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Development of a novel ophthalmic ciclosporin A-loaded nanosuspension using top-down media milling methods

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To develop a novel ciclosporin A (CsA)-loaded nanosuspension causing less ocular irritation, a range of nanosuspensions was prepared with various polymers using a media milling method. The effects of polymer, milling time, milling speed and bead material on the particle size of the nanosuspension were investigated. Stability and irritation tests in rabbits' eyes were then performed comparing the nanosuspension with a commercial product. Of the nanosuspensions prepared with various polymers, that with PVA showed no creaming or sedimentation phenomena and gave the smallest particles of about 530 nm. The particle size decreased abruptly as the milling time increased to 2 h and then hardly decreased further. As the milling speed was increased, the particle size of CsA in the nanosuspension also increased. Nanosuspensions prepared with zirconia beads gave significantly finer particles than those with polystyrene beads. In particular, the CsA-loaded nanosuspension with a CsA/PVA/water weight ratio of 0.5/1/100 prepared using the top-down media milling method with zirconia beads of 300 μm diameter at 1000 rpm for 2 h gave a minimum particle size of about 530 nm. This nansuspension was physcally and chemically stable for at least two months. In the Draize test, both this nanosuspension and the commercial product gave very slight ocular irritation. However, in the Schirmer tear test, this nanosuspension caused less irritation to the rabbits' eyes compared to the commercial product. Thus, the CsA-loaded nanosuspension prepared with PVA and water using the top-down media milling method could be a promising candidate for causing less ocular irritation.

1. Introduction

Ciclosporin A (CsA), a lipophilic cyclic undecapeptide of fungal origin, is a potent immunomodulator that acts selectively and locally when administered to the ocular surface (Kapoor and Chauhan 2008; Shen et al. 2009). Since 2002, 0.05% CsA-loaded ophthalmic emulsion (Restasis®; Allergan Co., Invine, CA, USA) has been used for treating keratoconjunctivitis sicca (Jadhav et al. 2006; Lallemand et al. 2003; Tang-Liu and Acheampong 2005). However, this commercial product, which is composed of solubilized CsA, oil and a surfactant for fine dispersion of the oil phase, induces a severe side effect: ocular burning in 17% of patients (Rx list 2010). Thus, the development of novel CsA-loaded products causing less ocular irritation is needed.

A nanosuspension is defined as a sub-micron colloidal dispersion of discrete drug particles which are stabilized with the help of polymers, surfactants or a mixture of both. The small size of the particles in a nanosuspension provides a large drug surface area and increases the dissolution rate of poorly soluble drugs, resulting in improved bioavailability and the rapid onset of action (Basa et al. 2008; Van Eerdenbrugh et al. 2008). No harsh chemicals or co-solvents are used in the preparation of nanosuspensions. They are easy to apply industrially due to the simplicity of their formulation, the high drug loading capability and the ease of scaling up production (Verma et al. 2009b).

Nanosuspension preparation processes are classified as top-down or bottom-up. The top-down process consists of reducing the particle size of large drug particles into smaller particles using various wet milling techniques such as media milling, microfluidization and high pressure homogenization. No harsh solvents are used in these techniques. However, all media milling processes 'involve a high energy input, making them highly inefficient' (Verma et al. 2009a, b). Furthermore, a considerable amount of heat can be generated, which may cause the degradation of heat-sensitive active pharmaceutical ingredients. In this study, in order to develop a novel CsA-loaded nanosuspension with reduced ocular irritation, a range of nanosuspensions was prepared with various polymers using a top-down media milling method. The effects of polymer, milling time, milling speed and bead material on the particle size of the nanosuspension were investigated. Furthermore, stability and irritation tests in rabbits' eyes were performed comparing the nanosuspensions to a commercial product, Restasis®. Since the CsA-loaded nanosuspension contains no solubilized drug, oil or surfactant, unlike the commercial product, it was expected to cause less ocular irritation.

2. Investigations, results and discussion

In order to select a suitable stabilizer for a CsA-loaded nanosuspension, nanosuspensions were prepared with a polymer such

Table 1: Effects of the polymers on the dispersibility of CsA-loaded nanosuspensions

Polymer	Creaming	Sedimentation
PVP	–	+
PVA	–	–
HEC	–	++
HPC	–	++
HPMC	–	++

No creaming (or sedimentation): -
Creaming (or sedimentation): ++ > +

as PVA, PVP, HPC, HEC and HPMC as the surface stabilizer, and dispersibility tests were performed (Table 1). The nanosuspensions with cellulose derivative polymers such as HPC, HEC and HPMC gave no creaming but a sedimentation phenomenon occurred. Nanosuspensions with PVP were similar, but with a smaller amount of sedimentation. On the other hand, the nanosuspension with PVA gave no creaming or sedimentation phenomena.

About 2.5 g of CsA was added to 500 ml of water containing 5 g of surface stabilizer and the mixture was homogenized and milled with zirconia beads of 300 μm diameter at 1000 rpm for 2 h. The effect of the polymers on the particle size of CsA in the nanosuspension was investigated. As shown in Fig. 1, the particle size of CsA in the nanosuspensions was ranked in the following order: PVA < HPMC = HPC = HEC < PVP. In particular, the nanosuspension with PVA gave the smallest particle size of about 530 nm. The cellulose polymers with carboxymethyl groups and the PVP with amide groups might not have been adsorbed on to the surface of CsA. However, PVA might have

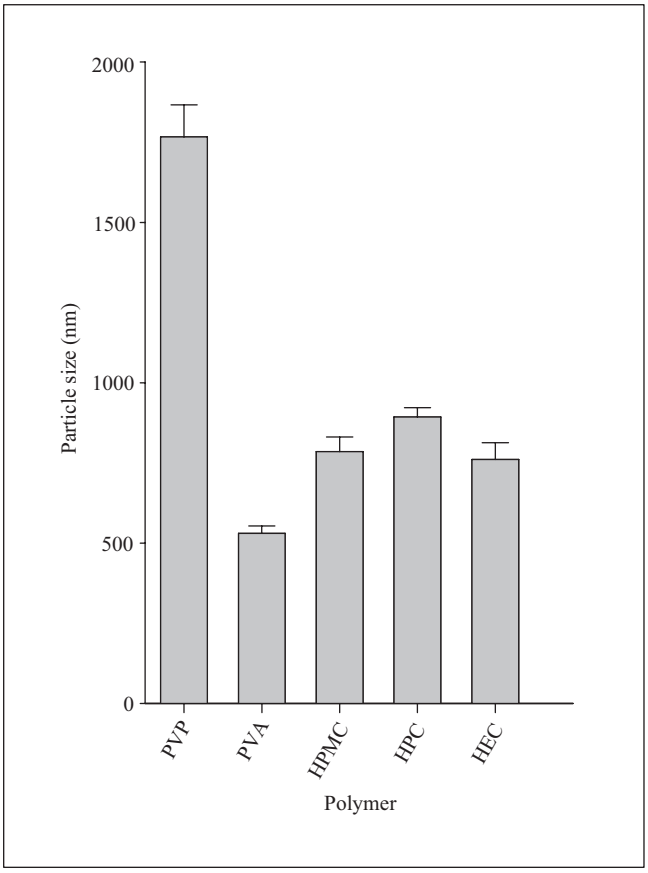

Fig. 1: Effecst of the polymers on the particle size of the nanosuspensions. Each value represents the mean ± S.D. (n = 3)

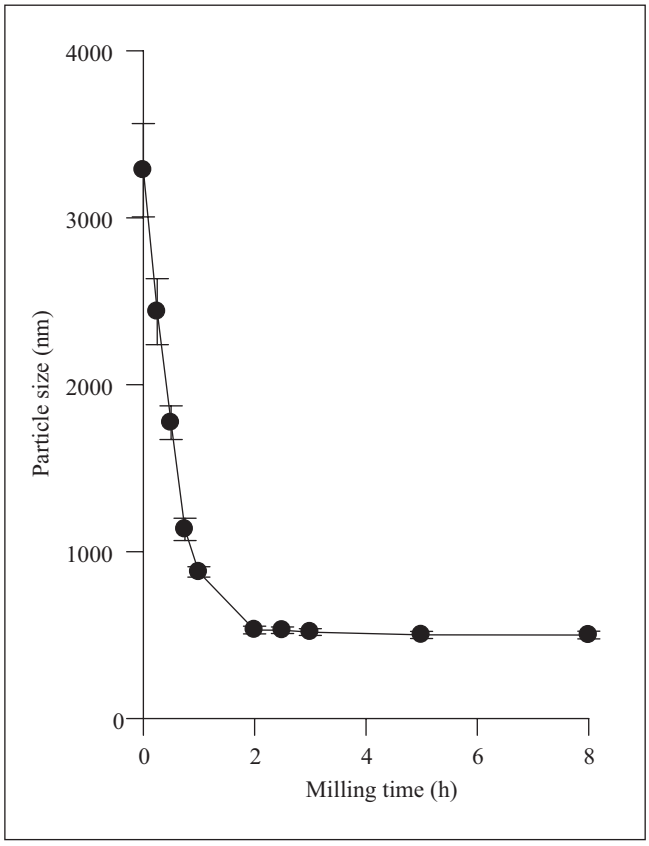

Fig. 2: Effect of the milling time on the particle size of the nanosuspensions. Each value represents the mean ± S.D. (n = 3)

been adsorbed on to the surface of CsA due to strong interactions of the hydrophobic (carboxyl) and hydrophilic (hydroxyl) groups (present in the polymeric chains), resulting in no creaming or sedimentation (Van Eerdenbrugh et al. 2009; Verma et al. 2009b). Thus, PVA was selected as the stabilizer for the CsA-loaded nanosuspension.

To investigate the effect of milling time on the particle size of CsA in the nanosuspension, about 2.5 g of CsA was added to 500 ml of water containing 5 g of PVA and homogenized and milled with zirconia beads of 300 μm diameter at 1000 rpm for various milling times. The particle size decreased abruptly as the milling time increased to 2 h, reaching a minimum of 533 nm and then hardly decreasing as the milling time increased further (Fig. 2) (Cerdeira et al. 2010). Our results suggested that the optimal milling time for the preparation of CsA-loaded nanosuspensions was 2 h.

Next, to investigate the effect of milling speed on the particle size of CsA in the nanosuspension, about 2.5 g of CsA was added to 500 ml of water containing 5 g of PVA and homogenized and milled with zirconia beads of 300 μm diameter at various milling speeds for 2 h. As the milling speed was increased, the particle size of CsA in the nanosuspension decreased (Fig. 3) (Van Eerdenbrugh et al. 2009). However, at a milling speed above 2000 rpm, broken beads were observed in the nanosuspension. Thus, for the preparation of the CsA-loaded nanosuspension, the milling speed was fixed at 1000 rpm.

Finally, to investigate the effect of bead materials on the particle size of CsA in the nanosuspension, about 2.5 g of CsA was added to 500 ml of water containing 5 g of PVA and homogenized and milled with various beads at 1000 rpm for 2 h. The nanosuspension prepared with zirconia beads gave significantly finer particles than that with polystyrene beads (Table 2). However, there was no significant difference in particle size between the two nanosuspensions prepared with different sized zirconia

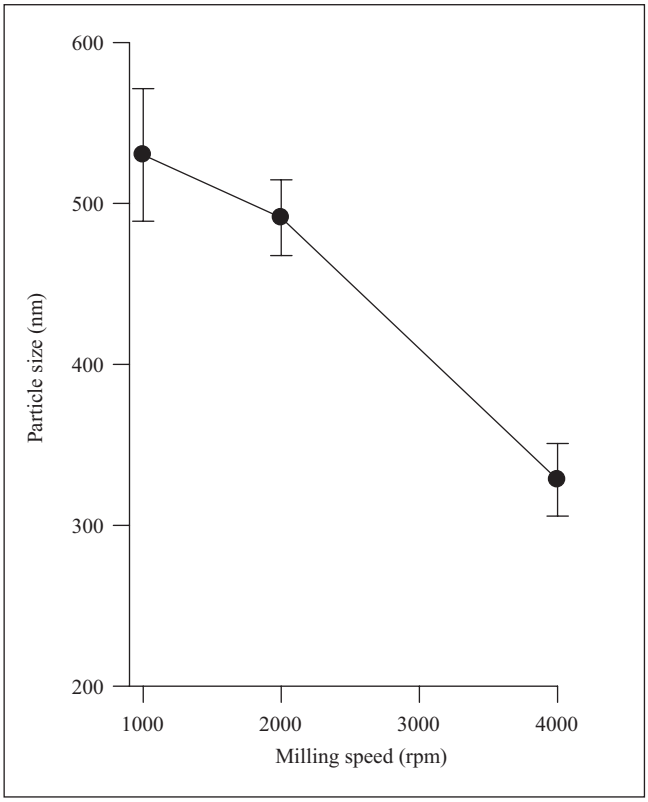

Fig. 3: Effect of the milling speed on the particle size of the nanosuspensions. Each value represents the mean ± S.D. (n = 3)

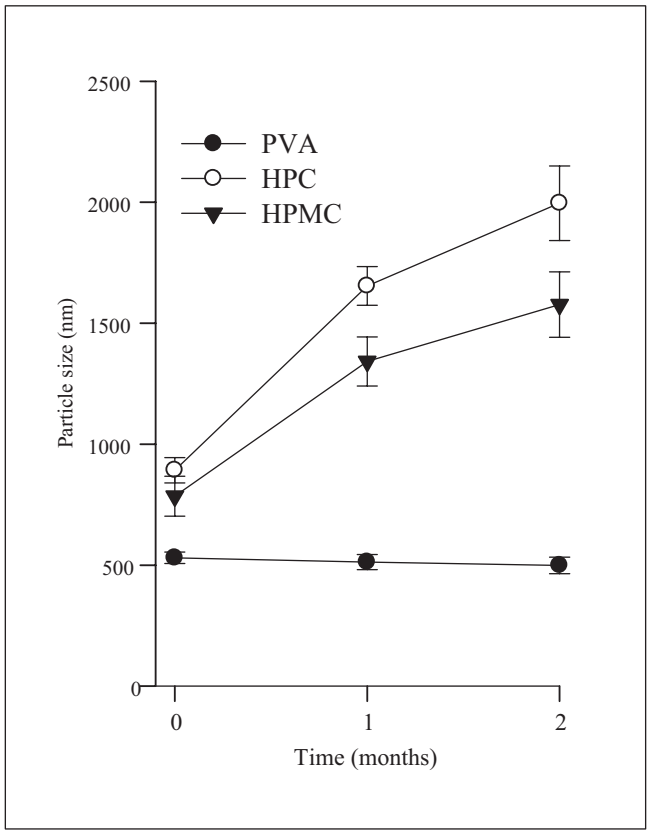

Fig. 4: Stability test. Each value represents the mean ± S.D. (n = 3)

beads. Thus, zirconia beads of 300 μm diameter were used in this study.

Based on these findings, a nanosuspension with a CsA/PVA/water weight ratio of 0.5/1/100 milled with 300 μm diameter zirconia beads at 1000 rpm for 2 h, which gave a minimum particle size of about 530 nm, was chosen for further study.

The stability of the drug in the nanosuspensions was evaluated by the particle size of the nanosuspension and the content of CsA in various nanosuspensions during storage for two months at 25 °C (Kobierski et al. 2009). The particle size increased in the CsA-loaded nanosuspensions prepared with HPC and HPMC and they showed a little sedimentation. Thus, these nanosuspensions were not physically stable. On the other hand, there was no noticeable change in the particle size of the CsA-loaded nanosuspensions prepared with PVA during this period (Fig. 4). Furthermore, they showed no sedimentation or creaming during the storage period. Thus, this CsA-loaded nanosuspension was physically stable for at least two months. The drug contents in all nanosuspensions decreased by less than 3% over this period, even at 25 °C (data not shown). Generally, in media milling processes, milling is thought to cause mechanical activation at the drug particle surfaces. Crystal defects due to disordering of the crystal surface and the generation of localized amorphous regions have been implicated in increased surface energy. Reordering of crystal defects and re-crystallization of amorphous regions has resulted in both physical and chemical instability of processed materials during storage. However, the presence of an appropriate stabilizer in the solution prevents rapid particle growth, resulting in particles of very small size (Verma et al. 2009b). Thus, PVA played a great role in physically and chemically stabilizing the CsA-loaded nanosuspension.

Ocular irritation bythe CsA-loaded nanosuspension was evaluated by comparing it with a commercial product, Restasis ®, using the Draize test and the Schirmer tear test. The Draize test used a 0 (absence) to 3 (highest) clinical evaluation scale of discharge and conjunctival redness, as shown in Table 3 (Lallemand et al. 2005; Said et al. 2007). In the Draize test, both the CsA-loaded nanosuspension and the commercial product resulted in very slight redness of the conjunctiva and no change of conjunctival discharge in the rabbits' eyes compared to the control (Table 4). Thus, both preparations caused very slight ocular irritation. The Schirmer tear test values obtained before and after instillation of the control, the CsA-loaded nanosus-

Table 2: Effect of bead materials on particle size of cyclosporin A

Material	Bead size (μm)	Particle size (nm)	PDI
Zirconia	100	463.9	0.218
	300	530.1	0.300
Polystyrene	300	909.2	0.362

Table 3: Draize's grading scale for the clinical evaluation of ocular irritation

Conjunctival redness	Score
Blood vessels normal	0
Some blood vessels definitely hyperaemic	1
Diffuse colour, individual vessels not easily discernible	2
Diffuse beefy red	3
Conjunctival discharge	Score
Normal	0
Slight discharge	1
Severe discharge covering a small area around the cornea	2
Severe discharge covering a large area around the cornea	3

Table 4: Results of the Draize test

Parameter	Parameter Test solution	Test times (day)					
		0	1	2	3	3.5	4
Conjunctival redness	Distilled water	0/2	0/2	0/2	0/2	0/2	1/2*
	Nanosuspension	0/2	0/2	1/2*	0/2	0/2	1/2*
	Distilled water	0/2	0/2	0/2	0/2	0/2	1/2*
	Commercial product	0/2	0/2	1/2*	0/2	0/2	1/2*
Conjunctival discharge	Distilled water	0/2	0/2	0/2	0/2	0/2	0/2
	Nanosuspension	0/2	0/2	0/2	0/2	0/2	0/2
	Distilled water	0/2	0/2	0/2	0/2	0/2	0/2
	Commercial product	0/2	0/2	0/2	0/2	0/2	0/2

*The rabbit's eye showed some blood vessels that were definitely hyperaemic (score 1).

pension and the commercial product in rabbits are shown in Fig. 5. The control and the CsA-loaded nanosuspension gave no significant differences in flow rates before and after administration. However, after the commercial product was instilled, a significant decrease in flow rate from pre-administration was observed. Our Schirmer tear test results suggested that the CsA-loaded nanosuspension caused less irritation to rabbits' eyes compared to the commercial product (Toshida et al. 2009). As the CsA-loaded nanosuspension contained no solubilized drug, oil or surfactant, unlike the commercial product, it might have given less ocular irritation compared to the commercial product (Van Eerdenbrugh et al. 2008).

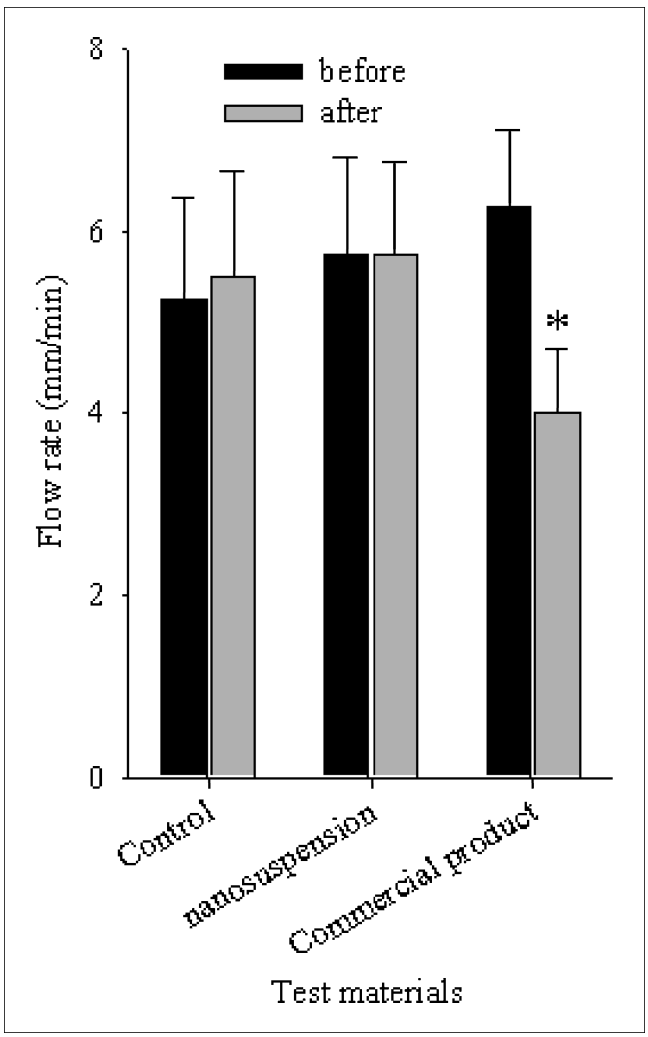

Fig. 5: Schirmer tear test: Flow rate of tear fluid before and at 3 h after instillation of control, CsA-loaded nanosuspension and commercial product to rabbits' eyes. The CsA-loaded nanosuspension was prepared with a weight ratio for CsA/PVA/water of 0.5/1/100 milled with zirconia beads of Φ300 μm at 1000 rpm for 2 h, which gave a minimum particle size of about 530 nm. * $P < 0.05$ compared with the flow rate before administration. Each value represents the mean ± S.D. (n = 4)

In conclusion, the nanosuspension with a CsA/PVA/water weight ratio of 0.5/1/100 milled using the media milling method with 300 μm diameter zirconia beads at 1000 rpm for 2 h gave a minimum particle size of about 530 nm. Furthermore, this CsA-loaded nanosuspension was stable for at least two months and caused less irritation to rabbits' eyes compared to a commercial product. Thus, a CsA-loaded nanosuspension prepared with PVA and water using a top-down media milling method would be a promising candidate with excellent stability and reduced ocular irritation. To develop a novel CsA-loaded nanosuspension, further efficacy tests in human subjects will be performed comparing this product to a commercial product.

3. Experimental

3.1. Materials

Ciclosporin A (CsA) was supplied by Dong-A Pharmaceutical Co. (Suwon, South Korea) and was of USP grade. Hydroxypropylcellulose (HPC), hydroxyethylcellulose (HEC) and hydroxypropylmethylcellulose (HPMC) were purchased from Shin-Etsu Co. (Tokyo, Japan). Polyvinyl alcohol (PVA) and polyvinyl pyrrolidone (PVP) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA) and Duksan Chemical Co. (Seoul, South Korea), respectively. The commercial product (Restasis ®; in an ophthalmic emulsion form) was purchased from Allergan Korea Co. (Seoul, South Korea). All other chemicals were of reagent grade and were used without further purification. Yttrium-stabilized zirconia beads (diameter 100 μm and 300 μm, YTZ® grinding media) were obtained commercially from Tosoh Co. (Tokyo, Japan).

3.2. Dispersibility

About 2.5 g CsA was added to 500 ml of water containing a polymer such as HPC, HEC, HPMC, PVA or PVP as the surface stabilizer. The solutions were homogenized using a homo mixer (T-50; IKA-Werke GmbH & Co. KG Staufen, Germany) at 7000 rpm for 30 min and then kept at room temperature for 24 h, after which their dispersibility properties such as creaming and sedimentation were evaluated by visual examination.

3.3. Preparation of CsA-loaded nanosuspension

About 2.5 g of CsA was added to 500 ml water containing 5 g surface stabilizer and the mixture was homogenized using a homo mixer at 7000 rpm for 30 min. Subsequently, zirconia beads were added to the mixing bowl as the milling agent. The mixture was then transferred to the grinding station of a Netzsch laboratory bead mill (Minicer, Germany). Milling was performed under various milling conditions, such as different milling times, rotation speeds, types of bead and bead size.

3.4. Determination of CsA content of nanosuspensions

To determine the CsA contents of the nanosuspensions, each nanosuspension (0.1 ml) was added to 5 ml of 50% ethanol, shaken in a water bath for 30 min and filtered through a membrane filter (0.45 μm). The concentration of CsA in the resulting solution was analysed by HPLC (Jasco PU-987, Japan) equipped with an Inertsil ODS-3 C_{18} column (GL science, 5 μm, 4.6 × 250 mm i.d.), a UV detector (Jasco UV-975) and an HPLC column temperature controller (Thermasphere® TS-130; Phenomenex, Torrance, CA, USA). The mobile phase consisted of acetonitrile/water (80:20) with a flow rate of 1.5 ml/min; the column was thermostated at 70 °C (Woo et al. 2007).

3.5. Determination of particle size

The particle size of all the CsA-loaded nanosuspensions was investigated by photon correlation spectroscopy using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK), performing 30 runs per measurement. Photon correlation spectroscopy yields the mean particle size and the polydispersity index (PI) as a measure of the width of the particle size distribution (Pardeike and Müller 2010). Prior to measurement, the samples were diluted to 0.1 mg/ml with purified water. No decrease in particle size was observed during the measurements on these nanosuspensions, indicating that no dissolution took place.

3.6. Stability

A CsA-loaded nanosuspension was stored for two months at 25 °C (Kobierski et al. 2009). At one-month intervals, the particle size was determined by photon correlation spectroscopy, and the CsA contents were analysed by HPLC as described above.

3.7. Ocular irritation study

3.7.1. Animals

Male New-Zealand rabbits weighing about 1.8 kg were fasted for 24 h prior to the experiments, but were allowed free access to water. The rabbits were kept at 20 °C and 70% RH with a normal 12 h light/dark cycle. Four rabbits were divided into two groups. The eyes of each rabbit were tested with a CsA-loaded nanosuspension and the commercial product. The protocols for the animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the Dong-A research center and were conducted according to the IACUC guidelines.

3.7.2. Draize test

The CsA-loaded nanosuspension or commercial product (40 μl) was instilled into the left eye of each of the four rabbits. The right eye of each of the four rabbits after the instillation of 40 μl distilled water was used as a control. Each eye was scored 5 min after instillation. At 6 h intervals for four days, test materials were instilled and their ocular irritation was scored at 0, 1, 2, 3, 3.5 and 4 days.

3.7.3. Schirmer tear test

At 6 h intervals for 4 days, the left eye of each of the four rabbits was instilled with 40 μl CsA-loaded nanosuspension or commercial product. The right eye of each of the four rabbits after instillation of 40 μl distilled water was used as a control. The Schirmer tear test was performed by measuring the flow rate of tear fluid before and at 3 h after instillation of the control, CsA-loaded nanosuspension and commercial product into the rabbits' eyes for 2 min using a Schirmer strip (Eagle vision Inc.; CA, Los Angeles, USA) (Toshida et al., 2009).

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