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# Positively charged nanoparticles for improving the oral bioavailability of cyclosporin-A

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**Abstract**

In this study, cyclosporin-A (Cy-A) a highly lipophilic, poorly absorbable drug can be prepared easily and reproducibly as positively and negatively charged nanoparticles with the aim of improving its bioavailability and reducing its inter- and intra-individual variability. The nanoparticles were prepared by emulsification solvent diffusion method, using lecithin and poloxamer 188 as emulsifiers, and chitosan HCl, gelatin-A or sodium glycocholate (SGC) as charge inducing agents. The prepared nanoparticles were evaluated with respect to particle size, zeta potential, drug content and encapsulation efficiency. The bioavailability Cy-A from nanoparticles in comparison with the currently available Cy-A microemulsion (Neoral®) were assessed in beagle dogs. The results obtained revealed that, it was possible to prepare Cy-A as nanoparticles with size range of 104–148 nm. Chitosan HCl and gelatin-A nanoparticles exhibited +31.2 and +23.1 mV zeta potential, respectively; while SGC-nanoparticles exhibited −41.6 mV zeta potential. The in vivo results showed that, chitosan-nanoparticles gave the highest $C_{max}$ (2762.8 ng/ml) of Cy-A after 2.17 h ($T_{max}$), while SGC-nanoparticles gave the lowest one (1202.4 ng/ml after 4.0 h). Furthermore, $AUC_{0-24}$ of Cy-A from chitosan-nanoparticles was markedly increased by about 2.6-fold when compared with SGC-nanoparticles and increased by about 1.8-fold when compared with the reference Neoral® microemulsion. However, in case of gelatin-nanoparticles the $AUC_{0-24}$ of Cy-A increased by about 1.8 and 1.2-fold when compared with SGC-nanoparticles and the reference Neoral® microemulsion, respectively. The relative bioavailability of Cy-A from chitosan-nanoparticles was increased by about 73%, and by about 18% from gelatin nanoparticles, while it was decreased by about 36% from SGC-nanoparticles.


*Keywords:* Bioavailability; Chitosan; Cyclosporin; Nanoparticles; Positively charged

## 1. Introduction

Cyclosporin-A (Cy-A) a highly lipophilic undecapeptide is commonly used as immunosuppressant to prevent allografit rejection in various organ transplantation such as kidney, liver, heart, lung and pancreas (Matzke and Luke, 1988). The drug has also been shown to be effective in the treatment of systemic and local autoimmune disorders (Borel and Gunn, 1986; Richardson and Emery, 1995; Sajjadi et al., 1994). However, in spite of the great

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therapeutic interest of this drug, the bioavailability after oral dosing is low (10–60%) with a higher variability (Ptachcinski et al., 1986; Lindholm et al., 1988). The incomplete and variable bioavailability of Cy-A has been attributed to higher molecular weight, higher lipophilicity, low intestinal permeability, first-pass metabolism by the liver and g.i. mucosa (Ptachcinski et al., 1986; Ismailos et al., 1991; Tjai et al., 1991; Taylor et al., 1993).

Cy-A is dispensed as an oily solution or microemulsion containing a high concentration of polyoxyethylated castor oil (Cremophor EL®). However, in spit of microemulsion enhance Cy-A absorption and reduce inter- and intra-subject variability (Kovarik et al., 1994), cremophor EL® has been reported to be nephrotoxic (Luke et al. 1987) and may cause anaphilactoid reactions (Cavanak and Sucker, 1986).

Many research efforts have been made to overcome the above mentioned difficulties and to increase the therapeutic efficacy of Cy-A, and to decrease its side effects. Alternative dosage forms have been suggested including incorporation of the drug into particulate carriers such as microspheres (Yanagawa et al., 1989; Urata et al., 1999) and liposomes (Venkataram et al., 1990; Lee et al., 1999). Nevertheless, liposomes have not been widely applied for clinical use, since they have a limited in vitro and in vivo stability (Yanagawa et al., 1989).

The formulation of Cy-A as nanoparticles or nanospheres have been received much attention over the last few years due to their ability to control drug release and distribution, and due to their biodegradability. Furthermore, these systems proven their potential to administer Cy-A or other drugs either by intravenous or oral routes, increasing their bioavailability and reducing the associated side effects (Guzmán et al., 1993; Molpeceres et al., 1996a,b; Ford et al., 1999; Zhang et al., 2000).

Recently, it has been shown that, enhancement of electrostatic interaction between the mucosal surfaces and drugs have a marked effect on their uptake and overall bioavailability. The epithelial cells in the various tissues including gastrointestinal tract, carry a negative charge on their surface due to the presence negatively charged residues of proteins in the outer membrane of the cells and the selective active ion pumps of the membrane. Therefore, all epithelia are selective to positively charged solutes (Rojanasakul et al., 1992). Accordingly, it is anticipated that positively charged delivery systems that will strong interact with the cells will result in better permeability and overall bioavailability of the drugs. One of the most successful approaches toward this aim has been the use of positively charged colloidal dispersions such as liposomes (Guo et al., 1989), submicron emulsions (Elbaz et al., 1993; Zeevi et al., 1994; Teixeira et al., 1999) and self-emulsifying oily formulations (Gershanik and Benita, 1996; Gershanik et al., 2000). A common conclusion from these previous studies is that, positively charged colloidal drug carriers increase the permeability and potential uptake of slightly soluble drugs when compared with neutral or negatively charged ones, thus improving their bioavailability and reducing their side effects. This behavior was attributed to the mucoadhesion mediated by electrostatic interaction between the positively charged colloidal particles and the negatively charged mucin on the mucosal surface.

On the basis of the above mentioned considerations, it was thought plausible to combine the advantages of nanospheres as oral delivery systems with the benefit of the presence of positive charge on their surfaces. Thus, the objective of the present study was to develop and to characterize Cy-A as positively charged nanoparticles as an attempt for improving its gastrointestinal uptake and its overall bioavailability. Chitosan hydrochloride and gelatin-A were selected as positively charge inducing agents. The bioavailability and pharmacokinetics of Cy-A from these nanoparticles in comparison with those carrying a negative charge and the currently available microemulsion (Neoral®) were assessed in beagle dogs.

## 2. Materials and methods

### 2.1. Chemicals

Cy-A and Neoral® microemulsion were kindly supplied from Sandoz Pharmaceutical (Novartis), Switzerland. Chitosan hydrochloride (SEACURE

CL 210, Pronova Biopolymer, Drammen, Norway). GA and Poloxamer 188 (Pluronic F-68) were purchased from Fluka Chemika (Switzerland). Sodium glycocholate (SGC, Sigma Chemical Co., USA). Soya lecithin (93% phosphatidylcholine, Nattermann, Cologne, Germany). Other chemicals and solvents were of pure analytical grades. Deionized twice-distilled water was used throughout the study.

## 2.2. Methods

### 2.2.1. Preparation of positively and negatively charged nanoparticles

The nanoparticles were prepared by emulsification solvent diffusion method (Niwa et al., 1993). The concentration of the drug and emulsifiers as well as the phase volume ratio required for preparing Cy-A nanoparticles are shown in Table 1.

The concentrations and the phase volume ratio listed in Table 1 were selected after preliminary experiments to determine the optimal concentration and phase volume ratio giving nanoparticles without any problems or complications.

Cy-A and lecithin were dissolved in methylene chloride and then mixed with acetone. The resulted solution was injected into an aqueous solution containing poloxamer in the presence of CS, GA or SGC, with magnetic stirring, followed by high pressure homogenization at a 1000 bar for 5 min (Microfluidizer TM110, Microfluidics Co., USA). Methylene chloride in the resulted emulsion was removed under reduced pressure using a rotavapor at room temperature and the whole dispersion system was then filtered through a 1.0 μm cellulose ester millipore filter to separate microspheres or agglomerates from the system. The resulting filtrate containing nanoparticles was diluted with 50 ml of distilled water to allow complete diffusion of acetone into the aqueous phase. The dispersion was then centrifuged for 15 min at 48 000 rpm and the supernatant was discarded. The sediment was resuspended in 20 ml of distilled water to remove the trace acetone from the nanoparticles. The resulting dispersion was recentrifuged again and the supernatant was discarded. The final volume of the resultant nanosuspension was adjusted to 10 ml with distilled water.

Table 1
Materials and their concentration used for preparing the nanoparticles

| Materials | Emulsion composition (% w/w) | | |
|---|---|---|---|
| | Positively charged | | Negatively charged |
| | CS | GA | SGC |
| Cy-A | 1.0 | 1.0 | 1.0 |
| Lecithin | 1.2 | 1.2 | 1.2 |
| Poloxamer 188 | 0.8 | 0.8 | 0.8 |
| Chitosan HCl (CS) | 0.5 | – | – |
| Gelatin-A (GA) | – | 0.5 | – |
| SGC | – | – | 0.5 |
| Methylene chloride–acetone (3:1) | 12.0 | 12.0 | 12.0 |
| Water to | 100 | 100 | 100 |

### 2.2.2. Particle size and zeta potential evaluation

Particle size analysis was performed by photon correlation spectroscopy (Malvern Zetasizer 4, Malvern Instr. UK). The zeta potential of nanoparticles was measured with the Malvern Zetasizer 4. The nanosuspensions were diluted properly with NaCl (1 mm/l) and placed in the cell where a potential of 150 mV was established.

### 2.2.3. Drug content

A specific volume (2 ml) of each of the prepared nanoparticle-suspensions was placed in a small petri-dish and dried under reduced pressure in a vacuum oven at 35 °C and then stored in a desiccator until a constant weight was achieved. The residue was dissolved in a specific volume of the mobile phase used for the HPLC analysis. After centrifugation at 5000 rpm, the concentration of Cy-A in the supernatant was determined by a reversed-phase HPLC method reported by Guzmán et al., 1993. The HPLC system consisted of an isocratic pump (LC-10 AS, Shimadzu, Japan), UV–visible detector (SPD 10A, Shimedzu, Japan) set at 210 nm. The chromatographic column used was a Spheriosorb ODS-2 (5 μm in 4.6 × 250 mm, Merck, Darmstadt, Germany) thermostated at 72 °C. The mobile phase consisted of acetoni-

trile/water (75:25 v/v) and the flow rate was 1 ml/min.

#### 2.2.4. Bioavailability study

For this study, six male beagle dogs weighing 12–15 kg were selected. Each formulation was orally administered to the dogs with 14-days washout period between dosing of the other formulations. The dogs were fasted for 24 h and continued for further 4 h after administration, only water was permitted during this period. Each dog received a single oral dose equivalent to 100 mg Cy-A which was diluted with distilled water just before oral administration. At predetermined time intervals (0, 1, 2, 3, 5, 8, 12 and 24 h), blood samples (3 ml each) were drawn from the cephalic vein in EDTA-containing tubes. The plasma was separated by centrifugation in a cooling centrifuge at 10 °C (Heraeus–Christ, GMBH Osterode) and stored in the frozen state for subsequent assays.

Cy-A in plasma were analyzed by a reversed-phase HPLC method reported by Guzmán et al., 1993, as mentioned above. The concentrations of the drug were determined from the calibration curve of beak areas which was obtained by analysis of drug-free plasma samples spiked with different amounts of Cy-A ranging from 200 to 3000 ng/ml.

The relevant pharmacokinetic parameters, $C_{max}$, $T_{max}$, $AUC_{0-24}$, $AUC_{0-\alpha}$, $K_{el}$ and $t_{1/2}$ were calculated based on the reported method of Gibaldi and Perrier, 1982. The data between different formulations were compared for statistical significance by the one-way analysis of variance (ANOVA). All data were expressed as means and standard deviation, $\pm$S.D.

## 3. Results and discussion

The present study was designed as a trial to: (a) overcome several problems associated with the oral absorption and bioavailability of Cy-A; (b) to study the efficiency of the method used for preparing the nanoparticles and the formulation factors affecting their characteristics and the availability of Cy-A.

### 3.1. Characterization of Cy-A nanoparticles

In this study, positively and negatively charged nanoparticles were prepared by emulsification solvent diffusion method using different formulations as shown in Table 1. From the results obtained in Table 2, it was generally noticed that, it was possible to prepare Cy-A nanoparticles of mean diameters ranging from 104 to 148 nm. The used method involves only stirring for emulsification followed by homogenization. The presence of acetone in organic phase facilitates the production of nanoparticles without using successive mechanical treatment, since homogenization of the system without acetone produced particles with an average diameter greater than 1.2 μm. The presence of acetone will reduce the interfacial tension between methylene chloride and the aqueous phase. In addition, the perturbation of the interface arising from the rapid diffusion of acetone across the interface between organic and aqueous phases spontaneously produces a much larger interface area resulting in much finer droplets. These results agree with those obtained by Niwa et al. (1993), who reported that, the addition of acetone to the organic phase was essential for effectively forming the nano-sized spheres. These advantages besides the formulation factors would be reflected on the encapsulation efficiency of Cy-A in the nanoparticles. The results in Table 2 show that, the encapsulation efficiency of the drug in the nanoparticles (the percentage of Cy-A encapsulated in respect to the total amount of Cy-A added to the system) was very high (from 88.60 to 94.33%). Since, Cy-A is a very poorly water soluble drug, it was preferentially partitioned in the organic phase of the emulsion and consequently, small amount of the drug is lost in the aqueous phase. Furthermore, it has been reported that, Cy-A is less soluble in poloxamer 188–water mixtures than in water alone at temperatures between 20 and 37 °C (Molpeceres et al., 1996a,b). Thus, the presence of poloxamer 188 in the formulation plays an important role not only as a co-surfactant for nanoparticle stability but also in achieving higher Cy-A encapsulation efficiency, because it reduces the solubility of Cy-A in the aqueous phase.

Table 2
Particle size, zeta potential, drug content and encapsulation efficiency of Cy-A nanoparticles ($n = 3$)

| Types of nanoprticles | Mean particle diameter (nm) ± S.D. | Zeta potential (mV) ± S.D. | Yeild (%) ± S.D. | Drug content (%) ± S.D. | Drug loading efficiency (%) ± S.D. |
|---|---|---|---|---|---|
| CS | 148 ± 29 | +31.2 ± 1.6 | 93.62 ± 3.41 | 24.21 ± 1.42 | 94.33 ± 2.86 |
| GA | 139 ± 23 | +23.1 ± 0.8 | 90.76 ± 4.52 | 23.04 ± 1.74 | 92.15 ± 3.77 |
| SGC | 104 ± 18 | −41.6 ± 1.1 | 85.85 ± 6.18 | 21.13 ± 1.86 | 88.60 ± 5.48 |

CS, chitosan HCl; GA, gelatin-A; SGC, sodium glycocholate.

In comparison between the mean diameters of different types of nanoparticles (Table 2), it was evident that, SGC-containing nanoparticles showed the lowest size (104 nm) than those of GA (139 nm) or CS (148 nm). The increased size of CS- or GA-nanoparticles may be attributed to the enlarged emulsion globules dispersed in the aqueous phase. Furthermore, CS and GA increase the viscosity of the aqueous phase, hence interfere with the interfacial hydrodynamic phenomena responsible for the spontaneous emulsification of the organic phase when mixed with the aqueous phase (Davis and Rideal, 1963).

The zeta potential values (Table 2) show the effect of CS, GA and SGC on the surface charge of nanoparticles. CS- and GA-containing nanoparticles were positively charged (+31.2 and +23.1 mV, respectively), while, SGC-nanoparticles exhibited a high negative charge (−41.6 mV). The results suggested that, a mixed interfacial film comprising the lecithin, poloxamer and each of these additives was formed at the o/w interface during the emulsification process with an overall resulting of the given charge over the nanoparticles. The relatively higher zeta potential of CS-nanoparticles to those of GA could be explained by the higher molecular weight of CS ( < 100 kD) with respect to that of GA ( > 50 kD), and thus the higher density of amine groups of CS.

### 3.2. Bioavailability study

The mean plasma levels of Cy-A after oral administration of a single dose of each type of nanoparticles and in comparison with those of the reference (Neoral® microemulsion) are shown in Fig. 1. The relevant pharmacokinetic parameters, $C_{max}$, $T_{max}$, $AUC_{0-24}$, $AUC_{0-\alpha}$, $K_{el}$ and $t_{1/2}$ are

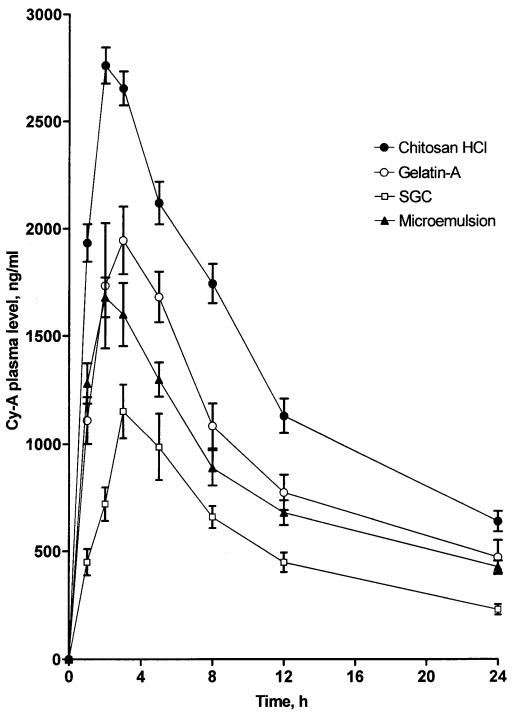


Fig. 1. Mean plasma level of Cy-A following oral administration to beagle dogs.

listed in Table 3. The results obtained revealed that, CS-nanoparticles showed the highest $C_{max}$ (2762.8 ± 106 ng/ml) of Cy-A at $T_{max}$ of 2.17 ± 0.408 h, while SGC-nanoparticles showed the lowest one (1202.4 ± 64 ng/ml) at $T$max of 4.0 ± 0.447 h. However, in case of GA-nanoparticles and Neoral® microemulsion, the $C_{max}$ of Cy-A was found to be 2035 ± 159.1 ng/ml at 2.67 ± 0.516 h and 1707 ± 72.1 ng/ml at 2.5 ± 0.548 h, respectively. Furthermore, the $AUC_{0-24}$ of Cy-A from CS-nanoparticles was markedly increased by about 2.6-fold when compared with SGC-nano-

Table 3
Pharmacokinetic parameters of Cy-A after oral administration of nanoparticles and Neoral® microemulsion to beagle dogs

| Pharmacokinetic parameters | CS nanoparticles | GA nanoparticles | SGC nanoparticles | Neoral® microemulsion |
|---|---|---|---|---|
| $C_{max}$ (ng/ml) ± S.D. | 2762.8 ± 106.0 | 2035.0 ± 159.1 | 1202.4 ± 64.0 | 1707.0 ± 72.1 |
| $T_{max}$ (h) ± S.D. | 2.17 ± 0.408 | 2.67 ± 0.516 | 4.0 ± 0.447 | 2.5 ± 0.548 |
| $AUC_{0-24}$ (ng/ml h) ± S.D. | 32 801.8 ± 2189.7 | 22 811.6 ± 2205.9 | 12 645.0 ± 678.5 | 18 875.0 ± 1280.7 |
| $AUC_{0-\alpha}$ (ng/ml h) ± S.D. | 39 992.0 ± 2058.3 | 27 241.5 ± 2095.6 | 14 813.2 ± 614.6 | 23 090.6 ± 1203.9 |
| $K_{el}$ (per h) ± S.D. | 0.089 ± 0.003 | 0.107 ± 0.011 | 0.107 ± 0.01 | 0.102 ± 0.002 |
| $t_{1/2}$ (h) ± S.D. | 7.77 ± 0.230 | 6.56 ± 0.304 | 6.54 ± 0.575 | 6.82 ± 0.122 |
| Fr (%) | 173 | 118 | 64 | – |

Fr, relative bioavailability = ($AUC_{0-\alpha}$ (Test) × 100)/($AUC_{0-\alpha}$ (Neoral®)).

particles and increased by about 1.8-fold when compared with the reference Neoral® microemulsion. However, in case of GA-nanoparticles the $AUC_{0-24}$ of Cy-A was increased by about 1.8 and 1.2-fold when compared with SGC-nanoparticles and the reference Neoral® micro-emulsion, respectively. The statistical analysis of data revealed that, the pharmacokinetic parameters, $T_{max}$, $K_{el}$ and $t_{1/2}$ show no significant differences ($P > 0.05$) among the four formulations, while the $C_{max}$, $AUC_{0-24}$ and $AUC_{0-\alpha}$ show significant differences ($P < 0.05$). The $C_{max}$ and AUC of Cy-A from CS-nanoparticles show a highly significant difference ($P < 0.01$) when compared with those of SGC-nanoparticles and microemulsion. These results indicated that, the type of charge (+ve) and the presence of inherent permeation enhancer (chitosan) are the main factors responsible for improving the oral absorption and overall bioavailability of Cy-A.

The relative bioavailability of Cy-A from CS-nanoparticles was increased by about 73%, and increased only by about 18% from GA-nanoparticles, while in case of SGC-nanoparticles, it was decreased by about 36%. Thus, from these results, it was evident that, in spit of SGC-nanoparticle has the smallest size (104 nm) relative to that of CS (148 nm) or of GA (139 nm), they gave the lowest $C_{max}$ and the lowest AUCs. These results could be attributed to the anionic charge conferred to the particles by SGC used in the formulation, since the interaction of the nanoparticles to the negatively charged surface of the intestinal mucosa might be hindered. Accordingly, coating of Cy-A nanoparticles with the cationic polymers such as CS or GA was intentionally tailored in order to facilitate the electrostatic interaction between the positively charged particles with the negatively charged mucosa.

Although, CS- and GA-nanoparticles displayed a similar positive surface charge, the higher $C_{max}$ and the greatest relative bioavailability of Cy-A from CS-nanoparticles indicated that, not only the positive charge or mucoadhesion would responsible for enhancing the gastrointestinal uptake of Cy-A. Nevertheless, for the interpretation of the favorable behavior of CS-nanoparticles, it is important to take into account the previous studies that showed the increase of permeability of nasal (Illum et al., 1994), ocular (Calvo et al., 1997) and intestinal (Lueßen et al., 1997; Kozté et al., 1999) epithelia by chitosan salts. The authors suggested a combined mechanism of mucoadhesion and ability to open the tight junctions of epithelial cells to allow for a paracellular transport pathway. Chitosan salts act primarily by an interaction between the positively charged amino groups on the C-2 position with the negatively charged sites on the cell membrane and tight junctions of the mucosal epithelial cells. In addition, chitosan induces a redistribution of cytoskeletal F-actin and the tight junction protein ZO-1 (Artursson et al. 1994; Schipper et al. 1997), thereby altering the integrity of the tight junctions which is associated directly or indirectly with the F-actin filaments, to allow an increase of the paracellular permeability (Madara, 1987). Furthermore, Kozté et al., 1998, reported that, chitosan salts caused an immediate and pronounced lowering in the transepithelial electric

resistance of the intestinal cell monolayers in a reversible way to allow for paracellular transport.

## 4. Conclusion

This study demonstrated that, Cy-A a poorly soluble and poorly absorbable drug can be prepared easily and reproducibly as positively and negatively charged nanoparticles of size range of 104–148 nm. The results revealed that, particle size reduction of Cy-A to positively charged nanoparticles using cationic polymers such as chitosan HCl or gelatin-A improved its absorption rate and overall bioavailability. The study also demonstrated that, the type of the charge, the nature of cationic polymers and their effect on the permeability of gastrointestinal mucosa play an important role in their ability to improve the absorption rate and the bioavailability of Cy-A. The greatest bioavailability of Cy-A from chitosan-coated nanoparticles suggests that, it is possible to use a lower dose of Cy-A with few side effects after its oral therapy. Moreover, maximizing the bioavailability of Cy-A will reduce the inter- and intra-individual variability; thereby improve its overall efficacy. Furthermore, this formulation approach can be used to improve the oral bioavailabilty of other poorly soluble and poorly absorbable drugs.

The stability of the prepared nanoparticles over pH range 1–6 at different temperatures and their incorporation into solid dosage forms have been the subject of my further studies.

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