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Preparation of an alternative freeze-dried pH-sensitive cyclosporine A loaded nanoparticles formulation and its pharmacokinetic profile in rats

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Received February 1, 2008, accepted July 11, 2008

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Pharmazie 64: 26–31 (2009) *doi: 10.1691/ph.2008.8029*

This work aimed to produce and develop new pH-sensitive cyclosporine A (CyA) loaded nanoparticles (CyA-NP) based on the enterically soluble copolymer Eudragit® S100 and to improve the poor bioavailability of lipophilic CyA. CyA-NP and freeze-dried nanoparticles (Lac-CyA-NP, 3% lactose as cryoprotectant) were prepared using a quasi-emulsion solvent diffusion technique and freeze-drying. The encapsulation efficiency, particle size and *in vitro* release characteristics from the vehicle of CyA were studied individually. The bioavailability of CyA-NP and Lac-CyA-NP was evaluated in rats at a dose of 15 mg/kg as compared to Neoral®. The mean particle size of CyA-NP was 44 ± 3 nm, while the encapsulation efficiency reached 99.7%. The particle size and encapsulation efficiency of the freeze-dried formulation remained relatively stable by using 3% lactose (W/V) as a cryoprotective agent before freeze-drying and after dissolving. Significantly pH-dependent release profiles were revealed when the pH of the medium was above 6.0. The relative bioavailabilities of CyA-NP and Lac-CyA-NP were 162.1% and 130.1% compared with Neoral® after oral administration at the same dosage. The results showed that the pH-sensitive CyA-loaded nanoparticles were a potential vehicle for developing a high performance CyA carrier system.

1. Introduction

Cyclosporine A (CyA), a cyclic oligopeptide, has been commonly used as a first line immunosuppressant to prevent allograft rejection in various organ transplant patients (Matzke and Luke 1988). It is also effective in the treatment of systemic and local autoimmune disorders such as uveitis or Crohn's disease (Thomson and Neild 1991). However, the oral bioavailability of CyA is usually very low with a high inter- and intra-patient variability due to its poor absorption. This has been attributed to its high molecular weight, high hydrophobicity (LogP = 3.0) (Taylor et al. 1993), poor aqueous solubility ($6.6\ \mu g \cdot mL^{-1}$) (Mithani et al. 1996), extensive metabolism by CYP 450 3A, and efflux by a transporter, P-glycoprotein present in the intestinal mucosa (Tjai et al. 1991). Moreover, cyclosporine A is a critical drug with a narrow therapeutic window (Kahan et al. 1999).

The available CyA oral formulations include Sandimmune Neoral® which is a microemulsion preconcentrate displaying low inter- and intra-individual variation (Kovarik et al. 1994). However, this formulation contains a high concentration of polyoxyethylated castor oil (Cremophor® EL) and has shown a series of biological and physiological effects, such as nephrotoxic (Luke et al. 1987) and anaphylactic reactions (Cavanak and Sucker 1986) in clinical trials.

In order to overcome the difficulties mentioned above, several strategies have been designed to minimize drug toxicity while optimizing its immunosuppressive ability. The formulation of nanoparticles is known to be an efficient approach to improving the oral bioavailability of some poorly bioavailable drugs with specialized uptake mechanisms (Bhardwaj et al. 2005; Hariharan et al. 2006). This formulation prevents P-gp efflux and protects incorporated drug molecules from gastro- intestinal tract degradation as well as gut wall metabolism. Simultaneously, the nanoparticles also bypass the liver and prevent first-pass metabolism of the incorporated drug. Many carrier materials have been used to prepare CyA nanoparticles including biodegradable polymers (such as poly (D,L-lactide-glycolide) copolymers (PLGA) (Italia et al. 2007), polycaprolactone (PCL) (Carmen Varela et al. 2001), poly (D,L-lactic-glycolic acid) (PLAGA) (Chacón et al. 1996); positively charged chitosan hydrochloride (El-Shabouri et al. 2002); the pH-sensitive soluble polymer hydroxypropyl methylcellulose phthalate (HPMCP) (Wang et al. 2004), etc.

Theoretically, pH-sensitive nanoparticles loaded with CyA (CyA-NP) would be expected to improve absorption and avoid first-pass metabolism after oral administration giving a highly dispersed drug within the gastrointestinal tract at a specific pH value, as close as possible to the absorption window of the drug. Poly(methacrylic acid-*co*-methyl methacrylate) copolymers are a series of enterically soluble polymers, and their nanoparticle carriers target different parts of the gastrointestinal tract. In this study, Eudragit® S100 was used to prepare nanoparticles as a alternative system for CyA delivery, and these were further characterized *in vivo* and *in vitro* as compared with

Sandimmune Neoral® (an available microemulsion system of CyA).
However, most nanoparticles are initially obtained as a milky suspension which shows acceptable short-term physical stability due to their reduced particle size. Because particle aggregation may occur after long periods of storage, surface active agents are usually added to stabilize the suspension by direct adsorption to the particle surface. Nevertheless, a little aggregation is often observed on storage (De Jaeghere et al. 1999). Furthermore, the biodegradable polymers degrade non-enzymatically in aqueous environments and drugs incorporated in such systems may be released during storage (Guterres et al. 1995; De Chasteigner et al. 1996). Lyophilization represents a good alternative to meet the requirement for long-term product stability. Nonetheless, previous studies of the freeze-drying of colloidal carriers (Crowe et al. 1996; Sun et al. 1996; Ozaki et al. 1997; Molpeceres et al. 1997) demonstrated that the addition of cryoprotectants is essential to maintain the initial formulation characteristics.
On the basis of the above mentioned considerations, it was thought plausible to introduce a freeze-drying technique to provide long-term stability. Thus, variations in bioavailability and pharmacokinetic parameters of CyA-NP after freeze-drying were investigated in the present study.

2. Investigations, results and discussion

2.1. Particle size, encapsulation efficiency and morphology of CyA-nanoparticles

The mean particle size of CyA-NP was 44.8 ± 3.2 nm, and the nanoparticles distributed between 20 and 82 nm as shown in Fig. 1. CyA-NP had a loading efficiency of $23.4 \pm 0.2\%$ and a maximum EE% of $99.7 \pm 0.1\%$.
Particle size is an important parameter as it can directly affect the physical stability, cellular uptake, biodistribution and drug release. In general, the smaller the particle size, the better the performance of nanoparticles (Mittal et al. 2007). For this reason, the effects of stirring speed, concentration of Poloxamer 188, and ratios of alcohol/water and CyA/Eudragit S100 were studied during our optimization procedures. It was demonstrated that the alcohol/water ratio plays a key role in particle size and distribution. That is understandable since the formation of the emulsion micro-droplets could affect the size of the nanoparticles.The high EE% values are due to the hydrophobicity of CyA.
The morphological properties of the nanoparticles are shown in Fig. 2. Most of the nanoparticles appear spherical under TEM.

2.2. Freeze-thawing studies

Freezing is considered to be the critical stage of lyophilization since a few large ice crystals may form at $-70\,^{\circ}C$ which can destroy the structure of the nanoparticles (Auvillain et al. 1989). As described for other particulate systems, the addition of cryoprotective agents induces non-regular packing of the molecules in the interstitial vitreous mass producing a larger internal space, and leading to decreased density compared with the more ordered structure built up by the ice crystals (Saez et al. 2000). This makes the frozen mass behave more like a fluid than a solid and provides better mechanical protection of the nanoparticles (Hancock et al. 1997). But not all the cryoprotectants can

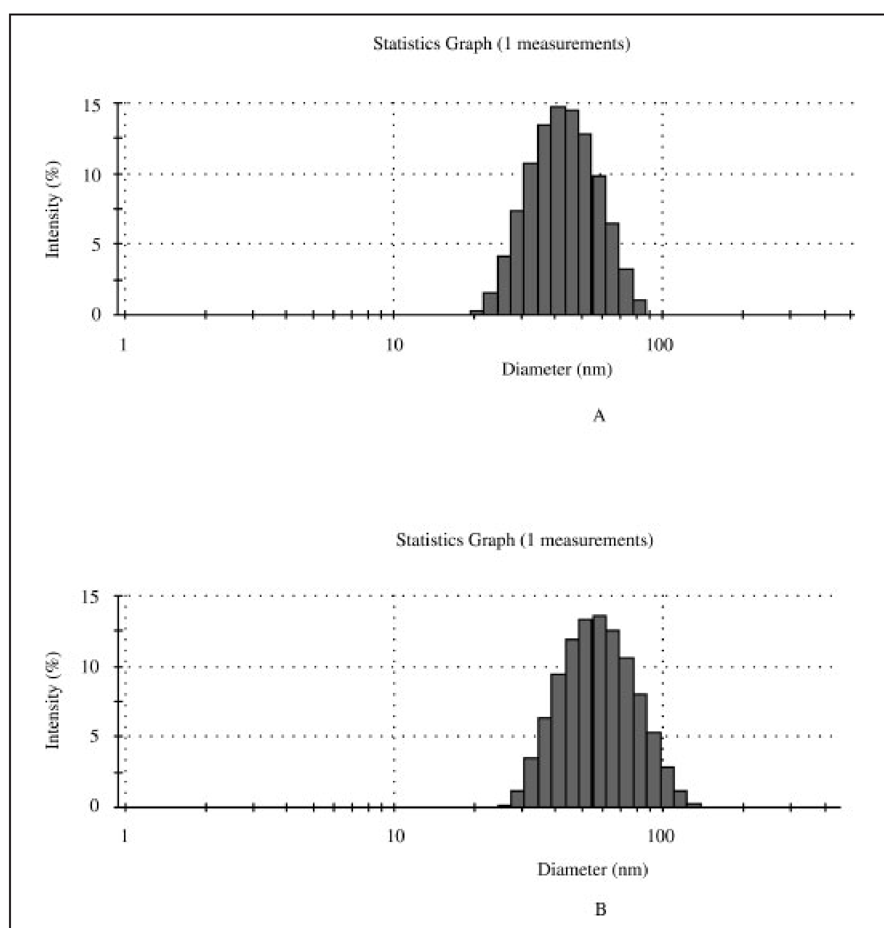

Fig. 1:
Size distribution of CyA-NP (A) and Lac-CyA-NP powders (B)

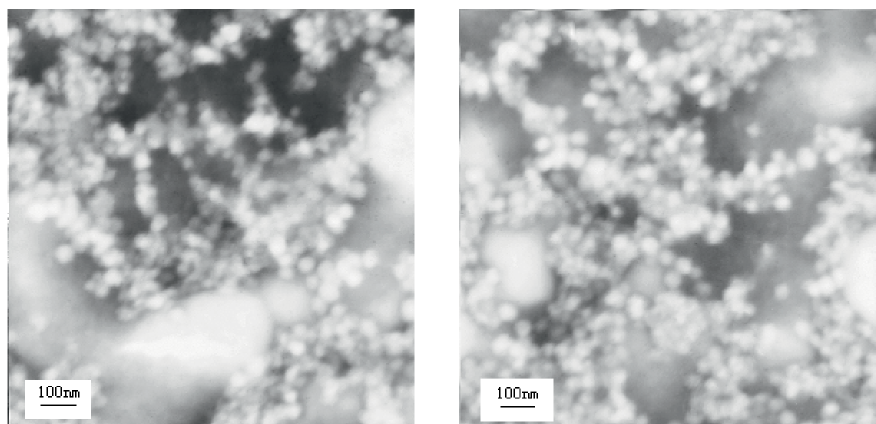

Fig. 2:
TEM photograph of CyA-NP (A) and Lac-CyA-NP powders (B). (magnification ×50,000)

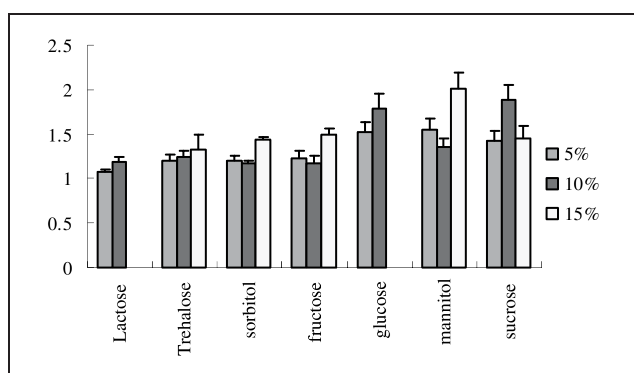

Fig. 3: Final to initial size ratio for CyA-NP frozen at −70 °C in the presence of variable amounts of cryoprotective agents (n = 5)

protect particles from aggregation and large variation in size. The values of $S_{frozen}/S_{unfrozen}$ are shown in Fig. 3. In the absence of cryoprotectants, significant aggregation was detected after freezing. As Fig. 3 shows, the value is below 1.3 for every concentration of trehalose, while for lactose, sorbitol and fructose, this is only the case at 5% and 10% concentrations. Nevertheless, others such as mannitol, sorbitol, glucose and sucrose result in high values above 1.5, and macroscopic aggregation was found occasionally. Accordingly, lactose, sorbitol, trehalose and fructose were selected for further study.

2.3. Freeze-drying studies

Table 1 summarizes the changes of particle size and CyA EE% after freeze-drying. The mean particle size of freeze-dried nanoparticles without cryoprotective agents increased to 164.9 ± 6.8 nm after reconstitution versus the initial 44.8 ± 3.2 nm for CyA-NP, indicating particle aggregation on freeze drying; the particle size was decreased to different degrees after adding three cryoprotective agents, the difference being significant ($P < 0.05$). Among these, the mean particle size was 55.7 ± 3.6 nm after reconstitution when using 3% lactose (W/V) as a cryoprotective agent. The size distribution was normal between 25 and 140 nm as shown in Fig.1. The morphological studies showed that most of the nanoparticles appear spherical without aggregation under TEM. As far as drug EE% is concerned, all formulations retained the amount of drug initially incorporated as shown by the ratio $EE_{frozen}/EE_{unfrozen}$, and the CyA EE% values were approximately 99.5%. The results proved that 3% lactose (W/V) was the best cryoprotective agent to stabilize the freeze-dried nanoparticle powder.

Furthermore, data on fructose used as the cryoprotective agent (not shown in Table 1) indicated that it resulted in considerable aggregation in the colliod suspension after reconstitution.

Table 1: Particle size of CyA-NP after freeze-drying without and with cryoprotective agents (n = 5)

Samples		Aggregation	$S_{frozen}/S_{unfrozen}$	$EE_{frozen}/EE_{unfrozen}$
Initial CyA-NP		0	1.00	1.00
Without cryoprotectants		2	3.43	–
Lactose	3%	0	1.16	1.00
	5%	0	1.28	1.00
	10%	0	1.34	1.00
Trehalose	5%	0	1.47	1.00
	10%	0	2.14	1.00
	15%	0	2.34	1.00
Sorbitol	5%	0	2.12	1.00
	10%	0	1.96	1.00
	15%	1	3.03	–

2.4. In vitro release studies

The sink condition was the major problem to be overcome in this study of the poorly soluble CyA. As Lee reported, 0.1 N HCl/acetonitrile (7 : 3) was chosen to meet the sink condition required for a release study of CyA-microspheres (Lee et al. 2001). However, Eudragit S100 is an enterically soluble polymer, and can dissolve completely at a high pH value. Given that a high concentration of organic solvent will increase drug release at low pH and then reduce the effects of the pH sensitive material on release, an ultracentrifugation method (Wang et al. 2004) was selected to determine the final drug release in mediums with different pH values. The results of the studies on CyA-NP, Lac-CyA-NP and Neoral® in mediums at different pH values are shown in Fig. 4. CyA-NP and Lac-CyA-NP displayed the same release behavior giving complete release in phosphate buffered saline at pH 6.8 and 7.4. This indicated that drug leakage was caused mainly by corrosion of the particle matrix. At pH values lower than 6.0, the release rates of the two preparations were below 30%. We can conclude that the end of the duodenum was the main site of drug release for CyA-NP and Lac-CyA-NP. In addition, Neoral®'s release behavior did not vary significantly at different pH values ($P > 0.05$) and the release rate was over 90% within 1 h.

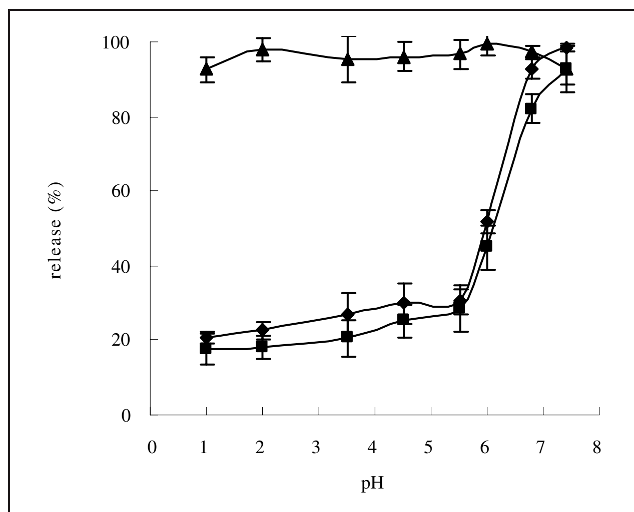

Fig. 4: *In vitro* drug release profiles from CyA-NP (●), Lac-CyA-NP (■) and Neoral® (▲), at different pH values (n = 3)

2.5. Bioavailability and pharmacokinetic studies

The bioavailability and pharmacokinetic parameters were determined after oral administration of Lac-CyA-NP to rats at a single dose of 15 mg/kg, and compared with those after oral administration of CyA-NP alone and Sandimmun Neoral®, respectively. The mean concentration-time curves of the three preparations are depicted in Fig. 5, and the pharmacokinetics of the three preparations were best fitted by a two-compartment model with a weight of $1/C^2$ assessed by AIC and correlation coefficient (r) of linear equation, and the corresponding pharmacokinetic parameters are summarized in Table 2.

As Table 2 shows, there are evident differences between the pharmacokinetic parameters of CyA-NP and Neoral® ($P < 0.01$), except for the mean residence time (MRT) and elimination constant of the central compartment (K_{10}). CyA-NP gave a significantly higher C_{max} of CyA ($3.38 \pm 0.34\ \mu g \cdot mL^{-1}$), than did Neoral® alone ($2.21 \pm 0.39\ \mu g \cdot mL^{-1}$), while the absorption constant decreased and T_{max} was delayed. The relative bioavailability from CyA-NP was 1.62-fold higher than that from Neoral® alone. As for Lac-CyA-NP, the T_{max} and C_{max} from Lac-CyA-NP (2.87 ± 0.35 h, $3.12 \pm 0.41\ \mu g \cdot mL^{-1}$) were also significantly different from those from Neoral® (1.75 ± 0.46 h), and the relative bioavailability from Lac-

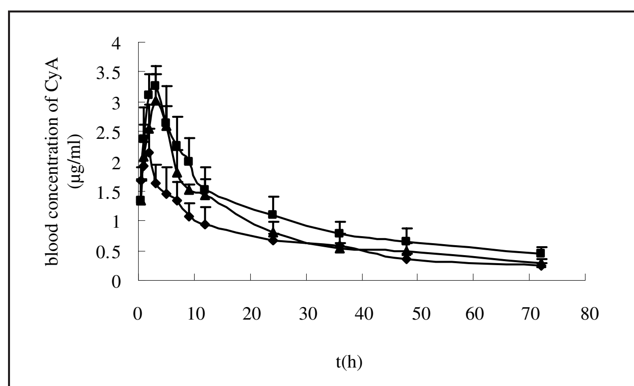

Fig. 5: Blood concentration profiles of CyA after oral administration of Neoral® (◆), CyA-NP(■) and Lac-CyA-NP (▲) into fasted rats at a dose of 15 mg/kg (n = 8, $\bar{x} \pm S$)

Table 2: Main pharmacokinetic parameters after oral administration of Neoral®, CyA-NP and Lac-CyA-NP in rats. Mean ± S.E. (n = 8)

Parameters	Neoral	CyA-NP	Lac-CyA-NP
C_{max} ($\mu g \cdot mL^{-1}$)	2.21 ± 0.39	$3.38 \pm 0.34^{*}$	$3.12 \pm 0.41^{\#}$
T_{max} (h)	1.75 ± 0.46	$2.75 \pm 0.52^{*}$	$2.87 \pm 0.35^{\#}$
AUC ($\mu g \cdot h \cdot mL^{-1}$)	46.86 ± 3.33	$75.97 \pm 6.85^{*}$	$60.95 \pm 3.04^{\#}$
MRT (h)	24.26 ± 0.25	$24.54 \pm 0.28^{**}$	$23.70 \pm 0.48^{\#}$
Ka (h^{-1})	2.49 ± 0.49	$1.04 \pm 0.20^{*}$	$0.86 \pm 0.14^{\#}$
K_{10} (h^{-1})	0.04 ± 0.01	$0.04 \pm 0.02^{**}$	$0.05 \pm 0.01^{\#}$
Fr (%)	–	162.1	130.1

Relative bioavailability: $Fr\% = (AUC_{test} \times D_{Neoral}/AUC_{Neoral} \times D_{test}) \times 100\%$
* $P < 0.01$ vs. Neoral®.; ** $P < 0.05$ vs. Neoral®; $^{\#}$ $P < 0.05$ vs. CyA-NP

CyA-NP was 1.30-fold higher than Neoral® ($P < 0.05$). On the other hand, there was no significant difference between the pharmacokinetic parameters of Lac-CyA-NP and those of CyA-NP. The AUC ($60.95 \pm 3.04\ \mu g \cdot h \cdot mL^{-1}$) of Lac-CyA-NP was less than that from CyA-NP ($75.97 \pm 6.85\ \mu g \cdot h \cdot mL^{-1}$), but there was no statistical difference between them. Accordingly, Lac-CyA-NP gave similar bioavailability to that of CyA-NP.

The results indicated that pH sensitive loaded CyA nanoparticles improve the bioavailability of CyA. This could be attributed to the protective effect of the pH sensitive nanoparticles. As Section 2.4 shows, the nanoparticles dissolve at pH 6.8 in order to target drug delivery at the end of duodenum. Thus, the nanoparticles could protect the CyA from degradation by gastric acid and enzymes, As reported (Drewe et al. 1992), CyA is absorbed primarily in the small intestine and the abrupt release creates a high concentration of CyA in the intestinal tract as close as possible to the absorption window. On the other hand, the absorption of particles from the intestine is a well known to be affected by a number of factors among which particle size is prominent (McClean et al. 1998). The uptake efficiency of 100 nm size particles by the intestine is 15 to 120-fold higher than for larger size microparticles, and these may be the primary factors responsible for the variation in absorption between CyA-NP and Lac-CyA-NP after oral administration at a fixed dose of 15 mg/kg. On the other hand, as Fig. 5 shows, the blood concentrations of CyA after a dose of Neoral® were at all times lower than those after a dose of CyA-NP or Lac-CyA-NP. However, the concentration-time curve of the three preparations showed wide variability, thus the incorporation of CyA into pH sensitive nanoparticles could not minimize the individual variation of pharmacokinetics. The long-term stability of Lac-CyA-NP and the variation of their pharmacokinetics during storage have been the subject of my further studies.

In summary, this study describes conditions for pH-sensitive CyA-loaded nanoparticles and freeze-dried powder. The results demonstrated that pH-sensitive nanoparticles improve the absorption and overall bioavailability of CyA after oral administration, due to particle size reduction and specific pH-dependent drug release. As regards lyophilization, the studies showed the necessity of adding cryoprotective agents to diminish aggregation of colloidal suspensions during the procedure. 3% lactose (W/N) was the best cryoprotective agent. The greater relative bioavailability of Lac-CyA-NP suggests that it is possible to develop a new CyA solid form for oral therapy.

3. Experimental

3.1. Materials

CyA and CyD were purchased from Fujian Kerui Pharmaceutical Co. Ltd (China). Sandimmune Neoral® capsules were obtained from Novartis (Switzerland). The pH-sensitive poly (methacrylic acid-*co*-methyl methacrylate) copolymers (Eudragit® S100) were from Röhm (Darmstadt, Germany). Poloxamer 188 was supplied from the pharmaceutical plant affiliated to Shenyang Pharmaceutical University (China). D-(+)fructose, D-(+)glucose, D-(+)sucrose, D-(+)dihydrated trehalose, D-mannitol, D-sorbitol and lactose were donated by the Shanghai Changwei Medicine Adjuvant Technique Group Co. Ltd (China). Acetonitrile, methanol and *n*-hexane were obtained from Fisher Chemical Group Co. Ltd (USA) and were of HPLC grade. All other reagents were of analytical grade.

Sprague-Dawley (SD) rats were obtained from the laboratory animals center of the Medical College, Soochow University. All the animal experiments adhered to the principles of care and use of laboratory animals and were approved by the laboratory animals center of the Medical College, Soochow University.

3.2. Preparation of CyA-nanoparticles

The CyA-NP were prepared by the quasi-emulsion solvent diffusion technique (Kawashima et al. 1989). Briefly, an oil phase containing CyA/Eudragit®S100 (1 : 4, w/w) dissolved in 50 mL of anhydrous ethanol was injected as quickly as possible into 125 mL of stirred water containing 125 mg of Poloxamer 188. The mixture was stirred for 10 min at 400 rpm and the CyA-NP was obtained by slowly evaporating residual ethanol in a 60 °C water bath for 3 h.

3.3. Particle size distribution and morphology

Particle size distribution analysis was performed by dynamic light scattering (Malvern Instruments Ltd., UK). The CyA-NP was dispersed in 10-fold ultrapure water; the wavelength and temperature were set at 670 nm and 25 °C respectively.

Analysis of morphology was carried out by transmission electron microscopy (TEM) (H600, Hitachi, Japan). CyA-NP were dropped on to the copper screen and stained with a 2% solution of phosphotungstic acid before analysis.

3.4. Encapsulation efficiency and loading efficiency

A freshly prepared suspension of CyA-NP was filtered through a 0.45 μm filter (Shanghai Xingya Cleaning Materials Co., China) to remove insoluble polymer residues and CyA microcrystals. Then, 5 mL of filtered suspension were ultracentrifuged (250 000 × g for 60 min, 10 °C) and the supernatant was sampled. The concentrations of CyA in the filtered suspension and in the supernatant were estimated by a reversed-phase HPLC method.

The HPLC system consisted of two pumps (LC-10AT VP, Shimadzu, Japan), and a UV-vis detector (SPD-M10A VP, Shimadzu, Japan) set at 210 nm. The chromatographic column used was a Luna C18 (5 μm in 4.6 mm × 250 mm, Phenomenex, USA) thermostated at 70 °C. The mobile phase consisted of acetonitrile/methanol/water (8 : 1 : 1) and the flow rate was 1.0 mL · min^{-1}. The encapsulation efficiency (EE%) and drug loading efficiency (LD%) were calculated by the formulate below:

$$EE\% = \frac{C - Cs}{C} \times 100\% \quad (1)$$

$$LD\% = \frac{(C - Cs) \times V}{W} \times 100\% \quad (2)$$

where C and Cs represent the drug concentrations in the filtered suspension and in the supernatant respectively, while V is the volume of the filtered suspension and W the theoretical amount of Eudragit S100 added.

3.5. Freeze-thawing

The CyA-NP were concentrated to 8 mg CyA per milliliter by an ultrafilter (Vivaflow 50, polysulfone ether membrane, 10 K) before use. The CyA-NP were freeze-thawed in the presence of 5%, 10% and 15% fructose, glucose, sucrose, dihydrated trehalose, mannitol, sorbitol and/or lactose. Aliquots (1 mL) were placed in sealed vials and frozen at −70 °C for 24 h. Then, the samples were thawed at room temperature and the process was repeated. Simultaneously, the experiments were performed in the absence of cryoprotective agents as a control. Particle size was determined for those preparations where no visible aggregation was observed and the final to unfrozen size ratio was calculated ($S_{frozen}/S_{unfrozen}$). Ratios of 1 ± 0.3 were considered as being acceptable (Saez et al. 2000).

3.6. Freeze-drying

Samples (1 mL) were frozen at −70 °C for 24 h. After freezing, the samples were immediately placed in a freeze-drying chamber (Lg-5A, Shanghai Centrifuge Institute Co., Ltd, China). Sublimation lasted 30 h without heating. Finally, glass vials were sealed under anhydrous conditions. Sample reconstitution was performed by adding 10 mL of ultrapure water to each powder sample. The ratios between the initial particle size and EE% of CyA to those of the powder were calculated but only for non-aggregated samples. Particle aggregation was quantified by the following numerical scale: (0) absence of aggregation, (1) few sizable aggregation, (2) extensive aggregation.

3.7. In vitro release studies

0.5 mL of a suspension of the nanoparticles or Neoral® microemulsion with a CyA concentration of 3 mg · mL^{-1} were dispersed in flasks filled with phosphate buffer containing 0.1% SDS at different pH values which were 1.0, 2.0, 3.5, 4.5, 5.5, 6.0, 6.8 and 7.4 respectively. Then the suspension was incubated in a shaker thermostated at 37 °C for 1 h at a speed of 100 rpm. The suspension was sampled and ultracentrifuged for 60 min (250 000 × g). The amount of CyA in the supernatant was determined by HPLC, as mentioned in section 3.4.

3.8. Bioavailability and pharmaceutics study

Twenty-four male SD rats (weighing 230–270 g) were requested from the laboratory animals center of Soochow University Medical College and randomly divided into three groups (eight animals for each group). They were maintained in metabolic cages for 2 days before the treatment, maintaining a 12 : 12 h dark/light cycle and free access to standard food and tap water. Before experiments, the animals were fasted overnight and had access to water ad libitum. A single oral dose (15 mg/kg) of Neoral®, CyA-NP or Lac-CyA-NP was given to rats by gavage from 09 : 00 to 10 : 00 am to avoid chronopharmacokinetic effects. After oral administration of the various formulations fasting continued for a further 4 h. At predetermined time intervals (0.5, 1, 2, 3, 5, 7, 9, 12, 24, 36, 48 and 72 h), blood samples (about 0.8 mL each) were drawn from the ocular vein into heparinized tubes and stored at −20 °C until analysis.

CyA concentration in whole blood was also estimated by a reversed phase HPLC method. Briefly, 0.5 mL of blood sample was added to a centrifuge tube, then 50 μL of internal standard (cyclosporine D, CyD) in methanol at a concentration of 6 μg · mL^{-1} and 1 mL of hydrochloric acid (180 mmol · L^{-1}) were added. After vortex mixing for 1 min, 5 mL of ether was added. The extraction was carried out by shaking the tube horizontally for 15 min at 100 rpm, centrifuging it for 15 min at 4000 rpm, and separating the ether phase to another centrifuge tube. After 1 mL of sodium hydroxide solution (95 mmol · L^{-1}) and 2.5 mL of 1% sodium pyrosulfite solution were added, the extraction was repeated by the same procedure as described above. The ether layer was transferred into a clean tube and evaporated to dryness under nitrogen at 40 °C. The residue was reconstituted with 100 μL of acetonitrile/water (70/30, v/v). The reconstituted samples were washed twice with 1 mL of n-hexane. 20 μL of residual solution was injected into the HPLC system. The peak area of CyA and CyD were recorded and the content of CyA was determined.

The HPLC system was the same as that mentioned in Section 3.4. The chromatographic column used was a Luna C18 (5μm in 4.6 mm × 250 mm, Phenomenex, USA) thermostated at 70 °C. The mobile phase consisted of acetonitrile/methanol/water (4 : 1 : 1) and the flow rate was 1.3 mL · min^{-1}.

50 μL CyA methanolic solution with concentrations of 1, 2, 5, 10, 20, 30 and 50 μg· m L^{-1} was added to 0.5 mL drug-free pooled whole blood, and vortex mixed for 1 min to provide samples with concentrations of 0.1, 0.2, 0.5, 1.0, 2.0, 3.0 and 5.0 μg · mL^{-1}, in the presence of the internal standard CyD (6 μg · mL^{-1}). Each blood standard was then taken through the sample preparation procedure described above. Quantitation was done by determining the peak-area ratio of CyA/CyD (A/Ai) against the drug concentrations. The concentrations of unknown samples were determined using the linear regression line (unweighted) of the concentration of the calibration standard versus peak-area ratio. The regression equation of the concentration of CyA in blood was: $C = 3.1165A/Ai + 0.0494$, with a good linear relationship in the range 0.1–5.0 μg · mL^{-1}, $r = 0.9997$. The mean extraction recovery rate of CyA was 95 ± 3.6% and the method recovery rate was 99.2–105.8%; the recovery rate of CyD was 90.2 ± 6.3%. The limit of quantification was 20 ng. There was no endogenous substance in the blood which would interfere with the determination of CyA and CyD, and there was no impurity introduced in the sample preparation procedure. CyD can be separated from CyA under these chromatographic conditions, the retention times of 9.3 min and 12.2 min giving good resolution of the two components and suitable appearance time.

3.9. Pharmacokinetic analysis

The zero-order moment area under the blood concentration-time curve (AUC) was calculated by the trapezoidal rule, while the maximum blood concentration (C_{max}) and the time of maximum blood concentration (T_{max}) were obtained directly by observation. The pharmacokinetic parameters were evaluated by 3P97 (a computer program produced by the Committee of Mathematical Pharmacology of the Chinese Society of Pharmacology).

The differences between pharmacokinetic parameters in the various test groups were estimated by the multiple comparison test.

Acknowledgements: The work was supported by the Social Development Foundation of Jiangsu Province (BS200522), the New High Technology Research Project of the department of education of Jinagsu Province (JHB05–46) and grants from the health department of Jinagsu Province (H200630).

References

Auvillain M, Cavé G, Fessi H, Devissaguet JP (1989) Lyophilisation de vecteurs collodaux submicroniques. STP Pharma 5: 738–744.

Bhardwaj V, Hariharan S, Bala I, Lamprecht A, Kumar N, Panchagnula R, Kumar MNVR (2005) Pharmaceutical aspects of polymeric nanoparticles for oral drug delivery. J Biomed Nanotech 1: 235–258.

Cavanak T, Sucker H (1986) Formulation of dosage forms. Progr Allergy 38: 65–72.

Hariharan S, Bhardwaj V, Bala I, Sitterberg J, Bakowsky U, Kumar MNVR (2006) Design of estradiol loaded PLGA nanoparticulate formulations: a potential oral delivery system for hormone therapy. Pharm Res 23: 184–195.

Chacón M, Berges L, Molpeceres J (1996) Optimized preparation of poly D,L(lactic-glycolic) microspheres and nanoparticles for oral administration. Int J Pharm 141: 81–91.

Crowe LM, Reid DS, Crowe JH (1996) Is trehalose special for preserving dry biomaterials? Biophys J 71: 2087–2093.

De Jaeghere F, Allemann E, Leroux JC, Stevels W, Feijen J, Doelker E, Gumy R (1999) Formulation and lyoprotection of poly(lactic acid-co-ethylene oxide) nanoparticles: Influence on physical stability and in vitro cell uptake. Pharm Res 16: 859–866.

De Chasteigner S, Cave G, Fessi H, Devissaguet JP, Puisieux F (1996) Freeze-drying of itraconazole-loaded nanosphere suspensions: a feasibility study. Drug Dev Res 38: 116–124.

Drewe J, Beglinger C, Kissel T (1992) The absorption site of cyclosporine in the human gastrointestinal tract. Br J Clin Pharm 33: 39–43.

El-Shabouri MH (2002) Positively charged nanoparticles for improving the oral bioavailability of cyclosporine-A. Int J Pharm 249: 101–108.

Guterres SS, Fessi H, Barrat G, Devissaguet JR, Puisieux F (1995) Poly(d,l-lactide) nanoparticles containing diclofenac: I Formulation and stability study. Int J Pharm 113: 57–63.

Hancock BC, Zoografi G (1997) Characteristics and significance of the amorphous state in pharmaceutical system. J Pharm Sci 86: 1–11.

Italia JL, Bhatt DK, Bhardwaj V (2007) PLGA nanoparticles for oral delivery of cyclosporine: Nephrotoxicity and pharmacokinetic studies in comparison to Sandimmune Neoral. J Control Release 119: 197–206.

Kahan BD, Podbielski J, Napoli KL, Katz SM (1998) Immunosuppressive effects and safety of a sirolimus/cyclosporine combination regimen for renal transplantation. Transplantation 66: 1040–1046.

Kovarik JM, Mueller EA, Van Bree JB, Tetzloff W, Kutz K (1994) Reduced inter- and intra-individual variability in cyclosporine pharmacokinetics from a microemulsion formulation. J Pharm Sci 83: 444–446.

Kawashima Y, Niwa T, Handa T, Takeuchi H, lwamoto T, Itoh K (1989) Preparation of controlled-release microspheres of ibuprofen with acrylic polymers by a novel quasi-emulsion solvent diffusion method. J Pharm Sci 78: 68–72.

Luke DR, Kasiske BL, Matzke GR, Awni WM, Keane WF (1987) Effect of cyclosporine on the isolated perfused rat kidney. Transplantation 43: 795–799.

Lee Eun-Jin, Lee Sa-Won, Choi Han-Gon, Kim Chong-Kook (2001) Bioavailability of cyclosporine A dispersed in sodium lauryl sulfate-dextrin based solid microspheres. Int J Pharm 218: 125–131.

Matzke GR, Luke DR (1988) Dialysis and renal transplant therapy. In: Herfindal ET, Gourley, DR, Hart LL (eds.) Clinical Pharmacy and Therapeutics. Williams & Wilkins, Baltimore, pp. 229–242.

Mithani SD, Bakatselou V, Tenhoor CN, Dressman JB (1996) Estimation of the increase in solubility of drugs as a function of bile salt concentration. Pharm Res 13: 163–167.

Molpeceres J, Aberturas MR, Chacon M, Berges L, Guzman M (1997) Stability of cyclosporine-loaded poly-ε-caprolactone nanoparticles. J Microencapsulation 14: 777–787.

Mittal G, Sahana DK, Bhardwaj V, Ravi Kumar MNV (2007) Estradiol loaded PLGA nanoparticles for oral administration: Effect of polymer molecular weight and copolymer composition on release behavior *in vitro* and *in vivo*. J Control Release 119: 77–85.

McClean S, Prosser E, Mechan E, O'Malley D, Clarke N, Ramtoola Z, Brayden D (1998) Binding and uptake of biodegradable poly-DL-lactide micro- and nanoparticles in intestinal epithelia. Eur J Pharm Sci 6: 153–163.

Ozaki K, Hayashi M (1997) The effects of glucose oligomers (maltodextrins) on freeze-drying liposomes. Chem Pharm Bull 45: 165–170.

Sun WQ, Leopold AC, Crowe LM, Crowe JH (1996) Stability of dry liposomes in sugar glasses. Biophys J 70: 1769–1776.

Saez A, Guzmán M, Molpeceres J, Aberturas MR (2000) Freeze-drying of polycaprolactone and poly(D,L-lactic-glycolic) nanoparticles induce minor particle size changes affecting the oral pharmacokinetics of loaded drugs. Eur J Pharm Biopharm 50: 379–387.

Thomson, N (1991) Cyclosporin: use outside transplantation. Brit Med J 302: 4–5.

Taylor NE, Mark AE, Vallat P, Brunne RM, Testa B, Van Gunteren WF (1993) Solvent dependent conformation and hydrogen bounding capacity of cyclosporine A: evidence from partition coefficient and molecular dynamics simulations. J Med Chem 36: 3753–3764.

Tjai JF, Webber IR, Back DJ (1991) Cyclosporin metabolism by the gastrointestinal mucosa. Br J Clin Pharmacol 31: 344–346.

Varela MC, Guzmán, M, Molpeceres J (2001) Cyclosporine-loaded polycaprolactone nanoparticles: immunosuppression and nephrotoxicity in rats. Eur J Pharm Sci 12: 471–478.

Wang XQ, Dai JD, Zhang Q (2004) Bioavailability and pharmacokinetics of cyclosporine A-loaded pH-sensitive nanoparticles for oral administration. J Control Release 97: 421–429.