed with a transmission electron microscope (CM12 Philips). The samples were stained with 2% (w/v) phosphotungstic acid and placed on copper grids for viewing by TEM.

2.10. Studies of enzymatic CsA release from SLN

The release studies were conducted in vials at 37 °C, with and without lipase/co-lipase mixture. 1 ml of lipase (2000 unit/ml) was mixed with 0.6 ml co-lipase solution (50 µg co-lipase/ml) and incubated at 37 °C for 15 min to form a complex. This complex is

necessary for the absorption on the particle surface. 50 µl SLN formulation and 350 µl of 0.01 M borate/boric acid buffer were added to this mixture. Borate/boric acid buffer was used to keep the pH at 7.4 for the activity of the enzyme complex (Olbrich et al., 2002). The amount of CsA released was determined by HPLC. The same mixture was maintained in the cuvette of the PCS at 37 °C and particle PS and PI was determined at predetermined time intervals for 90 min. The measurements were carried out for six samples per experiment.

2.11. In vitro permeation and penetration studies (RCE cells)

RCE were grown in a medium having the following composition: Dulbecco's Modified Eagle Medium (DMEM) mixed 1:1 with Ham's nutrient mixture F12, supplemented with L-glutamine (1%, v/v, 2 mM), a mixture of penicillin (100 IU/ml), streptomycin (0.1 mg/ml) and amphotericin B (0.25 µg/ml), foetal bovine serum (15%, v/v), epidermal growth factor (10 ng/ml) and insulin (5 µg/ml) (Sigma). The cells were incubated at 37 ± 0.5 °C in a humidified atmosphere, containing 5% CO₂.

2.11.1. Cytotoxicity study

RCE cells were seeded in 96-well plates with an area of 0.34 cm² at a density 10⁵ cells/cm² (Burgalassi et al., 2001). 24 h after the seeding, the toxicity study was performed using the neutral red (NR) assay (Tox Kit 4, Sigma–Aldrich) that determines the accumulation of the neutral red supravital dye in the lysosomes of viable, uninjured cells. Damages to cell membrane or lysosomes cause poor or no capability to pick up NR.

The cytotoxicity of different SLN formulations (blank, loaded, sterilized), surfactant solutions (0.2%, w/v P188 and 0.1%, w/v Tw 80) and CsA solution (0.05%, w/v) was evaluated using NR assay. Each well was washed with saline phosphate buffer (PBS) and 100 µl of each sample diluted 1:1 with HBSS (H9394 Sigma, Milan, I) at pH 7.4 were put in contact with the cells. After 2 h the samples were removed and the cell substrates were washed with PBS. 200 µl of NR solution (0.33 mg/ml in DMEM) were put in each well for another 3 h of contact time. Cell substrates were then washed with PBS to remove the NR not entrapped in the cells and the fixing medium (1% CaCl₂ and 0.5% formaldehyde aqueous solution) was added to fix the substrate. After removal of the fixing solution a solubilizing solution (1% of acetic acid in ethanol) was added to the cell substrates to cause cell disruption and to release NR captured by viable cells. The NR solution absorbance was determined by means of a plate reader (PerkinElmer) at wavelength of 490 nm. The absorbance read for each sample was compared with that of

HBSS, a positive control, that is not toxic and that was assumed to allow maximum cell viability (100%) (Popiolkiewicz et al., 2005; Fotakis and Timbrell, 2006).

Viability was expressed in percent compared to untreated cells in pure medium ($n=4$). Viability greater than 90% was considered absence of toxicity.

2.11.2. Permeation/penetration studies

RCE were seeded on tissue-culture-treated polycarbonate filters (filter area 113.1 mm²; inner diameter 13.85 mm) in 12 well plates (Greiner Bio-one, PBI International) at seeding density of 8×10^4 cells/cm² (Burgalassi et al., 2001). The cells were grown in standard conditions as reported in paragraph 2.11 for 7 days. On the 8th day the apical phase was removed and the cells were grown for another 8 days with cell substrates at basolateral medium/air interface. Such growth conditions allowed the cell substrate to form a multilayer cell substrate with tight junctions.

500 μ l of each sample were added to the cells (CsA and RhB loaded SLN, CsA and RhB suspension/solution at 0.05%) after dilution with 500 μ l of Hank's balanced salt solution without calcium and magnesium salts (HBSS H9394 Sigma, Milan, I: KCl 400 mg/l, KH₂PO₄ 60 mg/l, NaHCO₃ 350 mg/l, NaCl 8000 mg/l, Na₂HPO₄ 48 mg/l, D-glucose 1000 mg/l, Phenol Red 11 mg/l, buffered at pH 7.4). As the basolateral (receptor) phase of the monolayers, 2 ml of HBSS with calcium and magnesium salts (HBSS H9269 Sigma, Milan, I: CaCl₂ anhydrous 185 mg/l, MgSO₄ 7H₂O 98 mg/l, KCl 400 mg/l, KH₂PO₄ 60 mg/l, NaHCO₃ 350 mg/l, NaCl 8000 mg/l, Na₂HPO₄ 48 mg/l, D-glucose 1000 mg/l, Phenol Red 11 mg/l, buffered at pH 7.4 with) at pH 7.4 were used.

The amount of CsA permeated through the cell layer (permeation) and the amount retained in the cell layer (penetration) were evaluated for SLN colloidal dispersion loaded with CsA. CsA suspension at the concentration of 0.05% (w/w) was used as reference. At fixed time intervals the basolateral phase was withdrawn and replaced with 2 ml of fresh HBSS. At the end of the experiment the apical phase was replaced with 1 ml of Lucifer Yellow solution at 25 μ g/ml (LY, Sigma) using 1 h as contact time, to evaluate if the tight junctions were opened and the paracellular route was enlarged.

CsA permeated across the cell substrate was extracted from the basolateral phase using chloroform and assayed by means of the HPLC method while the CsA penetrated and retained into the cell substrate was assayed by means of the HPLC method after the cell digestion. The digestion was performed by adding 1 ml of NaOH solution (0.5 M) to the cell substrate and shaking the system for 12 h at 37 °C (De Campos et al., 2003). At the end of this time the drug was extracted using 1 ml of chloroform. The chloroform was evaporated and the residue of CsA was solubilized using HPLC mobile phase and assayed as previously described (paragraph 2.6.). A calibration curve was made following extraction of different amounts of CsA. (Midelfart et al., 1999) according to the above-mentioned protocol (De Campos et al., 2003).

LY permeated across the cell substrate was quantified using a spectrofluorimetric method ($\lambda_{\text{exc}}=428$ nm and $\lambda_{\text{emm}}=521$ nm) (LS50B, PerkinElmer) (Suzuki and Hara, 2004; Wright et al., 1992).

2.12. Ex vivo permeation/penetration studies (excised pig cornea)

Pig cornea was excised immediately after the animal sacrifice (local slaughter-house) and put in an iced (4 °C) Krebs buffer in which oxygen was continuously bubbled. Each cornea was placed between the donor and the acceptor chamber of Valia-Chien diffusion cells (PermeGear) and oxygen was bubbled into Krebs buffer in both chambers. 200 μ l of each sample (CsA and RhB loaded SLN,

CsA and RhB suspension/solution at 0.05%), was added to the donor phase. After 1 h of contact time each cornea was washed.

Each cornea was digested using 500 μ l of 0.5 M NaOH for 12 h at 37 °C in a shaking-bath (De Campos et al., 2003) and CsA was extracted using chloroform (1 ml) and analyzed following the procedure previously described (Midelfart et al., 1999). A calibration curve was made following extraction of different amounts of CsA according to the above-mentioned protocol (De Campos et al., 2003).

2.13. Confocal laser scanning microscopy studies

RCE cells grown on filters in 12 well plates and subjected to permeation/penetration measurements were analyzed using CLSM to visualize Rhodamine loaded SLN entrapped into the cells. The cell substrates were fixed in ethanol and dried overnight.

Corneas subjected to permeation/penetration measurements of the RhB samples were rinsed in physiological solution, included in the OTC compound (Leica Microsystem) and frozen in liquid N₂ and stored at –80 °C. Slices perpendicular to the mucosa surface, 25 μ m in thickness, were cut using a cryostat (Leica CM1510, Leica Microsystem) at –20 °C. Each slice was placed on a microscope slide, dehydrated for 12 h and subsequently fixed by dipping the microscope slides in acetone.

Cell nuclei of cell substrate and cornea tissue were stained by dipping the biological substrates into a solution (1:100,000) of Hoechst 33258 (Sigma, Milan, I). Each cell filter was mounted using PVA-DABCO, polyvinyl alcohol mounting medium with DABCO antifading (mixture of tris (hydroxymethyl) aminomethane/tris (hydroxymethyl) aminomethane hydrochloride, polyvinyl alcohol 22,000 Da, glycerol anhydrous and 1,4-diazabicyclo[2.2.2]octane; BioChemika, Fluka, I) and covered with cover glass.

A confocal microscope (Leica DM IRBE/Leica TCS SP2) was used to observe the cellular uptake of SLNs () at a wavelength of $\lambda_{\text{ex}}=543$ nm and $\lambda_{\text{emm}}=570$ nm/620 nm for RhB; $\lambda_{\text{ex}}=346$ nm and $\lambda_{\text{emm}}=460$ nm for Hoechst 33258.

The acquired images were processed by means of specific software (Leica Microsystem).

2.14. Statistical analysis

Statistical differences were determined using ANOVA followed by Tukey's test for comparisons between groups. The significance level was taken as 95% ($P < 0.05$).

3. Results and discussion

3.1. Preparation of CsA and RhB loaded SLNs

The studies have shown that production parameters have a significant effect on the particle size of the SLN systems (Almeida et al., 1997). As the PS decrease, the total area of the active substance and the interaction with the body fluids increase. This phenomenon results in the enhancement of bioavailability (Sjöstrom et al., 1993). In our study, SLNs were prepared by high shear homogenization and ultrasound method that does not involve large amounts of surfactants which must be avoided in ophthalmic formulations. It has been shown that there is a relationship between the high shear homogenization and the kinetic energy of the system (Boistelle and Astier, 1988). This kinetic energy causes the system to load up static energy causing an agglomeration of the small particles (Jenning et al., 2000b). Table 1 shows the results of particle size and polydispersity index of SLN formulations prepared using different production conditions (mixing time (MT), dilution temperature (DT), ultrasonication time (UT) and total volume of formulation (VF)). 5 min

mixing time allowed us to obtain SLN formulations characterized by the lowest particle size and polydispersity index.

The re-crystallization of the melted lipid and hydrophobic active substance during emulsification was identified, according to the literature, as the most critical parameter of the nanoparticle formulation (Siekman and Westesen, 1994). Therefore, the system was quickly cooled down to avoid the agglomeration during emulsification. A dilution temperature of +4 °C allowed us to obtain SLN formulation characterized by the lowest particle size and polydispersity index (Table 1).

Ultrasonication time did not show any significant effect on the PS of the formulations. However, ultrasonication plays a key role in obtaining a formulation characterized by unimodal narrow distribution: without ultrasonication, the average PS increased from 224.8 to 268.3 nm (Table 1). 10 min ultrasonication was considered suitable for SLN production procedure.

The total volume of the preparation is important for the resulting PS because the basic factor in high shear homogenization method is the Ultra-Turrax mixing efficiency (Mehnert and Mäder, 2001), which in turn is affected by the preparation volume. A total volume of 25 ml in the present case corresponded to the best results in terms of PS and PI of the SLNs (Table 1).

Finally, the ratio of lipid and surfactants was evaluated. It was seen that the increment in the PS was directly proportional to the amount of lipid in SLN formulations. F6 formulation (blank, without CsA), characterized by the smallest (206.7 nm) and most uniform particle size, was chosen as the more suitable system for ophthalmic applications (Table 2). However, when CsA was loaded in F6 SLNs, after 2 days of storage, particle aggregates, visible to the naked eye, occurred. Therefore, Tw 80 was added as co-surfactant. In an optimization study on lipid nanospheres, Quintanar-Guerrero et al. (2005) evidenced that a suitable surfactant or the addition of co-surfactant (Tw 80, P 188 and P 407) played a key role in avoiding particle aggregation and in stabilizing the formulation. Moreover, particle dimensions were dependent on the surfactants used (Kim et al., 2005; Müller et al., 1995). Given the hydrophilic character of Tw80 (HLB = 15) (Ren et al., 2008; Lo, 2003), it was presumed that the addition of Tw 80 to SLN formulations contributed to make SLN systems more hydrophilic, modifying their HLB towards hydrophilic values and causing a narrow size distribution. Such a consideration is supported by previous studies by Törsten and

Müller (2005) in which it was stated that surfactant concentration and the HLB values played an important role on both particle size distribution and surface adsorption properties: the addition of Tw 80 in polysorbate-stabilized SLNs increased the ability of surfactants to be adsorbed into hydrophobic surfaces (Törsten and Müller, 2005).

CsA loaded F12 formulation, characterized by the lowest particle size and polydispersity index (PS: 225.9 nm ± 5.5 and PI: 0.253 ± 0.05) and by the absence of visible particle aggregates, was considered as the optimum formulation and employed in further release and toxicity studies. The unimodal distribution of F12 is shown in Fig. 1.

RhB was used in many imaging studies to mimic hydrophobic substances (Guss et al., 1984). CsA was replaced with RhB in F12 formulation for confocal microscopy imaging studies. PS and PI were determined in this case as 228.6 ± 4.27 and 0.211 ± 1.12 nm respectively. No significant difference in PS and PI could be detected between CsA and RhB loaded SLNs ($P > 0.05$).

Table 2 shows pH values of SLN systems that range between 6.87 and 7.0. The pH of ophthalmic preparation is important for patient compliance: although the pH of tears is 7.2–7.4, it is believed that preparations with pH values between 6.8 and 8.2 can be tolerated by the eye without irritation (Bodor and Buchwald, 2005).

3.2. Measurement of zeta potential

The zeta potential is the overall charge a particle acquires in a specific medium. The magnitude of the zeta potential gives an indication of the potential physical stability of a colloidal system. If all the particles have a large negative or positive zeta potential they will repel each other and dispersion is considered to be stable (Riddick, 2007).

Since F10 and F13 formulations, containing the lowest Tw 80 concentration (0.1%), showed visible aggregates, the zeta potential of these systems was not evaluated (Table 2). The decrease of Tw 80 concentration (non-ionic surfactant) from 0.4% (F8) to 0.2% (F9) caused a decrease of zeta potential from -20.8 ± 0.4 to -23.6 ± 0.6 . However, in CsA loaded SLNs, no significant difference in zeta potential was detected between F11 and F12 characterized by zeta potentials of -16.1 ± 0.6 and -16.9 ± 0.7 , respectively. High concentrations of the emulsifier reduce the surface tension and

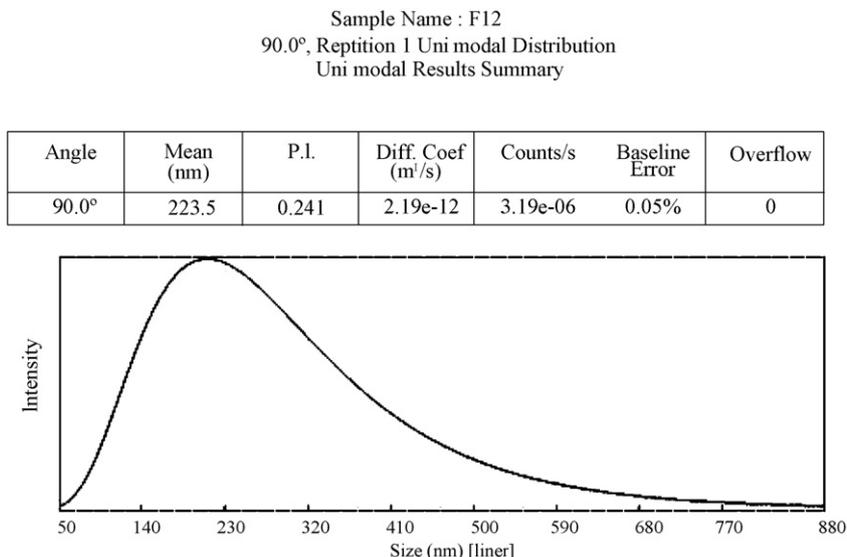


Fig. 1. The unimodal particle size distribution of F12 SLN formulation (consisting of 0.4% C 888, 0.2% P 188, 0.1% Tw 80 and 0.05% CsA) measured at 25 °C by Photon Correlation Spectroscopy (Beckman Coulter N5) at an angle of 90°.

facilitate the particle partition during homogenization. Since the decrease in particle size is connected with a tremendous increase in surface area, higher Tw 80 concentrations have a stabilization effect: in fact the emulsification process implies a primary coverage of the new surfaces by the surfactant molecules, competing with the agglomeration of uncovered lipid surfaces (Mehnert and Mäder, 2001).

3.3. Sterilization of SLNs

Since all ophthalmic formulations have to be sterile, one of the challenges of SLN production is the sterilization process. Although sterile filtration was proposed as an alternative method, filtration-sterilization of dispersed systems (using membrane with pore size of 0.22 μm) requires high pressure and is not applicable to particles greater than 0.2 μm (Mehnert and Mäder, 2001).

Steam sterilization resulted in an increment in particle sizes for both the temperatures considered in the present study (Table 2). The PS of blank and loaded formulations without Tw 80 increased to micron levels and the system was no longer unimodal, when they were sterilized in an autoclave at 121 °C for 15 min. This result

was thought to be due to the partial collapse of polymer adsorption layer. Tw 80 conferred a protective effect in the sterilization against the PS growth when added to SLN formulation. Tw 80 added to CsA loaded formulations, F11 and F12, showed an increase in PS of 79.9% and 15.9%, respectively.

In a study of Schwarz (Schwarz and Mehnert, 1995) long term sterilization at lower temperatures was proposed instead of 121 °C for 15 min. On that basis, F12 was sterilized at 110 °C, for 30 min and an increment in PS limited to 11% was obtained. After sterilization process at 110 °C, the zeta potential decreased from -16.9 ± 0.7 to -20.5 ± 0.5 , pointing out a better stability in comparison to loaded but not sterilized F12 formulation. In fact the measurement of the zeta potential allows for predictions regarding the storage stability of colloidal dispersion: in particular, particle aggregation is generally less likely to occur for charged particles (high zeta potential) due to electric repulsion (Mehnert and Mäder, 2001).

A temperature increase affects the mobility and the hydrophilicity of all emulsifiers to a different extent. Steam sterilization causes the formation of an o/w-emulsion due to the melting of the lipid particles and solid particles are reformed after lipid re-crystallization (Mehnert and Mäder, 2001). A system desta-

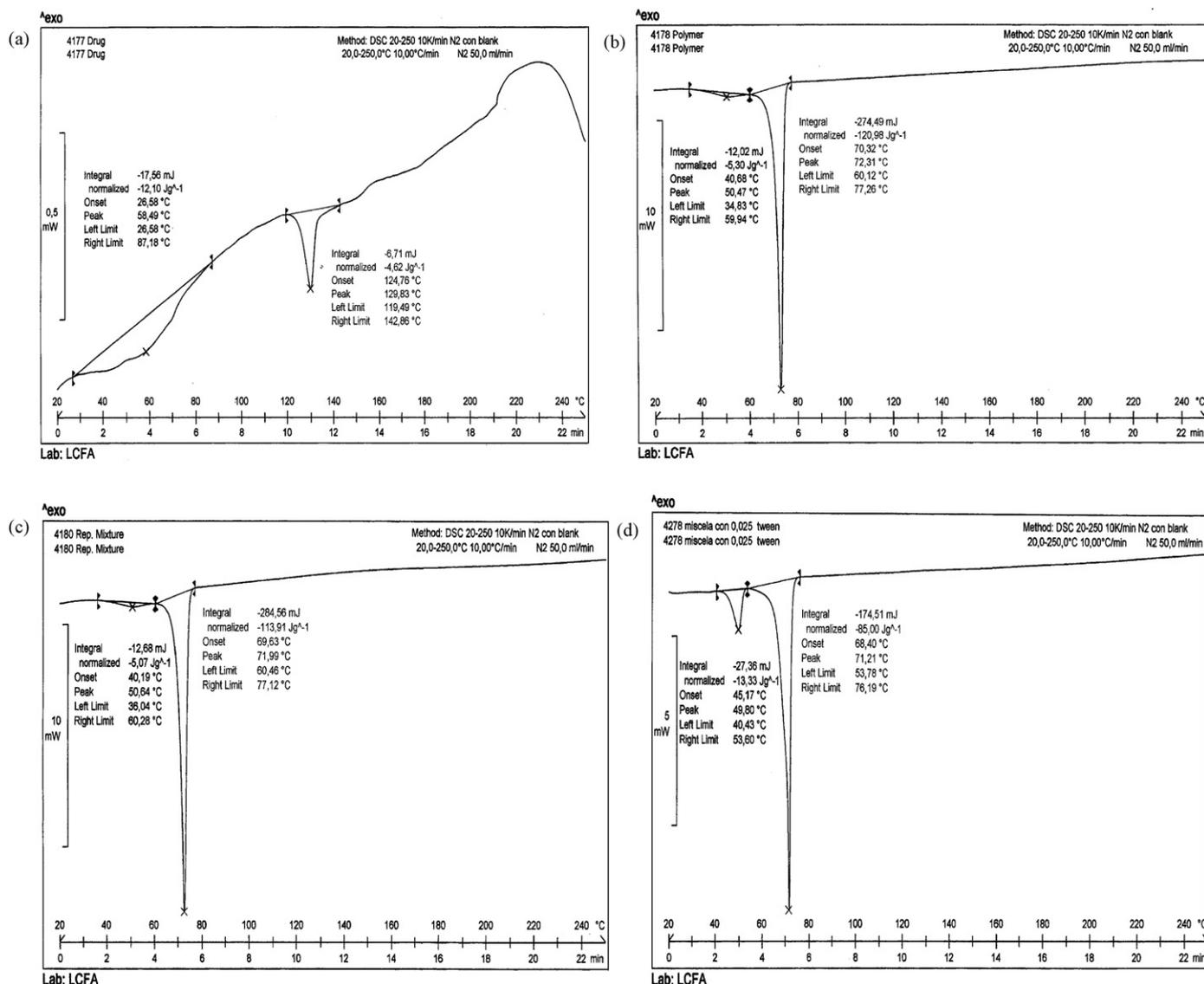


Fig. 2. DSC thermograms of (a) CsA, (b) bulk C 888, (c) physical mixture of C 888: CsA (10:1), and (d) F12 SLN formulation.

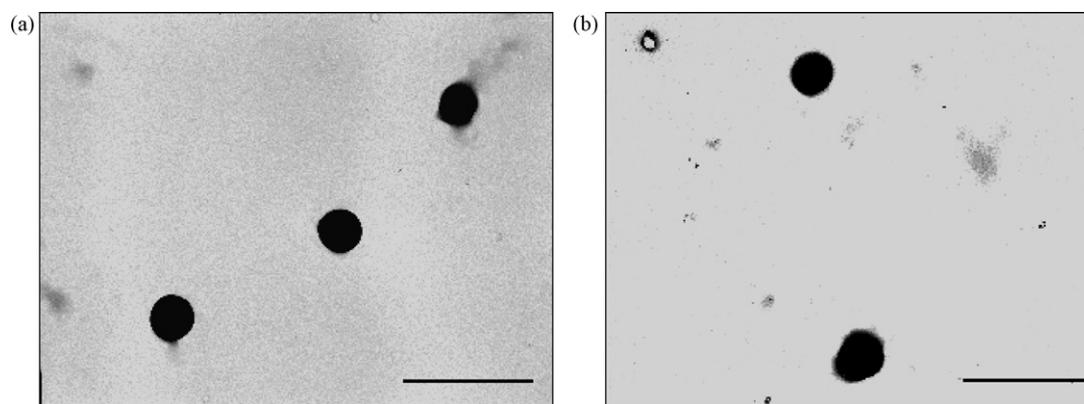


Fig. 3. Transmission Electron Microscopy photomicrographs of (a) F12 SLN formulation containing 0.05% CsA before sterilization (b) F12 SLN formulation after being sterilized in a thermo-controlled autoclave at 110 °C for 30 min. (Bar = 500 nm.)

bilization (particle size increase, aggregation) was attributed to a distortion of the mechanical properties of the surfactant film (Mehnert and Mäder, 2001). It is conceivable that as the temperature increased, the reformation of nanoparticles encountered more difficulty due to the possible influence of lipids on crystallization time and an interaction of surfactants.

3.4. Solubility studies of CsA with surfactants employed

The solubility of CsA (mg/ml \pm S.D.) was affected by the different systems according to the following rank order:

P 188 + Tw 80 (0.163 mg/ml \pm 0.001)

> Tw 80 (0.087 mg/ml \pm 0.002)

> No Surfactant (0.04 mg/ml \pm 0.004)

\geq P 188 (0.035 mg/ml \pm 0.005)

It has been reported that CsA is less soluble in P 188/water mixtures than in water alone at temperatures between 20 and 37 °C (Molpeceres et al., 1996). Our data was in accordance with these previous solubility results. In a study of Ran et al. (2001), Tw 80 increased the solubility of CsA. A synergistic effect was also determined in our study by the concomitant use of P 188 and Tw 80.

3.5. Drug entrapment efficiency

A validated HPLC method (Husek, 1997) was used to measure the concentration of CsA in the aqueous phase. EE% of CsA (the percentage of CsA encapsulated with respect to the total amount of CsA added to the system) was as high as 95.6%. This result was expected because of the high solubility of the drug in lipid matrices. Since CsA is a very poor water soluble drug, it was preferentially partitioned in the lipid phase of the emulsion and a consequently small amount of the drug was lost in the aqueous phase. Although high pressure homogenization method was used to prepare SLN dispersions, a similar amount of CsA entrapment (96%) was determined in the study of Müller et al. (2006).

3.6. DSC analysis

DSC was used to investigate the melting and crystallization behavior of materials and SLNs. The thermograms of bulk lipid C888, CsA, lipid:drug physical mixture (10:1) and F12 formulation were shown in Fig. 2. The melting process for C 888 and CsA took place with maximum peaks at 72.31 and 129.83 °C, respectively. It

was seen that lipid polymer C 888 was completely in crystalline form. The CsA peak was lost in the formulation, pointing out the solubilization of CsA in the lipid (Fig. 2).

3.7. TEM analysis

TEM analysis confirmed the spherical shape, the smooth surface and colloidal sizes of SLNs as can be seen in Fig. 3. The regular round shapes of the nanoparticles have changed into more amorphous geometrical shapes after sterilization probably due a SLN re-crystallization during the cooling down. Even though during the 110 °C sterilization temperature the particle flocculation was avoided, as confirmed by particle size evaluation by PCS, TEM suggested that this process partially modified the particle shape making it become less regular (see for example SLN in the lower part of Fig. 3b). A possible explanation is that adsorption layer of P188 (polymeric surfactant) around SLN was altered (Mehnert and Mäder, 2001).

The reason for this result is probably due to a lack of additional high shear mixing force during the re-crystallization process.

3.8. Studies of enzymatic CsA release from SLN

No release could be detected under the conditions of our studies without lipase and co-lipase enzyme complex. After addition of the enzyme mixture to loaded SLN dispersion (F12), a release could be observed that is well correlated with particle size decrease.

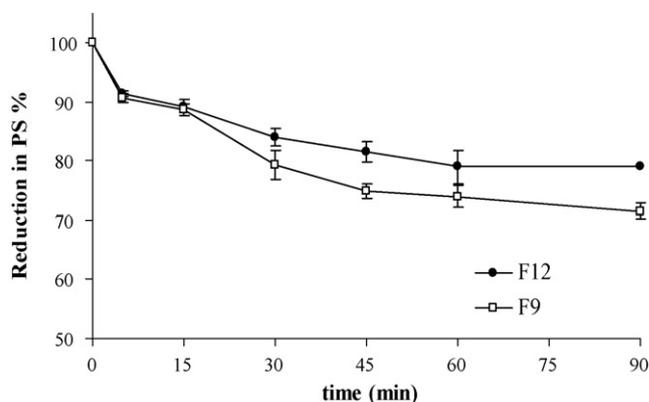


Fig. 4. The particle size reduction profile of 0.05% CsA loaded SLN (F12) and blank SLN (F9) in borate/boric acid buffer (pH 7.4) with the presence of lipase/co-lipase enzyme complex, at 37 °C. The results are the means \pm S.D. (n = 6).

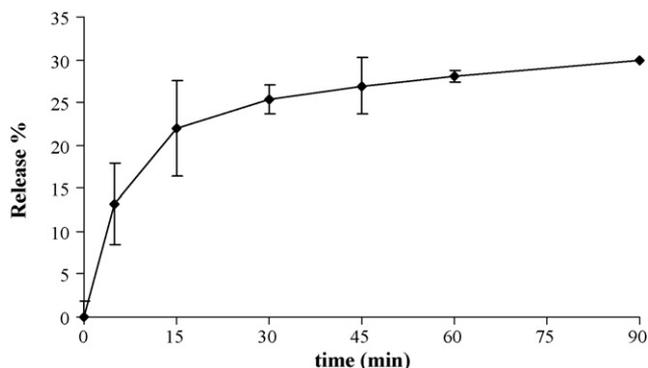


Fig. 5. Drug release profile of 0.05% CsA loaded SLN (F12) in borate/boric acid buffer (pH 7.4) with the presence of lipase/co-lipase enzyme complex, at 37 °C. The results are the means \pm S.D. ($n = 6$).

ment (Fig. 4). The decrease in the dimension of unloaded and loaded SLNs was calculated as 29% and 21%, respectively. As the enzyme degraded the lipid matrix into fatty acids, 28% of CsA was released (Fig. 5). These results showed that drug release from CsA loaded SLNs was enzymatic degradation dependent. In a study of Olbrich et al. (2002), it was similarly shown that SLNs prepared with Dynasan were degraded by lipase.

3.9. Toxicity studies

The toxicity was determined by means of Neutral Red assay. As cells lose viability, their membrane permeability properties change and they lose the ability to take up Neutral Red (Babich and Borenfreund, 1990). As can be seen in Fig. 6, the blank and loaded formulations were biocompatible. P 188 solution is characterized by cell viability comparable to that of the growth medium used as control. There was also no toxicity when formulation was sterilized at 110 °C. The viability decreased down to 60% at the end of 2 h, when the cells were treated with Tw 80 solution alone (Table 3). The SLNs sterilized at 121 °C also showed toxicity similar to that of the Tw 80 solution. The difference between the two sterility processes was significant ($P < 0.05$). The cell damaging effect of the surfactants depends on whether they are free in solution or bound to a surface (Müller et al., 1997). It was supposed that Tw 80 might be released during sterilization to a higher extent at 121 °C than at 110 °C, causing cell damage.

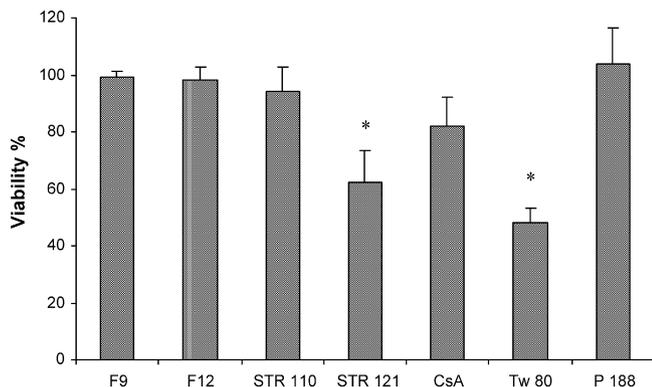


Fig. 6. Viability % of rabbit cornea epithelial cells after 2 h treatment with 100 μ l of blank SLN (F9), 0.05% CsA loaded SLN (F12), F12 sterilized at 110 °C (STR 110), F12 sterilized at 121 °C (STR 121), 0.05% CsA solution (CsA), 0.1% Tween 80 solution (Tw 80), 0.2% Poloxamer solution (P 188). The viability of cells treated with STR 121 and Tw 80 was significantly lower ($P < 0.05$) when compared to the other groups. The results are the means \pm S.D. ($n = 4$).

Table 3

The cellular viability percentage of blank (F9), loaded (F12) and sterilized SLN formulations at 110 °C (STR 110) and 121 °C (STR 121), 0.05% of Cyclosporin A solution (CsA), 0.1% of Tween 80 solution (Tw 80) and 0.2% of Poloxamer 188 solution (P 188)

Code	Viability% \pm S.D.
F9	99.0 \pm 2.12
F12	98.3 \pm 4.19
STR 110	94.0 \pm 8.92
STR 121	62.4 \pm 10.70
CsA	81.9 \pm 10.00
Tw 80	48.1 \pm 4.88
P 188	103.9 \pm 12.60

The results are the means \pm S.D. ($n = 4$).

3.10. In vitro and ex vivo permeation/penetration studies

The in vitro evaluation on RCE cells evidenced that CsA loaded SLN formulation did not show significantly different drug permeation and penetration results with respect to those obtained using CsA suspension (reference). In both cases (SLN and drug suspension) the drug% permeated or penetrated was close to the limit of detection.

Different results were obtained using excised pig cornea as biological substrate. Fig. 7 shows the profiles of CsA permeated across pig cornea as a function of time. CsA was not able to permeate across pig cornea tissue without a suitable carrier. The colloidal system, CsA loaded SLN, was characterized by the 24% of CsA permeated after 2 h of contact time.

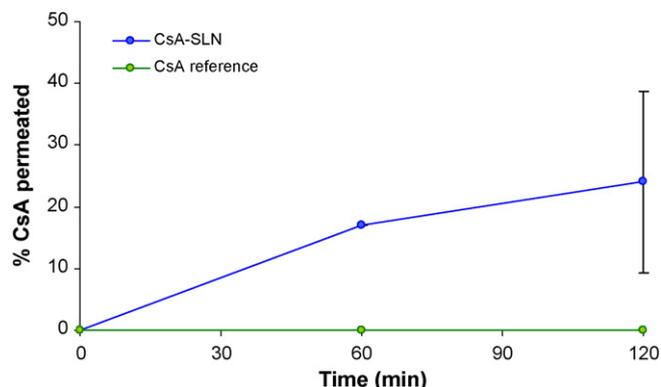


Fig. 7. The profiles of CsA (as percentage of the amount put in contact with the tissue) permeated across pig cornea as a function of time. The results are the means \pm S.D. ($n = 3$).

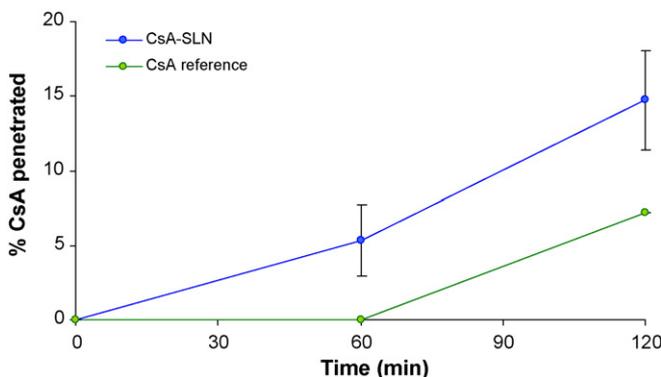


Fig. 8. The profiles of CsA (as percentage of the amount put in contact with the tissue) penetrated in the cornea tissue as a function of time. The results are the means \pm S.D. ($n = 3$).

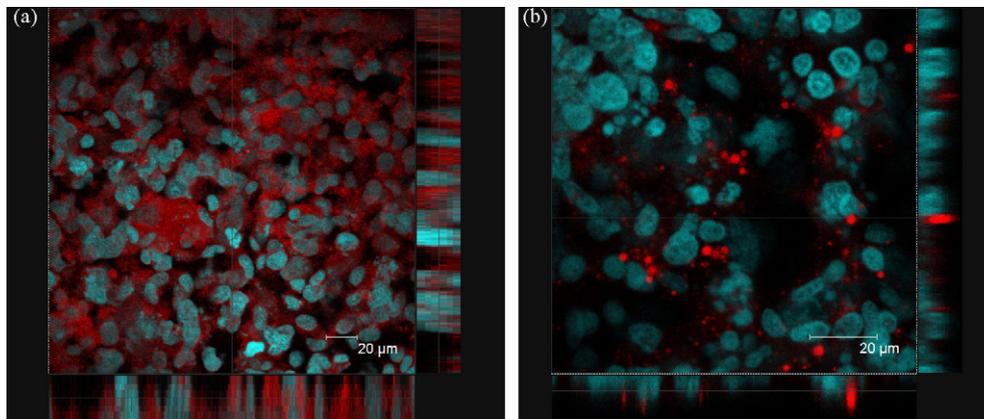


Fig. 9. Confocal images of (a) RCE cells exposed to 0.05% Rhodamine B suspension as reference. (b) RCE cells exposed to 0.05% Rhodamine B loaded SLNs. The blue fluorescence corresponds to nuclei. The SLN formulation can be seen as red spots.

Fig. 8 reports the profiles of CsA penetrated in the excised cornea as a function of time. After 1 h of contact between sample and tissue no CsA penetration into the cornea could be detected for CsA suspension (reference) while after 2 h the CsA penetrated was 7% of the total amount put into contact with the tissue.

CsA loaded SLNs were characterized by higher penetration profiles with respect to the reference, and 15% of CsA was detected inside the tissue after 2 h of contact time.

No correlation of results can be evidenced between the *in vitro* experiments (RCE cell substrate) and the *ex vivo* substrate (excised pig cornea): this discrepancy is conceivably due to the different structures of the biological substrates: the RCE cells were just epithelial ones while the cornea was obviously more complex and consisted not only of the epithelial cells but also of immune cells which have higher affinity with lipidic particles.

3.11. CLSM studies

To evaluate the eventual cellular internalization (uptake), the RCE cell substrate (*in vitro*) and excised pig cornea (*ex vivo*) were kept in contact with SLNs loaded with RhB, a fluorescent probe, which allowed the visualization of the nanosystems by means of CLSM.

Fig. 9 shows the microphotographs of the RCE cell substrate after contact with 0.05% RhB suspension (reference) (a) and RhB loaded SLNs (b). The blue spots are the cell nuclei.

The RhB suspension (**Fig. 9a**) used as a reference causes a diffuse red fluorescence inside the cells due to the natural tendency of RhB to penetrate into the biological substrates.

A high number of RhB SLNs (**Fig. 9b**) can be seen as red spots of intense fluorescence showing that they are able to interact with the RCE cells. The confocal projection along the z-axis evidences that the interaction of nanoparticles with the biological substrate is not only localized at the membrane surface of the cells. In fact the SLNs are visible in depth inside the substrate. Even if the amount of CsA penetrated into the cell layers could not be quantified (being lower than the detection limit), it can be noticed that there is a captation/internalization of SLNs and not just a simple diffusion of the fluorescent dye as in the reference.

The same evaluation was performed on excised pig cornea after contact with RhB loaded SLNs and RhB suspension as reference.

Fig. 10 illustrates the microphotographs of cornea section obtained after contact with 0.05% RhB suspension used as reference (a) and RhB loaded SLNs (b). The blue spots are the cell nuclei.

Also with this substrate, RhB suspension as reference (**Fig. 10a**) caused a slight red fluorescence inside the tissue depth, indicating a limited diffusion of RhB into the tissue.

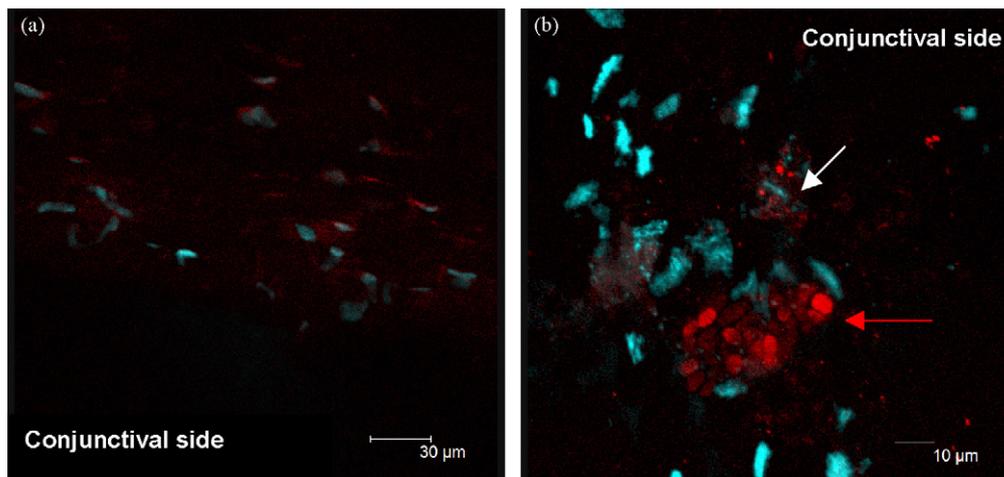


Fig. 10. Confocal images of (a) cornea section exposed to 0.05% Rhodamine suspension as reference. (b) Cornea section exposed to 0.05% Rhodamine B loaded SLNs. White arrow: spots attributable to SLNs. Red arrow: spots probably due to Rhodamine B released from SLNs. The blue fluorescence corresponds to nuclei.

The section corresponding to the cornea after contact with RhB SLNs (Fig. 10b) evidences two kinds of red spots. The smallest spots (white arrows) can be attributed to SLNs penetrated into the cornea tissue, to confirm the penetration properties evidenced in the RCE substrate. The biggest spots (red arrows) are characterized by a size greater than the SLN dimensions. It can be supposed that they are due to an intracellular release of Rhodamine B from SLN, probably due to endosomal enzymatic activity.

4. Conclusion

CsA loaded SLNs were prepared by means of high shear homogenization and ultrasound method with high drug loading efficiency. The combination of Tw 80 and P 188 as surfactants improved the physical stability of the system avoiding aggregation. Particle size, polydispersity index and formulation pH are suitable for ophthalmic application and good patient compliance can be expected as blurred vision and irritation are avoided. The viability of rabbit cornea epithelial (RCE) cells is not affected by SLN formulation, but different results can be obtained depending on sterilization temperature: in particular 110 °C was a more suitable temperature for steam sterilization to maintain an unaltered particle size and polydispersity index, in comparison to 121 °C. Moreover, steam sterilization increased the zeta potential of drug loaded SLN formulation with a positive effect on physical stability: in fact particle aggregation is generally less likely to occur for charged particles (high zeta potential) due to electric repulsion (Mehnert and Mäder, 2001).

It was found that the release of CsA from SLNs, under the conditions of our study, was dependent on the presence of lipase/co-lipase.

The in vitro studies showed that the CsA amounts permeated/penetrated across/into the RCE cell substrate were below the detection limits, both for SLN formulation and for drug suspension reference. In the ex vivo studies, SLNs improved penetration of CsA across/into excised cornea tissue. Apparently no correlation can be evidenced between the results of in vitro experiments (RCE cell substrate) and those of ex vivo substrate (excised pig cornea), probably due to the different structure of the substrates. However, the CLSM observation performed on both the biological substrates after contact with Rhodamine suspension and SLN formulation, show that SLN are able to penetrate into both the biological substrates by means of a captation/internalization mechanisms of SLNs into the cells.

The optimized CsA SLN demonstrates to be a promising formulation to target CsA to the cornea.

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References

- Almeida, A.J., Runge, S., Muller, R.H., 1997. Peptide-loaded solid lipid nanoparticles (SLN): influence of production parameters. *Int. J. Pharm.* 149, 255–265.
- Babich, H., Borenfreund, E., 1990. Applications of the neutral red cytotoxicity assay to in vitro toxicology. *Altern. Lab. Anim.* 18, 129–144.
- Bodor, N., Buchwald, P., 2005. Ophthalmic drug design based on the metabolic activity of the eye: soft drugs and chemical delivery systems. *AAPS Pharm. Sci. Tech.* 7, E820–E833.
- Boistelle, R., Astier, J.P., 1988. Crystallization mechanisms in solution. *J. Cryst. Growth* 90, 14–30.
- Bourlairs, C.L., Acar, L., Zia, H., Sado, P.A., Needham, T., Leverage, R., 1998. Ophthalmic drug delivery systems—recent advances. *Prog. Retin. Eye Res.* 17, 33–58.
- Burgalassi, S., Chetoni, P., Monti, D., Saettone, F., 2001. Cytotoxicity of potential ocular permeation enhancers evaluated on rabbit and human corneal epithelial cell lines. *Toxicol. Lett.* 122, 1–8.
- Burgalassi, S., Monti, D., Brignoccoli, A., Fabiani, O., Lenzi, C., Pirona, A., Chetoni, P., 2004. Development of cultured rabbit corneal epithelium for drug permeation studies: a comparison with excised rabbit cornea. *J. Ocul. Pharmacol. Ther.* 20, 518–532.
- Cavalli, R., Gasco, M.R., Chetoni, P., Burgalassi, S., Saettone, M.F., 2002. Solid lipid nanoparticles (SLN) as ocular delivery system for tobramycin. *Int. J. Pharm.* 238, 241–245.
- Cavalli, R., Morel, S., Gasco, M.R., Saettone, M.F., Chetoni, P., 1995. Preparation and evaluation in vitro of colloidal lipospheres containing pilocarpine as ion-pair. *Int. J. Pharm.* 117, 243–246.
- De Campos, A., Sanchez, A., Gref, R., Calvo, P., Alonso, M.J., 2003. The effect of a PEG versus a chitosan on the interaction of drug colloidal carriers with the ocular mucosa. *Eur. J. Pharm. Sci.* 20, 73–81.
- Ding, S., 1998. Recent developments in ophthalmic drug delivery. *PSTT* 1, 328–335.
- Fotakis, G., Timbrell, J.A., 2006. In vitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicol. Lett.* 160, 171–177.
- Guo, J., Wu, T., Ping, Q., Chen, Y., Shen, J., Jiang, G., 2005. Solubilization and pharmacokinetic behaviors of sodium cholate/lecithin-mixed micelles containing cyclosporine. *Adv. Drug Deliv. Rev.* 12, 35–39.
- Guss, R., Johnson, F., Maurice, D., 1984. Rhodamine B as a test molecule in intraocular dynamics. *Invest. Ophthalmol. Vis. Sci.* 25, 758–762.
- Husek, A., 1997. High performance liquid chromatography cyclosporin A and its oral solution. *J. Chromatogr. A* 759, 217–224.
- Italia, J.L., Bhardwaj, V., Kumar, M.N., 2006. Disease, destination, dose and delivery aspects of cyclosporine: the state of the art. *Drug Discov. Today* 11, 846–854.
- Jenning, V., Gysler, A., Schafer-Korting, M., Gohla, S.H., 2000a. Vitamin A loaded solid lipid nanoparticles for topical use: occlusive properties and drug targeting to the upper skin. *Eur. J. Pharm. Biopharm.* 49, 211–218.
- Jenning, V., Mäder, K., Gohla, S., 2000b. Solid lipid nanoparticles (SLNTM) based on binary mixtures of liquid and solid lipids: a H-NMR study. *Int. J. Pharm.* 205, 15–21.
- Kim, B.D., Na, K., Choi, H.K., 2005. Preparation and characterization of solid lipid nanoparticles (SLN) made of cacao butter and curdlan. *Eur. J. Pharm. Sci.* 24, 199–205.
- Kuwano, M., Ibuki, H., Morikawa, N., Ota, A., Kawashima, Y., 2002. Cyclosporine A formulation affects its ocular distribution in rabbits. *Pharm. Res.* 19, 108–111.
- Lallemant, F., Felt-Baeyens, O., Besseghir, K., Behar-Cohen, F., Gurny, R., 2003. Cyclosporine A delivery to the eye: a pharmaceutical challenge. *Eur. J. Pharm. Biopharm.* 56, 307–318.
- Lo, Y., 2003. Relationships between the hydrophilic–lipophilic balance values of pharmaceutical excipients and their multidrug resistance modulating effect in Caco-2 cells and rat intestines. *J. Control. Release* 90, 37–48.
- Manjunath, K., Reddy, J.S., Venkateswarlu, V., 2005. Solid lipid nanoparticles as drug delivery systems. *Methods Find. Exp. Clin. Pharmacol.* 27, 127–144.
- Mehnert, W., Mäder, K., 2001. Solid lipid nanoparticles: production, characterization and applications. *Adv. Drug Deliv. Rev.* 47, 165–196.
- Midelfart, A., Dybdahl, A., Krane, J., 1999. Detection of dexamethasone in the cornea and lens by NMR spectroscopy. *Graefes Arch. Clin. Exp. Ophthalmol. Lab. Invest.* 237, 415–423.
- Molpeceres, J., Guzman, M., Bustamante, P., del Rosario Aberturas, M., 1996. Exothermic–endothermic heat of solution shift of cyclosporin A related to poloxamer 188 behavior in aqueous solutions. *Int. J. Pharm.* 130, 75–81.
- Müller, R.H., Rühl, D., Runge, S., Schulze-Forster, K., Mehnert, W., 1997. Cytotoxicity of solid lipid nanoparticles as a function of the lipid matrix and the surfactant. *Pharm. Res.* 14, 458–462.
- Müller, R.H., Mehnert, W., Lucks, J.S., Schwarz, C., zur Muhlen, A., Weyhers, H., Freitas, C., Rühl, D., 1995. Solid lipid nanoparticles (SLN)—an alternative colloidal carrier system for controlled drug delivery. *Eur. J. Pharm. Biopharm.* 41, 62–69.
- Müller, R.H., Runge, S., Ravelli, V., Mehnert, W., Thunemann, A.F., Souto, E.B., 2006. Oral bioavailability of cyclosporine: Solid lipid nanoparticles (SLN[®]) versus drug nanocrystals. *Int. J. Pharm.* 317, 82–89.
- Müller, R.H., Mäder, K., Gohla, S., 2000. Solid lipid nanoparticles (SLN) for controlled drug delivery: a review of the state of the art. *Eur. J. Pharm. Biopharm.* 50, 161–177.
- Olbrich, C., Kayser, O., Müller, R.H., 2002. Lipase degradation of Dynasan 114 and 116 solid lipid nanoparticles (SLN)—effect of surfactants, storage time and crystallinity. *Int. J. Pharm.* 237, 119–128.
- Popiolkiewicz, J., Polkowski, K., Skierski, J.S., Mazurek, A.P., 2005. In vitro toxicity evaluation in the development of new anticancer drugs—genistein glycosides. *Cancer Lett.* 229, 67–75.
- Qaddoumi, M.G., Ueda, H., Yang, J., Davda, J., Labhasetwar, V., Lee, V.H., 2004. The characteristics and mechanisms of uptake of PLGA nanoparticles in rabbit conjunctival epithelial cell layers. *Pharm. Res.* 21, 641–648.
- Quintanar-Guerrero, D., Tamayo-Esquivel, D., Ganem-Quintanar, A., Allemann, E., Doelker, E., 2005. Adaptation and optimization of the emulsification–diffusion technique to prepare lipidic nanospheres. *Eur. J. Pharm. Sci.* 26, 211–218.

- Ran, Y., Zhao, L., Xu, Q., Yalkowsky, S.H., 2001. Solubilization of Cyclosporin A. *AAPS PharmSciTech.* 2: Article 2.
- Reichl, S., Muller-Goymann, C.C., 2003. The use of a porcine organotypic cornea construct for permeation studies from formulations containing befunolol hydrochloride. *Int. J. Pharm.* 250, 191–201.
- Ren, S., Park, M.J., Sah, H., Lee, B.J., 2008. Effect of pharmaceutical excipients on aqueous stability of rabeprazole sodium. *Int. J. Pharm.* 350, 197–204.
- Riddick, T.M., 2007. Control of colloid stability. In: *Zeta Potential*. Zeta-Meter Inc., 765 Middlebrook Ave, Staunton, VA 24401, pp. 198–200.
- Schwarz, C., Mehnert, W., 1995. Sterilization of drug-free and tetracaine-loaded solid lipid nanoparticles (SLN). In: *Proc.1st World Meeting APGI/APV*, Budapest, pp. 485–486.
- Siekmann, B., Westesen, K., 1994. Thermoanalysis of the recrystallization of melt-homogenized glyceride nanoparticles. *Colloids Surf. B. Bioint.* 3, 159–175.
- Sjöstrom, B., Bergenstahl, B., Kronberg, B., 1993. A method for the preparation of submicron particles of sparingly water-soluble drugs by precipitation in oil-in-water emulsions. II: influence of the emulsifier, the solvent, and the drug substance. *J. Pharm. Sci.* 82, 584–589.
- Suzuki, T., Hara, H., 2004. Various nondigestible saccharides open a paracellular calcium transport pathway with the induction of intracellular calcium signaling in human intestinal Caco-2 cells. *J. Nutr.* 134, 1935–1941.
- Törsten, M., Müller, R.H., 2005. Polysorbate-stabilized solid lipid nanoparticles as colloidal carriers, for intravenous targeting of drugs to the brain: Comparison of plasma protein adsorption patterns. *J. Drug Target.* 13, 179–187.
- Wright, K.M., Davies, T.G.E., Steele, S.H., Leigh, R.A., Opark, K.J., 1992. Development of a probenecid-sensitive Lucifer Yellow transport system in vacuolating oat aleurone protoplasts. *J. Cell Sci.* 102, 133–139.