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Research paper

Freeze-drying of polycaprolactone and poly(D,L-lactic-glycolic) nanoparticles induce minor particle size changes affecting the oral pharmacokinetics of loaded drugs

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Abstract

The present study was geared at identifying the conditions to stabilize poly (D,L-lactic-glycolic) (PLGA) and polycaprolactone (PCL) nanoparticles (NP) by freeze-drying with several cryoprotective agents. Differential scanning calorimetry and freeze–thawing studies were used to optimize the lyophilization process. These studies showed that all samples were totally frozen at −45°C and evidenced the necessity of adding sucrose, glucose, trehalose or gelatine to preserve the properties of NP regardless of the freezing procedure. However, only 20% sucrose and 20% glucose exerted an acceptable lyoprotective effect on PLGA and PCL NP, respectively. Nonetheless, the final to initial size ratios (~1.5) indicated that particle size was slightly affected in both cases. In vivo studies with CyA-loaded PCL NP whose sizes matched those obtained after NP preparation (100 nm) and after being lyophilized (160 nm) showed that the changes of particle size might have some relevance on drug pharmacokinetics. The MRT was significantly ($P < 0.05$) modified after an oral CyA dose of 5 mg/kg and the treatment with 160-nm sized CyA-loaded NP produced a higher drug partition into the liver of Wistar rats potentially affecting the toxic and immunosuppressive profile of the drug. Therefore, although the particle size changes induced by NP lyophilization were slight, they need to be carefully evaluated and cannot be neglected. © 2000 Published by Elsevier Science B.V.

Keywords: Nanoparticles; Freeze drying; Cryoprotectives; Pharmacokinetics, Cyclosporine

1. Introduction

The advantages shown by nanoparticles (NP) in the pharmaceutical field have been previously reported in many papers [1,2]. However, the extensive application of NP might be limited due to problems for maintaining the integrity of the liquid suspension for a prolonged time period. Despite the colloidal nature of NP, surface active agents are usually added to stabilize the suspension by direct adsorption to the particle surface. Nevertheless, a little aggregation is often observed upon storage [3,4]. In addition to the improvement of physical stability, the chemical stability of the polymeric material forming the NP [5] and the encapsulated drug needs to be considered as well, thus avoiding a premature drug release and the formation of undesired degradation products. Hence, the improvement of NP stability represents an important issue in the development of these drug carriers. Freeze-drying appears as one of the most suitable methods to stabilize and facilitate the handling of colloidal systems, which otherwise stored as suspensions would suffer alteration in a brief period of time. This fact has been broadly discussed in many papers, but mainly in terms of liposomes [6–9]. In contrast, the few references to the freeze-drying of polymeric NP are relatively recent [4,10–15]. Moreover, these papers lead to some controversy about the factors involved in maintaining the integrity of NP after re-hydrating the lyophilized samples. Therefore, it would appear to be useful to conduct systematic studies focused on the optimization of the freeze-drying of NP. Several authors [13,14] have claimed that additives are necessary, and of these saccharides have been frequently used to achieve successful results. Their protective mechanism for liposomes (proposed by Crowe et al. [6]) involved the formation of hydrogen bonds between the saccharides and the polar head groups of phospholipids, thus inhibiting the fusion of liposomes and the depression in the transition temperature of the dried lipids. Although this may not be valid for the resistant and rigid structure of the polymeric matrix forming the NP, the ability of saccharides to work as spacing matrix between the particles may prevent their aggregation [16].

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However, there is a considerable variation among saccharides in their ability to stabilize colloidal systems, and in some cases is not completely clear how they protect against the water crystallization stress during the freezing step [8]. Recently, we investigated the application of freeze-drying to enhance the stability of polymeric NP but conflicting results were obtained with poly-D,L-lactide-glycolide (PLGA) NP [3,17]. Differential scanning calorimetry (DSC) studies offer the possibility of identifying the thermal events that take place during sample freezing. As it is considered to be a critical step of lyophilization, the first part of the present paper investigated the cryoprotective ability of various additives such as saccharides, polyalcohols and high molecular weight compounds. The optimum freeze-drying conditions applied to the stabilization of polymeric NP made from two biodegradable polymers, poly-ε-caprolactone (PCL) and PLGA, were also addressed.

In vivo, NP are captured by the cells of the reticuloendothelial system (RES) upon intravenous dosage, thus making them specially suitable for the passive targeting of immunosuppressive drugs such as cyclosporine (CyA). Additionally, the oral bioavailability of this drug was improved by its incorporation into polymeric NP [18]. So then, CyA-loaded NP may be an alternative to other existing CyA formulations. Nonetheless, the difficulties in maintaining the initial particle size after freeze-drying and the size dependent behaviour exhibited by NP in vivo [19] led us to evaluate the effect that the slight NP size variations induced by freeze-drying have on the oral bioavailability and pharmacokinetics of CyA loaded into PCL NP.

2. Materials and methods

2.1. Materials

CyA was kindly donated by Sandoz Pharma, S.A.E. PLGA (Resomer RG-504, molecular weight 45 kDa) was purchased from Boehringer lngelheim (Germany) and PCL (molecular weight 50 kDa) from Aldrich-Chemie (Germany). Poloxamer 188 (PF-68) was obtained from Fluka (Spain). Chemicals used were all of analytical grade, except those for HPLC assay which were of HPLC grade. D-(+) anhydrous glucose, D-(+) sucrose, D-(+) dihydrated trehalose, D-mannitol and D-sorbitol were supplied by Fluka. Lactose and dextran (average molecular weight 71 kDa) were obtained from Sigma (USA) and gelatine from Merck (Germany). All other solvents and chemicals were purchased from Scharlau (Spain).

2.2. NP preparation

The method used to prepare the NP is based on the nanoprecipitation method but has been slightly modified [20,21]. The preparation conditions were selected to obtain NP of a predetermined size (approximately 100 nm), exposed to be partially capable of crossing the biological barriers of the gastrointestinal tract [19,22]. CyA-loaded PCL NP of a mean size about 100 and 160 nm were used for in vivo studies. They were prepared by the same method but the global injection rate of the organic phase into the aqueous medium was decreased to increase the particle size.

2.3. NP characterization

The average particle size and size distribution profiles were determined at 25°C by dynamic light scattering in a Microtrac UPA (Leeds & Northrup, Ireland) for each batch immediately after preparation and after being freeze–thawed or freeze-dried.

Previous to in vivo administration, the amount of CyA incorporated by PCL NP was determined by HPLC. The suspension and the supernatant obtained after centrifugation at 40 000 × *g* and 4°C for 1 h were analyzed and drug encapsulation percentage and encapsulation efficiency were calculated according to previous reports [23].

2.4. Cryo/lyo-protectives

The effects of eight cryo/lyo-protectives at different concentrations on the stability of PCL and PLGA NP were assayed. Glucose, sucrose, trehalose and lactose were used at 5, 10, 20 and 30% concentrations; mannitol (15%), dextran and gelatine (2.5 and 5%); and sorbitol (30%) except for PLGA NP formulations that only tolerated a maximum sorbitol concentration of 5%. Solutions of each cryoprotective agent and NP with no additives were taken as reference samples.

2.5. DSC studies

The thermal behaviour of NP during freezing and rewarming was studied using a differential scanning calorimeter (DSC-30, TA4000 Mettler-Toledo, Switzerland). Indium platinum and zinc were used for calibration, which was further verified using double-distilled water and the sodium chloride/water eutectic. The effect of the sample weight, the cryoprotective concentration, and the cooling and heating rates were evaluated in order to establish the optimum conditions of the process, thus avoiding anomalous results due to supercooling. The samples (30 μl) were cooled down to −100°C at a rate of 10°C/min and maintained for 5 min before applying a constant heating rate of 2°C/min. Then, glass transition temperatures (T_g) and melting enthalpies (ΔH_m) were measured during the warming of frozen solutions of each cryoprotective alone or added to PCL and PLGA NP suspensions. All T_g values were reported as the midpoint of the transition whereas ΔH_m was calculated as the area under the curve of the melting peak divided by the sample weight.

2.6. Freeze–thawing

The influence of the cooling procedure on the cryoprotective ability of each additive was established by freeze–

thawing cycles. Aliquots (1 ml) were placed in sealed vials and frozen at −70°C for 72 h or immersed in liquid nitrogen for 1 h and further maintained at −70°C for 72 h to allow for the crystalline re-organization of the system. Sample thawing took place at room temperature. The visual appearance of the suspensions after thawing was evaluated and compared to the reference unfrozen samples that were initially free of aggregates and displayed a Tyndall effect. Particle size was also determined but only for those preparations where no visible aggregation was observed and the final to initial size ratio calculated (S_f/S_i). Ratios of 1 ± 0.3 were considered as not being significant.

2.7. Freeze-drying

The freeze-dryer used for these experiments was a Telstar L-1 model. The samples (1 ml) were dispensed in 5-ml semi-stoppered glass vials with slotted rubber closures and frozen during 24 h on the shelves of the lyophilization chamber at its minimum temperature (−45°C). Sublimation lasted 48 h at a vacuum pressure of 4×10^{-5} atm and without heating, being maintained at the condenser surface temperature of −60°C. Finally, glass vials were sealed under anhydrous conditions and stored until being re-hydrated (within two days) by using the same initial volume (1 ml) of ultrapure water. The lyoprotection provided by each additive was established by following the same procedure applied for freeze–thawing studies. Particle aggregation was quantified by the following numerical scale: (0) absent, (1) scarce and (2) significant aggregation.

2.8. In vivo studies

In order to evaluate the effect that the NP size changes induced by freeze-drying might have on the pharmacokinetics of encapsulated drugs a comparative in vivo study was developed in rats by using CyA as a model hydrophobic compound. The study was approved by the local ethical committee. Eight male Wistar rats weighing 280–320 g were requested from the Central Stabulary of the University (homologation number EC 28005-22A) and randomly divided into two groups (four animals each). They were maintained in metabolic cages for 24 h before the experiments, with a preserved 12:12-h dark/light cycle and free access to standard food and tap water. Before treatment, the animals were fasted overnight and had access to water ad libitum. Two batches of CyA-loaded PCL NP were prepared under experimental conditions adjusted to obtain mean particle sizes of 100 and 160 nm [20], that corresponded to the NP size observed before and after freeze-drying, respectively. A single CyA oral dose (5 mg/kg) equivalent to 0.5 ml of PCL NP was given to each animal by gavage between 09:00 and 10:00 h to avoid chronopharmacokinetic effects. Group 1 received 100-nm sized PCL NP and group 2 received 160-nm sized PCL NP. Whole blood samples (150 μl) were withdrawn from the jugular vein of lightly isoflurane anaesthetized animals at 0 (predose), 0.5, 1, 2, 4, 6, 8, 24, 32 and 48 h post-treatment. Samples were collected in polyethylene vials over 20 μl disodium EDTA (22 mg/ml), thoroughly mixed and 100 μl separately frozen at −20°C until analysed. Urine was collected from under each cage; the urine volume was recorded and a 1.5-ml aliquot stored at −20°C until drug content assay. The total amount of faeces excreted as well as those retained in the intestine were collected and allowed to dry under ambient temperature. After animal exsanguination, the liver, kidneys and spleen were perfused and processed to determine CyA contents as previously described [24].

2.9. Drug analysis

CyA concentrations in whole blood, liver, spleen, kidney, faeces and urine were determined by using a monoclonal antibody fluorescence polarization immunoassay (monoclonal FPIA, TDx, Abbot Laboratories). The method was slightly modified by adding 33% of acetonitrile (MeCN) in order to make it suitable for CyA determination in tissues where an extraction procedure with organic solvents is necessary. Calibration curves in each tissue or sample matrix were obtained each time a set of samples was analysed. Under these conditions, percentage recovery of CyA in the blood and urine samples was $100 \pm 5\%$ and the within-day and between-day coefficients of variation did not exceed 5% for the same batch of reagents. The recovery obtained for the rest of sample matrices amounted on average to $83.62 \pm 12.15\%$ as compared to drug solutions in the absence of tissue or faeces. The limit of quantification was in all cases 25 ng/ml and no interference of the compounds used in NP preparation was observed.

2.10. Pharmacokinetic analysis

The pharmacokinetic parameters were estimated by non-compartmental methods. The zero-order moment area under the curve (AUC) and the first-order moment mean residence time (MRT) were determined by standard methods applying the linear trapezoidal rule. The elimination rate constant (λz) was derived from the slope of the terminal phase estimated by log-linear regression of at least four experimental blood concentrations. The apparent body clearance (CL/F) and the apparent volume of distribution ($V\beta/F$) were calculated by using the following equations: $CL/F = Dose/AUC$ and $V\beta/F = CLx\lambda z/F$. The differences found between pharmacokinetic parameters in both groups were statistically evaluated by the t-test.

3. Results and discussion

3.1. DSC studies

Thermal analysis is a commonly used technique for the

optimization and characterization of freeze-drying cycles, but finding the optimal experimental conditions is critical and depends on the nature of the samples assayed [25,26]. Therefore, we established the optimal experimental conditions for the DSC analysis. A quick enough cooling rate followed by a slow sample heating allowed for a more accurate estimation of T_g and ΔH_m, thus avoiding the appearance of enthalpic relaxation phenomena. As an example, Fig. 1 shows the DSC thermograms recorded during the heating process of the following aqueous dispersions which had been previously cooled at −100°C: 10% PF-68 aqueous solution (a), PLGA NP as such (b), or in the presence of 30% dextran (c) or sucrose (d). All of them show a large endothermic transition peak at about 0°C corresponding to the melting of ice. The inflexion point observed at −33°C in the baseline of thermogram (d) indicates a discontinuity in the heat capacity of the frozen sample and it can be ascribed to the T_g of a water containing sucrose system, which is formed during freezing of the solution. This thermal behaviour is characteristic of substances forming an amorphous mass when they are frozen [26,27]. Those NP containing glucose and trehalose gave similar thermograms (not shown) to that of sucrose. Thermogram (c) shows an inflexion point just before the initiation of the melting of ice which starts at higher temperatures than those observed in thermogram (d). No baseline inflexion is observed for NP without cryoprotectant (b), but as in thermogram (c), a small endothermic peak at −19.9°C was observed, which corresponds to the eutectic temperature of the PF-68 contained in the formulation (thermogram (a)). In this sense, previous studies suggest that the surfactant (PF-68) used as stabilizer of NP crystallizes upon freezing (thermogram (a)) impairing the maintenance of NP properties in the absence of cryoprotectives (thermogram (b)). On the contrary, their presence dehydrate the surfactant in the bulk solution forcing it to the particle surface, and thereby acting as a cryoprotective agent [12,15]. Recently, De Jaeghere et al. [4] reported that poly-ethylene-oxide (PEO) polymers located at the carrier surface were adequate cryoprotectants for NP but rather poor lyoprotectants unless saccharides were present. Assuming the same mechanism, the PEO groups of PF-68 oriented towards the aqueous phase might undergo hydrogen bonding with the lyoprotective agent keeping the surfactant in a pseudo-hydrated state, thus preventing the crystallization of PF-68. However, the absence of this peak at −19.9°C in the thermograms of glucose, sucrose and trehalose containing formulations does not indicate that the eutectic mixture is not formed. In fact, since the melting endotherm begins at lower temperatures it is not possible to establish whether the peak of PF-68 is masked by the depression in the baseline as consequence of ice melting, or it does not appear because of interactions between the surfactant and the other solutes. It must be taken into account that these interactions between components in a solution may substantially alter the physical properties of the frozen system, and affect the characteristics of the lyophilized product [28].

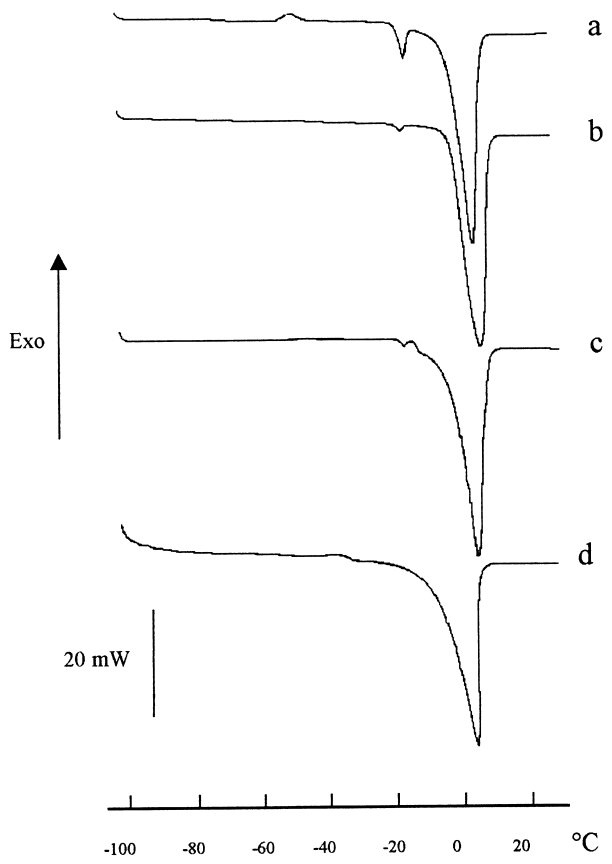

Fig. 1. DSC thermograms plotted during the heating cycle corresponding to: (a) PF-68 solution (10%), (b) PLGA NP without cryoprotectives, (c) PLGA NP with dextran (30%), and (d) PLGA NP with sucrose (30%).

Table 1 summarizes the T_g values obtained for each cryoprotectant as aqueous solutions or when added to NP. These data are in accordance with previous studies [27] and the values obtained with PCL and PLGA NP formulations were almost the same as the corresponding aqueous solutions. Therefore the changes that occurred during NP freezing are, apparently, independent of the polymer forming the

Table 1
Experimentally determined glass transition temperatures (T_g) of the cryoprotective agents in aqueous solutions and when added to NP formulations[a]

	PCL NP (°C)	PLGA NP (°C)	Aqueous solutions (°C)
Glucose 30%	−43.8	−44.0	−43.6
Sucrose 30%	−33.3	−33.5	−32.7
Trehalose 30%	−30.5	−30.7	−29.8
Lactose 15%	−29.9	−30.3	−28.7
Sorbitol 30%	−44.4	ND	−43.9
Manitol 15%	−30.3	−31.1	−28.9
Dextran 15%	−12.1	−12.4	−11.1
Gelatine 5%	ND	−19.9	ND

[a] ND, Not detected.

Table 2
Melting enthalpies of ice for the cryoprotector aqueous solutions and for the NP formulations with and without cryoprotectors

	Melting enthalpy (J/g)			% (w/w) Over-cooled water		
	PCL NP	PLGA NP	Aqueous solutions	PCL NP	PLGA NP	Aqueous solutions
No cryoprotector	309.7	306.4	326.9[a]	5	6	0
Glucose 30%	251.4	243.2	223.3	23	28	32
Sucrose 30%	283.5	282.8	237.1	13	13	28
Trehalose 30%	269.1	267.4	252.7	18	18	23
Lactose 15%	301.2	308.1	306.3	8	6	6
Sorbitol 30%	246.2	281.2[b]	230.7	25	14	19
Manitol 15%	315.5	318.2	304.7	3	3	7
Dextran 15%	301.6	256.6	281.7	8	19	14
Gelatine 5%	244.4	271.1	308.0	25	17	6

[a] Pure water.
[b] Only tolerated 5% of sorbitol.

NP, and exclusively depend on the cryoprotective agent. The lowest T_g corresponded to solutions containing glucose and sorbitol. Sucrose, trehalose, lactose and mannitol exhibited intermediate values. The highest T_g were found for dextran and gelatine. The transition peak observed in all thermograms at about 0°C represents the melting of ice and it accounts for the amount of water not associated to the solutes in the samples. The values of ΔH_m of ice for solutions containing cryoprotectives as well as those for PCL and PLGA formulations with and without cryoprotectors are shown in Table 2. In all cases, these experimental ΔH_m values were lower than that of pure water. If the water contained in the samples becomes 'free' water, the value of the ΔH_m of ice should be of the same order as that obtained from pure water (326.9 J/g). Lower ΔH_m suggest that part of the water in the sample is associated to the solutes. It is thus possible to calculate the portion of water forming pure ice, according to the ratio ΔH_m of ice in each sample/ΔH_m of pure ice. Assuming that there is no mass loss during the process, the remaining quantity should correspond to over-cooled water; that is water undergoing supercooling with the incorporated solutes, thus forming an internal phase of increasing viscosity and concentration. Consequently, a plasticizing effect is offered as mechanical protection to the NP contained in the mass, thus decreasing the possibility of aggregation [6,27,29]. Hence, systems with low melting enthalpies of ice should contain a correspondingly higher proportion of over-cooled water acting as a plasticizer. In accordance, glucose, for both types of NP, and sorbitol and gelatine for PCL NP, would offer the best cryoprotective effect, and then trehalose and sucrose. The widely different thermal behaviour between solutions of two diastereoisomers, such as sorbitol and mannitol, should be noted. In particular, mannitol solutions form a metastable amorphous mass, clearly detected in the thermogram (not shown) suggesting that the characteristics of the solutes during freezing do not exclusively depend on their chemical structure.

3.2. Freeze–thawing studies

Freezing is considered to be a critical stage of lyophilization. Hence, prior to freeze-drying, the cryoprotective ability of different compounds under two different freezing conditions was tested. The samples were frozen either at a slow (−70°C) or a rapid rate (liquid nitrogen) leading to the growth of small number of large ice crystals or a high number of small ice crystals, respectively [10]. The results for both types of NP and cooling rates are shown in Figs. 2 and 3. Values of $S_f/S_i \sim 1 \pm 0.3$ indicate the initial size was maintained, while larger values approaching 2 or more suggest a significant aggregation of NP. In the absence of cryoprotectives, a macroscopic and irreversible aggregation was detected regardless of the cooling procedure and the polymer forming the NP. On the other hand, the additives used in this study showed a significant cryoprotective effect of variable intensity. In fact, glucose, sucrose and trehalose at concentrations as low as 5% maintained the properties of the system independently of the cooling conditions and the

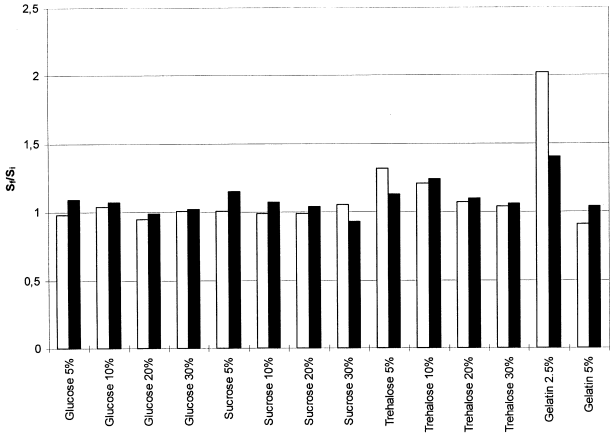

Fig. 2. Chart diagrams representing the final to initial size ratio for PCL NP frozen at −70°C (open bars) and −196°C (filled bars) in the presence of variable amounts of cryoprotectives.

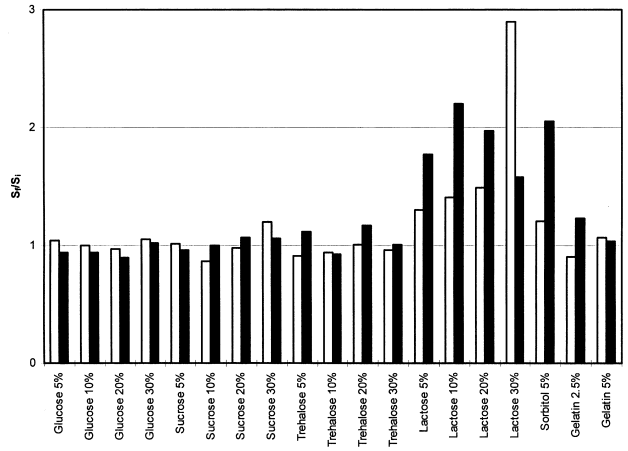

Fig. 3. Chart diagrams representing the final to initial size ratio for PLGA NP frozen at −70°C (open bars) and −196°C (filled bars) in the presence of variable amounts of cryoprotectives.

polymer type. On the other hand, polyalcohols such as sorbitol and mannitol did not show the same behaviour during the freezing step, only sorbitol proved to be an effective cryoprotective when PLGA NP were frozen at −70°C. Nevertheless, sorbitol also prevented the formation of macroscopic aggregates during the freezing of PCL NP at −70°C and PLGA NP at −196°C, although the final to initial size ratios were 4.5 and 2.1, respectively. It has been suggested that the different stereochemical conformation of these polyalcohols may be the reason for a modified interaction between the cryopreservative and the structure of the frozen mass. Mannitol and dextran exhibited cryoprotective action neither for PLGA NP nor in the case of PCL NP, and was lactose only moderately effective for preserving the characteristics of PLGA NP. These results are in agreement with the predictions made from the DSC analysis (Table 2) except for sorbitol. Nevertheless, it may be possible that the different molecular conformation of saccharides (ring disposition) and the less compact structure of polyalcohols (i.e. their linear dispositions) have some influence.

As described for other particulate systems, the congealing stage during lyophilization determines the structure of the frozen mass [10,12]. The addition of cryoprotective agents induce a non-regular packing of the molecules in the interstitial vitreous mass producing a great inner space, thus leading to a decreased density as compared to the more ordered mass built up by the ice crystals. This makes the frozen mass behave more as a fluid than a solid [29] and it provides better mechanical protection of the NP. Consequently, NP aggregation or any alteration due to the pressure developed by the growth of crystals is avoided.

3.3. Freeze-drying studies

The macroscopic observation of the lyophilized product showed slight differences between formulations, which consisted in variations of aspect and volume. Most of the cakes were brittle and white with a fibrous aspect. PCL NP formulations containing glucose, 20% of lactose and gelatine, and PLGA NP with glucose, 10 and 20% of sucrose, 20% of lactose and gelatine showed porous cakes. In contrast, the remaining samples showed a shiny surface attributed to the migration of solute or particles towards the surface [29]. In a global sense, NP preparations lyophilized in the presence of glucose, sucrose and trehalose

Table 3
Characteristics of freeze-dried PCL and PLGA NP in the presence of different cryoprotective substances[a]

	PCL NP			PLGA NP		
	Aggregation scale	Tyndall effect	S_f/S_i	Aggregation scale	Tyndall effect	S_f/S_i
No cryoprotector	2	−	ND	2	−	ND
Glucose 5%	2	+	ND	2	+	ND
Glucose 10%	0	++	2	2	+	ND
Glucose 20%	0	++	1.5	0	++	>2
Sucrose 5%	2	−	ND	1	++	ND
Sucrose 10%	1	+	ND	1	++	ND
Sucrose 20%	0	++	>2	0	++	1.5
Trehalose 5%	1	+	ND	1	+	ND
Trehalose 10%	1	+	ND	1	++	ND
Trehalose 20%	0	++	>>2	1	++	ND
Lactose 5%	2	−	ND	2	−	ND
Lactose 10%	2	−	ND	2	−	ND
Lactose 20%	2	−	ND	2	−	ND
Sorbitol 15%	2	−	ND	2[b]	−	ND
Manitol 15%	2	−	ND	2	−	ND
Dextran 15%	2	−	ND	2	−	ND
Gelatine 2.5%	2	−	ND	2	−	ND

[a] Aggregation scale: (0) absent, (1) scarce, (2) significant aggregation. ND, not determined.
[b] Only tolerated 5% of sorbitol.

preserved the Tyndall effect upon re-dispersion, suggesting that at least some of the particles retained their nanometric size (Table 3). However, only PCL NP containing 20% glucose and PLGA NP containing 20% sucrose showed acceptable products upon reconstitution with no macroscopic aggregation, the ratio being $S_f/S_i \sim 1.5$. The initial size of PCL NP (134 ± 58 nm) was modified to 197 ± 92 nm by freeze-drying with 20% glucose and the size of PLGA NP changed from 117 ± 55 to 178 ± 68 nm when lyophilization took place in the presence of 20% sucrose. In spite of the changes induced by freeze-drying in the average particle size, the diagrams representing the size distribution profiles of PCL NP containing 20% glucose and PLGA NP containing 20% sucrose under initial conditions and after freeze-drying shows a significant overlap. In fact, 99.62 and 97.48% of freeze-dried PCL NP and PLGA NP, respectively, were within their initial size ranges. Furthermore, the statistical analysis, using the *t*-test, showed no significant differences between the initial and final particle size distributions for both NP formulations ($P > 0.05$).

3.4. In vivo studies

The incorporation of CyA into polymeric NP had been initially thought as a way of increasing its oral bioavailability and to target the drug towards the immune system in order to improve its therapeutic effectiveness. Recently, it was demonstrated that the first objective can be reached despite the fact that a significant amount of drug is released in a short time period [18,30]; however, a stable association between the drug and the carrier is necessary to achieve the second objective. The absorption of particles from the intestine is a well-known process [2] affected by a number of factors among which particle size is prominent. These studies are usually conducted with latex particles with an extremely narrow size distribution, which is unfortunately quite far removed from the size profiles obtained when NP manufacturing follows drug loading. Furthermore, a wide size range, from nm to μm [19], is normally studied so that the influence of slight particle size changes is not usually addressed. The in vivo studies described here were conducted to evaluate the potential influence of the size changes due to freeze-drying on the oral pharmacokinetics of a model drug. To this end, CyA loaded PCL NP, whose mean sizes (100 ± 41 and 160 ± 38 nm) matched those obtained under initial conditions and after freeze-drying, were manufactured according to an experimental design previously developed [20].

HPLC analysis revealed that CyA-loaded PCL NP contained on average 95.6 ± 0.8% of the drug initially added to the preparation reaching drug concentrations of 2.81 and 2.44 mg/ml for 100- and 160-nm sized NP, respectively. The encapsulation efficiency expressed as μg of encapsulated CyA per mg of polymer was 133 and 129 μg/mg for NP of 100 and 160 nm, respectively. The mean pharmacokinetic profiles obtained for each group receiving

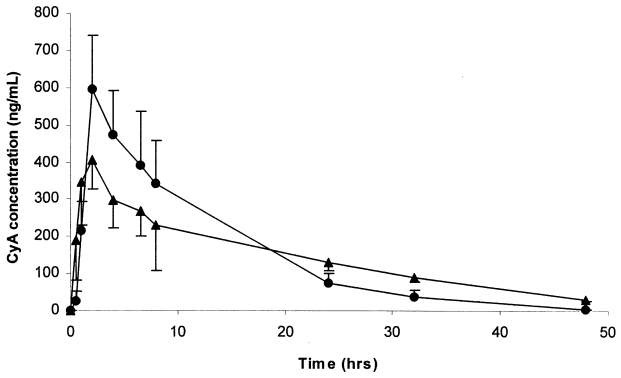

Fig. 4. Whole blood concentration–time profiles of CyA (mean ± SEM, $n = 4$) following a single 5 mg/kg oral administration to male Wistar rats as drug-loaded PCL NP of 100 nm (circles) and 160 nm (triangles) in size.

CyA orally as PCL NP of 100 or 160 nm are depicted in Fig. 4. Table 4 shows the corresponding pharmacokinetic parameters derived by non-compartmental analysis. The concentration-time curves showed a wide variability, especially for those time points describing the absorption phase. In all cases the C_{max} was reached at 2 h after dosing, but the average value with 100 nm NP (596.59 ± 143.70 ng/ml) was 46.5% higher than that corresponding to 160-nm sized NP (407.04 ± 81.01 ng/ml). The variability associated with the AUC and related parameters (CL/*F* and $V\beta/F$) was also higher after treatment with NP of 100 nm. Only the MRT exhibited significant differences probably due to a slightly different capacity in the elimination of the drug among groups. Some nephro- and hepatotoxicity are the major side effects of CyA and the liver and the spleen play an important role in the immune reaction. Therefore drug levels in the liver, the spleen and the kidneys may be considered as a rough index of potential toxicity and immunosuppressive ability. Likewise, the liver and the kidneys represent an important percentage of total solid organ transplantation. Therefore, the effect of particle size variations on the drug distribution towards these organs was evaluated as well. The CyA hepatic levels obtained 48 h after the administration of 5 mg/kg in the form of 100-nm sized NP (Table 5) showed no difference to those reached in the kidneys or the spleen but they were about 3- to 4-fold lower than the

Table 4
Average (±SD) pharmacokinetic parameters derived from the kinetic profiles by using a non-compartmental approach

	NP 100 nm	NP 160 nm
λz (h^{-1})	0.08 ± 0.02	0.06 ± 0.01
$t_{1/2}$ (h)	8.73 ± 2.11	11.04 ± 1.75
MRT (h)	12.95 ± 1.96	19.84 ± 3.72[a]
AUC (ng h/ml)	8857.01 ± 3563.90	7381.12 ± 1753.75
CL/*F* (ml/h per kg)	710.93 ± 292.97	628.63 ± 131.16
V_β/F (ml/kg)	7775.31 ± 1355.99	9991.24 ± 2623.42

[a] $P < 0.05$.

Table 5
Mean (SD) tissue levels of CyA after the oral administration of 5 mg/kg as 100-nm and 160-nm sized NP to male Wistar rats

	100 nm	160 nm
Liver (ng/g)	179.97 (130.87)	685.51 (113.38)[a]
Kidney (ng/g)	168.61 (128.45)	171.86 (43.03)
Spleen (ng/g)	173.17 (163.83)	204.74 (49.59)
Faeces (μg)	22.54 (6.89)	42.32 (26.64)
Urine (μg)	12.22 (2.62)	10.42 (3.09)

[a] $P < 0.05$.

hepatic concentrations achieved with 160 nm NP. In contrast, the kidneys and the spleen in the latter group contained the same drug amounts as those found in the group receiving NP of 100 nm. As previously indicated for drug levels in the blood a huge variability there was in the drug tissue contents in the group receiving 100 nm NP. With respect to drug excretion, the amounts of unchanged CyA found in the urine after 48 h were 12.22 ± 2.62 and 10.42 ± 3.09 μg for rats treated with 100 and 160 nm NP, respectively, which represented 0.83 ± 0.15 and $0.67 \pm 0.17\%$ of the administered dose, respectively. The amounts of parent CyA excreted in faeces were two and four-fold higher than those excreted in urine after 100 and 160 nm NP treatment, respectively (Table 5).

Globally, these in vivo data are in agreement with previous studies [31], suggesting that part of the CyA encapsulated into 100 nm PCL NP is surface adsorbed, the rest being entrapped by the polymeric matrix. In addition dissolution studies have shown that approximately 40% of the CyA associated to the carrier is released within the first 15 min [30]. Therefore, the larger specific surface area of 100 nm NP makes if possible that relatively more CyA is adsorbed on the particle surface, thus producing a higher initial release of CyA from these NP. Consequently, higher C_{max} values should be expected for smaller NP as opposed to NP of 160 nm where probably a more sustained release led to therapeutic CyA concentrations even at 24 h post-treatment (Fig. 4). Nonetheless, it is not possible in the present study to establish whether CyA in blood or tissues remains associated to NP or is in its free form. Hence, a contribution of NP crossing the gastrointestinal barrier cannot be neglected. Concerning the passage of polymeric NP through the intestinal mucosa different mechanisms have been proposed and most evidence suggests that particle size dramatically affects the extent of absorption [2,19]. In fact, the importance of the paracellular pathway is inversely correlated to the particle size whereas the absorption through the Peyer's patches gains more importance as particle size increases until 5 μm. Then, the higher CyA blood and hepatic levels reached at the end of the study with 160-nm sized NP may support the hypothesis that the liver could have captured CyA associated to NP. Furthermore, the amount of unchanged CyA excreted in faeces is higher with 160-nm NP, suggesting that less CyA is absorbed with this formulation. Alternatively since CyA is mainly cleared by hepatic and intestinal metabolism, it may be that 160-nm NP provide more protection to absorbed and non-absorbed drug against biotransformation processes.

4. Conclusion

In summary, this paper describes conditions for freeze-drying PCL and PLGA NP. DSC showed that all studied cryoprotectives (glucose, sucrose, trehalose, lactose, gelatine and dextran) formed an amorphous mass at very low temperatures. However, not all solutes forming a vitreous mass were able to stabilize NP suspensions, suggesting the contribution of additional physicochemical processes. The freeze–thawing studies have evidenced the necessity of adding cryoprotectives to preserve the integrity of the colloidal suspensions during freezing. Sucrose, glucose, trehalose and gelatine maintained the integrity of PLGA and PCL NP suspensions, without aggregation, independently of the freezing procedure. Concerning NP lyophilization, glucose and sucrose were the best cryoprotective agents but the final to initial size ratio ($S_f/S_i \sim 1.5$) exceeded the limit for non-significant changes after lyophilization-reconstitution. In vivo studies indicated that this particle size increment may have implications for the oral pharmacokinetics of loaded drugs. Using CyA as a model drug, the pharmacokinetic analysis revealed that 160 nm NP induced a larger MRT and higher drug hepatic levels. These changes can be considered useful if a selective accumulation of CyA in the liver is sought but a potential increase in its hepatotoxicity could be produced. Consequently, the effect of particle size changes due to freeze-drying of PCL and PLGA NP on the pharmacokinetics of loaded drugs should be carefully evaluated.

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