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Oral evaluation in rabbits of cyclosporin-loaded Eudragit RS or RL nanoparticles

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Abstract

The hydrophobic cyclic undecapeptide cyclosporin A (CyA) used in the prevention of graft rejection and in the treatment of autoimmune diseases was encapsulated by nanoprecipitation within non-biodegradable polymeric nanoparticles. The effect of polymers (Eudragit® RS or RL) and additives within the alcoholic phase (fatty acid esters and polyoxyethylated castor oil) on the size, zeta potential and the encapsulation efficiency of the nanoparticles was investigated. The mean diameter of the various CyA nanoparticles ranged from 170 to 310 nm. The size as well as the zeta potential increased by adding fatty acid ester and polyoxyethylated castor oil within the organic phase. No significant differences in surface potential were observed for all formulations tested. Probably due to the very low water solubility of the drug, high encapsulation efficiencies were observed in a range from 70 to 85%. The oral absorption of CyA from these polymeric nanoparticles was studied in rabbits and compared to that of Neoral® capsule. Based on comparison of the area under the blood concentration–time curve values, the relative bioavailability of CyA from each nanoparticulate formulation ranged from 20 to 35%.

Keywords: Nanoparticle; Cyclosporin; Oral administration; Eudragit®

1. Introduction

Cyclosporin A (CyA), a potent immunosuppressive agent, is widely used for the prevention of graft rejection in transplanted patients (Matzke and Luke, 1988) as well as in the treatment of autoimmune diseases (Richardson and Emery, 1995).

CyA was first marketed as an oil-based oral solution, or an injectable solution containing polyoxyethylated castor oil. However, the oral bioavailability of CyA from these conventional preparations displayed considerable inter- and intra-individual variability (Fahr, 1993; Molpeceres et al., 1998, 2000), probably because of poor drug absorption and intestinal metabolism.

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Moreover, CyA exhibits a broad toxicity profile, including nephrotoxicity and hepatotoxicity. The marketing of a pre-microemulsion of CyA allows a better reproducibility of the absorption, but the bioavailability, although improved, is still low (Klyashcchitsky and Owen, 1998).

Various dosage forms have been studied to reduce the toxicity and to increase the absorption of CyA. Particulate polymeric drug delivery systems such as micro- and nano-particles have been studied extensively. CyA has been associated to poly(isohexylcyanoacrylate) nanospheres (Bonduelle et al., 1992), or encapsulated within poly(isobutylcyanoacrylate) or poly-ε-caprolactone nanoparticles (Guzman et al., 1993). Sanchez et al. (1993) demonstrated the feasability of efficiently encapsulating CyA into poly(DL-lactide-co-glycolide) micro- and nano-spheres allowing a controlled release of the drug. CyA loaded nanocapsules composed of an oily core (mygliol®) and a poly-ε-caprolactone coat were interesting carriers for ocular delivery; this dosage form resulted in a better absorption of the drug through the cornea (Calvo et al., 1996). Poly(acrylic acid) polymeric gels and poly(isobutylcyanoacrylate) nanocapsules of CyA also showed, ex vivo, an increased absorption of the drug in the bovine cornea model (Le Bourlais et al., 1997). More recently, CyA loaded poly(DL-lactic acid) microspheres prepared in the presence of fatty acid esters as additives exhibited a significant inhibitory effect on the edema after subcutaneous administration in rats with adjuvant-induced arthritis (Urata et al., 1999).

CyA delivery systems were also prepared without particle-forming polymers. CyA nanospheres were prepared by precipitation in an aqueous surfactant solution; however, after oral administration in dogs, the absorption of CyA and the relative bioavailability were poor when compared to the Neoral® microemulsion (Ford et al., 1999). On the contrary, liposomes and mixed micelles containing CyA (Lee et al., 1999) as well as lecithin micelles (Guo et al., 2000) and CyA loaded stearic acid nanoparticles (Zhang et al., 2000) showed that CyA was available after topical, intravenous or oral administration. However, these formulations were not stable enough and the bioavailability was lower than the currently available marketed dosage forms of CyA.

In this study, CyA nanoparticles were prepared by nanoprecipitation with non-biodegradable positively-charged polymers (Eudragit® RS and RL), with or without fatty acid esters (Maisine®) and polyoxyethylated castor oil (Cremophor®). Maisine and Cremophor are excipients already used in the marketed dosage form Neoral®. The association of colloidal particles and polycationic polymers was supposed to improve the interaction with the negatively-charged mucus of the gastro-intestinal tract. The formulations were characterized in vitro with regard to encapsulation efficiency, size and surface potential and were evaluated in vivo after oral administration to rabbits, in comparison to both, a Neoral® capsule administered orally and a marketed CyA solution administered intravenously.

2. Materials and methods

2.1. Materials

Eudragit® RS 100 and RL 100 as well as Maisine® (glyceryl monolinoleate), Cremophor® RH40 (polyoxyethylated castor oil) and Pluronic® F68 were kindly supplied, respectively, by Röhm GmbH (Darmstadt, Germany) and BASF (Ludwigshafen, Germany). Cyclosporin A powder and absolute ethanol were purchased from Sigma (St. Louis, MO, USA). Marketed cyclosporin (injectable solution, Sandimmum®) and Neoral® capsules (25 mg) were obtained from commercial sources. The standard kit (Emit®) used for the assay of free cyclosporin recovered in the aqueous solution for the determination of the encapsulation efficiency, as well as the absorbed cyclosporin recovered in blood for the pharmacokinetic study was provided by Dade Behring (Paris, France). All other chemical reagents were of analytical grade and used as supplied.

2.2. Preparation of nanoparticles

The preparation of the nanoparticles was carried out by the nanoprecipitation method previously described by Fessi et al. (1989) and Bodmeier et al. (1991) and adapted as follows: Eudragit® RS or RL (0.2 g) and cyclosporin A powder (50 mg) were dissolved in a sealed vial containing ethanol (1 ml) in an ultrasound bath for 10 min. Then, an aqueous solution (9 ml) of Pluronic® F68 (0.5% m/V) was added to this organic solution under magnetic stirring (500 rpm) for 2 min.

Nanoparticles prepared with Maisine® (0.75% m/V) and Cremophor® (0.25% m/V) within the alcoholic solution were formulated in the same way.

2.3. Cyclosporin encapsulation efficiency

The amount of cyclosporin entrapped within the polymeric nanoparticles was determined by an enzyme immunoassay (EIA) by indirectly measuring the amount of CyA in the aqueous phase with the Emit® test on a Cobas Mira® automate (Dade Behring) ($n = 3$). Briefly, the amount of non-entrapped cyclosporin in the hydro-alcoholic solution recovered after centrifugation ($50{,}000 \times g$ for 30 min) and washing of the nanoparticles was determined according to the procedure described by the supplier of the kit. The actual encapsulation rate was based on the difference between the initial CyA amount and the free (non-encapsulated) drug detected in the hydro-alcoholic solution.

2.4. Particle size and zeta potential

The nanoparticles were analyzed for their mean size and size distribution as well as for their surface potential by using a Zetasizer® (Malvern Instruments, UK) ($n = 3$). The results were all normalized with respect to a polystyrene standard suspension (Malvern Instruments).

2.5. Pharmacokinetic study

Male adult New Zealand rabbits with a mean body weight of 3000 ± 500 g were fasted overnight prior to the experiments with free access to water. The hydro-alcoholic suspension of cyclosporin-loaded nanoparticles (10 ml) was administered orally through a cannula to heparinized (1000 IU/kg) rabbits. The Sandimmum® solution (50 mg of cyclosporin) (administered intravenously) and Neoral® capsule (50 mg of cyclosporin) (administered orally) were used as controls. In each case, blood samples (500 μL) were withdrawn from the marginal ear vein of the rabbits at 0 (pre-dose) and 0.5, 1, 2, 3, 4, 5, 6, 8, 10 and 24 h post-administration. Whole blood samples were stored at 4 °C until assayed by the EIA previously described. The sensitivity of the EIA method was 40 ng mL^{-1} and the linearity ranged from 40 to 500 ng mL^{-1}.

The areas under the curve (AUC) of the concentration-time profile were calculated with the linear trapezoidal method. The absolute bioavailability was calculated as $(AUC_{oral}/AUC_{i.v.}) \times 100$.

All statistical analyses were carried out with the software package Sigmastat 2.0. Each pharmacokinetic parameter value was analyzed by the nonparametric Mann–Whitney test. Data are reported as mean ± S.D. A value of $p < 0.05$ was considered to be statistically significant.

3. Results and discussion

CyA-loaded nanoparticles were prepared by nanoprecipitation. Briefly, an ethanolic drug-containing polymer phase was combined with an external aqueous phase resulting in the formation of the nanoparticles because of the water-insolubility of both cyclosporin and the Eudragit® RS/RL polymers. The polymers carry cationic functional groups, which are also responsible for the stabilization of the nanoparticle dispersion. The method was slightly modified in order to form the nanoparticles from a more concentrated and therefore viscous drug/polymer phase. The aqueous Pluronic® solution was directly poured under magnetic mixing into the alcoholic drug/polymer phase and not vice versa, as is normally done. The amount of ethanol had to be minimized because the nanoparticles were administered without evaporating ethanol. It has to be noted that ethanol is also present in the two control dosage forms, Sandimmum® for intravenous administration and Neoral® soft capsules for the oral route.

The size of CyA-loaded nanoparticles of all formulations ranged from 170 to 310 nm, with a relative monodisperse distribution (Table 1).They exhibited significant size differences according to their composition. Larger particles were obtained when Maisine® and Cremophor® were added to the alcoholic solution containing both the polymer and the drug. As reported previously (Urata et al., 1999), polymeric particles containing a fatty acid ester have the structure of a composite matrix with channels formed by the ester. Water can diffuse through the channels resulting in a slight swelling of the particles in the aqueous phase during their preparation and consequently a larger size. No significant size and surface potential differences were observed between unloaded and drug-loaded nanopar-

Table 1
Mean diameter, zeta potential and encapsulation efficiency of CyA-loaded nanoparticles prepared with Eudragit® RS or RL with or without Maisine® (M) and Cremophor® (C)

Formulations	Mean diameter (nm)	Zeta potential (mV)	Encapsulation efficiency (%)
NP Eudragit® RS	176 ± 5	49 ± 3	70 ± 3
NP Eudragit® RS/M + C	290 ± 8	63 ± 5	73 ± 5
NP Eudragit® RL	180 ± 7	55 ± 2	85 ± 5
NP Eudragit® RL/M + C	310 ± 5	86 ± 2	78 ± 6

Data are expressed as mean ± S.D. ($n = 3$).

ticles (data not shown). These results demonstrate that the modification in the manufacturing process (addition of the aqueous phase to the organic one) did not significantly change the mean diameter of the nanoparticles (Fessi et al., 1989).

The zeta potential values (Table 1) show the effect of Eudragit® RS and RL with or without Maisine® on the surface charge of nanoparticles. All formulations were positively-charged with a zeta potential in the range of 49–86 mV because of the quaternary ammonium groups of Eudragit® (between 4.5–6.8 and 8.8–12% for RS and RL, respectively).

If a part of the drug was adsorbed onto the polymeric particles, a part of the positive charges of the polymers would be masked involving the decrease of the surface potential value. Since it did not occur, it could consequently be concluded that CyA was not adsorbed onto the polymeric particles, but rather encapsulated within nanoparticles. In addition, due to the low water solubility of CyA, it was preferentially partitioned into the polymer phase, thus preventing the drug to locate at the outer surface of the particles. Only a small amount of the drug was lost in the aqueous phase, resulting in high encapsulation efficiencies (70–85%) for all formulations (Table 1). A slightly higher entrapment efficiency was observed when Maisine® was added to the polymers Eudragit® RS or RL, probably due to higher solubility of the drug in the polymer/Maisine® matrix. The drug has to have a high affinity (solubility) for the nanoparticle matrix, otherwise it would diffuse in the external aqueous phase and potentially precipitate after solvent evaporation. In addition, larger particles (Eudragit® RS or RL nanoparticles) have a smaller overall surface area and thus drug leakage is smaller.

For the pharmacokinetic evaluation, a dose of 50 mg CyA was administered both orally and intravenously to rabbits. Due to potential stability problems with the pre-microemulsion, it was prefered not to open the capsules for adjusting the dose to rabbit weight. Despite being carried out with only a small number of animals ($n \geq 3$), the pharmacokinetic data obtained after intravenous administration of the Sandimmum® solution were reproducible and thus allowed the calculation of the absolute bioavailability.

The results obtained with the marketed Neoral® and the tested formulations showed large differences in terms of AUC and C_{max}. The absorption profiles were similar regardless of the dosage form, with

Table 2
Pharmacokinetic parameters after oral administration of CyA-loaded nanoparticles in rabbits (50 mg) vs. the oral Neoral® microemulsion (50 mg) and the Sandimmum solution administered intravenously (50 mg)

Formulations	C_{max} (ng mL^{-1})	t_{max} (min)	AUC/kg (ng mL^{-1} kg^{-1} h)	Relative F (%)	Absolute F (%)
Eudragit® RS nanoparticles	142 ± 74	45 ± 17	363 ± 164	25 ± 11	5.6 ± 2.5
Eudragit® RS/M + C nanoparticles	335 ± 52	60 ± 0	335 ± 52	34 ± 9[a]	7.5 ± 2.2
Eudragit® RL nanoparticles	139 ± 56	70 ± 45	392 ± 162	27 ± 11	6.0 ± 2.5
Eudragit® RL/M + C nanoparticles	173 ± 21	60 ± 0	293 ± 29	20 ± 2	4.5 ± 0.4
Neoral microemulsion	803 ± 63	60 ± 0	1141 ± 8	100	22.2 ± 0.1
Sandimmum solution, i.v.	ND	ND	6490 ± 715	–	100

AUC, M and C indicate area under the curve, Maisine® and Cremophor®, respectively. Data are mean ± S.D. ($n \geq 3$).
[a] Statistically different from Eudragit® RL/M + C nanoparticles at $p < 0.05$.

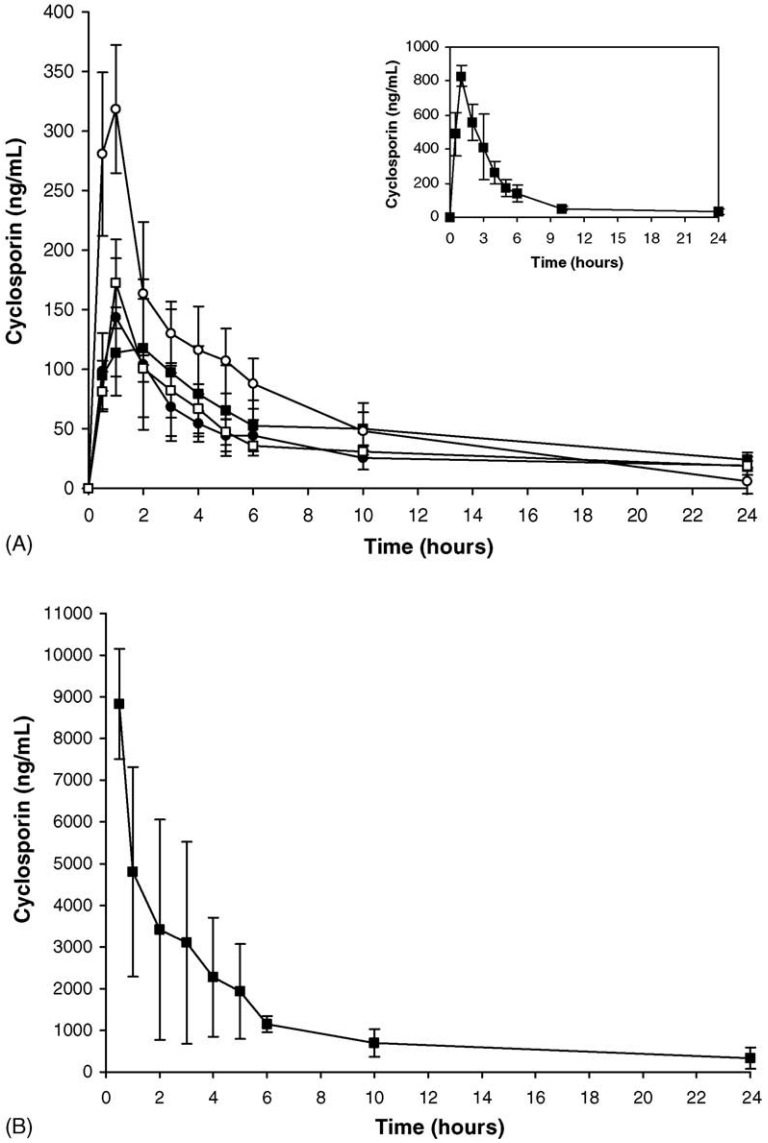

Fig. 1. CyA concentration in blood after (A) a single oral administration in rabbits of CyA (50 mg)-loaded nanoparticles prepared with Eudragit® RS without and with Maisine®/Cremophor® (full circle and open circle, respectively) and Eudragit® RL without or with Maisine®/Cremophor® (full square and open square, respectively), and (B) Sandimmum® solution (50 mg) administered intravenously in rabbits. Inset in Fig. 1A: mean CyA concentration in blood vs. time after oral administration of 2 capsules (25 mg) of Neoral® microemulsion in rabbits. Data are mean ± S.D. ($n \geq 3$).

a t_{max} at 1 h (Fig. 1). However, the C_{max} values are not similar for each formulation (Table 2). This also reflects the differences in relative bioavailabilities. The highest relative bioavailability (compared to Neoral®) was 34% for nanoparticles prepared with Eudragit® RS and Maisine®. The lowest was obtained after administration of nanoparticles consisting of Eudragit® RL and Maisine® (20%). In this case, it has to be noted that the standard deviation was much lower than for the other formulations.

These trends in differences are not fully supported by the statistical analysis of relative bioavailability. Indeed, Eudragit® RL nanoparticles were statistically different from Eudragit® RS/Maisine®/Cremophor RH40 nanoparticles ($p = 0.028$). All other formulations were not statistically different. Presumably, when the drug was dissolved within the polymeric particles, it did not distribute into the aqueous environment of the gastro-intestinal tract but remained mostly entrapped. Since Eudragit® RS or RL particles were obviously not absorbed to a considerable extent, the entrapped drug was not available in the systemic circulation.

Our bioavailability results display a much better absorption than the 3% obtained after oral administration of pure CyA nanospheres in dogs (Ford et al., 1999). This demonstrates that the presence of polymers with or without fatty acid esters increased CyA absorption.This is supported by an increase in oral absorption (in dogs) of CyA of 73 and 18% after administration of chitosan and gelatine nanoparticles, respectively, compared to Neoral® (El-Shabouri, 2002).

As suggested by El-Shabouri (2002) and other groups (Thanou et al., 2001; Jiao et al., 2002), positively-charged polymers such as chitosan and Eudragit® RS and RL may interact with the negatively-charged mucus and open up the tight junctions of epithelial cells to allow the paracellular transport pathway, resulting in an increase in bioavailability.

On the other hand, it has to be noted that nanodispersed systems usually do not display a better bioavailability than Neoral®. For instance, Bekerman et al. (2004) prepared CyA lipid nanoparticles ranging from 25 to 400 nm. The best results were obtained for the formulations with particle size below 60 nm. However, these authors found a good correlation in human volunteers between the formulation particle size and the bioavailability. Lipid nanoparticles with diameters ranging from 150 to 400 nm displayed relative bioavailabilities of around 49 and 16% versus Neoral, respectively. Although different in nature (polymers versus lipids), the results obtained in our study are pretty close since the bioavailability figures range from 20 to 34% (Table 2) for nanoparticles ranging from 170 to 310 nm. Therefore, it can be concluded that size, more than constitution, is a very critical parameter in nanoparticle suspensions with regard to CyA delivery.

4. Conclusion

The oral absorption of the poorly absorbable CyA was shown by using polycationic nanoparticles. However, the results are still lower than those observed with the marketed Neoral® premicroemulsion. Nevertheless, further work has to be carried out to increase the amount of absorbed CyA since the absolute bioavailability figures ranged from 22% for Neoral® down to 4.5–7.5% for Eudragit® nanoparticles with or without Maisine®.

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