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Long-term studies on the stability and oral bioavailability of cyclosporine A nanoparticle colloid

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Abstract

The present study was geared at the long-term stability and the changes in oral bioavailability of CyA Eudragit® S100 nanoparticles stabilized by suspending agents. CyA Eudragit® S100 nanoparticle colloids were prepared by quasi-emulsion solvent diffusion technique and they were mixed with Xanthan gum to obtain suspended nanoparticle colloids. The suspended nanoparticle colloids were preserved at different temperatures for different period of time, as long as 18 months. During the storage period, the CyA concentration, particle size, pH and viscosity were determined. The results indicated that CyA concentration, particle size and viscosity of the colloids had no obvious change. However, the pH increased slightly from 5.5 to about 6.4. The results of bioavailability and pharmacokinetic study revealed that all formulations of nanoparticles showed higher C_{max} and higher $AUC_{0–24}$ values than that of reference (Neoral®). The relative bioavailability of S-CyA-S100 NP initial compared with Neoral® was 162.8%. The C_{max} and $AUC_{0–24}$ values of nanoparticle formulations at 12 and 18 months were both lower than that of the initial. The bioequivalency was suggested between the tested nanoparticle formulations at the initial and 12 months. It was deduced by surface analysis, TEM observation, in vitro release as well as the characteristics of Eudragit® S100 that the decrease in bioavailability might be due to the pH change of the nanoparticle colloid.

Keywords: Cyclosporine A; Nanoparticles; Stability; Bioavailability; Pharmacokinetics

1. Introduction

Cyclosporine A (CyA) is a cyclic undecapeptide with a potent immunosuppressive activity that has been used to prevent allograft rejection in various organ transplantations, such as kidney, liver, heart, lung and pancreas (Matzke and Luke, 1988). The drug has also been applied in the treatment of patients with selected autoimmune diseases, such as rheumatoid arthritis (Richardson and Emery, 1995) and Behçet's disease (Sajjadi et al., 1994).

Despite the great therapeutic interest of this drug, the bioavailability of oral administration is low with a high variability (Lindholm et al., 1988). The current available CyA oral formulation includes Sandimmun Neoral® (Neoral®), which is a microemulsion containing a high concentration of polyoxyethylated castor oil (Cremophor EL®). Cremophor EL®, a formulation vehicle used for various lipophilic drugs, is not an inert vehicle and it exerts a series of biological and physiological effects, such as nephrotoxic (Luke et al., 1987) and anaphylactoid reactions (Cavanak and Sucker, 1986).

Many efforts have been made to increase the bioavailability of CyA and decrease its side effects. Alternative approaches have been suggested, including the incorporation of the drug into particulate carriers such as microspheres (Yanagawa et al., 1989; Urata et al., 1999) and liposomes (Venkataram et al., 1990). The formulations of CyA nanoparticles have received much attention over the last few years, due to their ability to increase the absorption of drugs and control drug release (Sánchez and Alonso, 1995; Bonduelle et al., 1995; Zhang et al., 2000). The main carrier materials used to prepare CyA nanoparticles include biodegradable polyalkyl-cyanoacrylate, polycaprolactone (Molpeceres et al., 1996), poly(D,L-lactide-

Abbreviations: CyA, cyclosporine A; CyA-S100 NP, CyA Eudragit® S100 nanoparticle colloids; S-CyA-S100 NP, suspended CyA Eudragit® S100 nanoparticle colloids

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glycolide) copolymers, poly(D,L-lactic acid) (Desai et al., 1996; Lee et al., 2002; Saez et al., 2000), positively charged chitosan hydrochloride (El-Shabouri, 2002), pH-dependent dissolving polymer Eudragit® (Dai et al., 2004) and hydroxypropyl methylcellulose phthalate (Wang et al., 2004). Most of these studies have improved the bioavailability of CyA. For instance, CyA nanoparticles with Eudragit® S100 as carrier showed a relative bioavailability of 132.5% versus Neoral® (Dai et al., 2004).

However, the bioavailability is not the only obstacle for a nanoparticle formulation which is expected to be developed into a pharmaceutical product. The stability of such a system brings another serious challenge. Due to its large specific interfacial area, nanoparticle dispersion is a typical thermodynamically unstable system. After a period of storage, particle aggregation often occurs. Some efforts have been made to increase the physical stability of such a system, and lyophilization is an important technique. But lyophilization has some disadvantages. Firstly, the particle size could increase during the procedure of freeze drying, which may affect the drug pharmacokinetic characteristics, such as AUC (Saez et al., 2000). Secondly, lyophilized nanoparticles may still aggregate after some time of storage and therefore will not be satisfactorily reconstituted (Chacón et al., 1999; Dai et al., 2005).

We have demonstrated the improvement in stability of nanoparticle colloid by adding some suspending agents (Wang et al., 2004). In this study, we prepared suspended CyA Eudragit® S100 nanoparticles and focused on their long-term stability at different temperatures, evaluating by CyA concentration, morphology, particle size, surface analysis, pH, viscosity, and bioavailability. Although few studies of such investigation have been reported, our understanding is that the long-term stability of nanoparticle colloid is a crucial problem to overcome before its application in the market.

2. Materials and methods

2.1. Materials

CyA was kindly donated by North China Pharmaceutical Group, China. Neoral® microemulsion was purchased from Sandoz Pharmaceutical (Novartis), Switzerland. The pH-sensitive poly(methacrylicacid-co-methyl methacrylate) copolymers (Eudragit® S100) were the gifts from Röhm (Darmstadt, Germany). Poloxamer 188 (Pluronic F_{68}) was purchased from the pharmaceutical plant affiliated to Shenyang Pharmaceutical University, China. Xanthan gum (Vanzan™ NF-C) was a product of R.T. Vanderbilt Company Inc., USA. Other chemicals and solvents were of analytical grade, except those for HPLC assay which were of HPLC grade. Sprague-Dawley (SD, male, weighing 240–280 g) rats were obtained from Animal Institute of Peking University Health Science Center.

2.2. Preparation of CyA Eudragit® S100 nanoparticle colloids

The preparation method of CyA Eudragit® S100 nanoparticles (CyA-S100 NP) applied in the study was same as Dai et al. (2004) reported. Briefly, CyA and Eudragit® S100 were co-dissolved in anhydrous ethanol. The solution was quickly injected into the stirring aqueous solution of poloxamer 188 with a 7# needle for bone marrow puncture. Afterward, the mixture was evaporated in a 60 °C water bath to drive off the ethanol, then it was concentrated to about 8–10 mg CyA/ml with an ultrafilter (Millipore 8200, regenerated cellulose membrane, 10 K).

2.3. Preparation of suspended CyA nanoparticle colloids

Xanthan gum was scattered onto distilled water over night, then a Xanthan gum solution was obtained by gently stirring. The Xanthan gum solution was mixed with CyA-S100 NP to obtain an oral formulation with 5 mg/ml CyA, which was coded as S-CyA-S100 NP.

2.4. Evaluation of CyA content in the suspended nanoparticle colloids

CyA concentrations were determined by reversed-phase HPLC. S-CyA-S100 NP were dissolved in mobile phase and filtered through organic membrane with 0.2 μm pores. Then they were applied to a HPLC system (HP1100, Agilent Co. Inc, USA). The mobile phase consisted of acetonitrile/methanol/water (8:3:3, v/v/v). The determination was achieved by using a reversed-phase column (Zorbax SB-C_{18}, 4.6 mm × 250 mm, Agilent Co. Inc, USA) thermostated at 70 °C with a flow rate of 1.5 ml/min and detecting at 210 nm. The content of CyA was calculated by external standardization method.

2.5. Particle size, viscosity and pH assessment

We determined the CyA concentration, particle size, viscosity and pH at 30 °C at the initial, 1, 2, 3 and 6 months; at 25 °C at the initial, 1, 2, 3, 6, 9, 12 and 18 months and 4 °C at the initial, 3, 6, 9, 12 and 18 months.

The analysis of particle size was performed by dynamic light scattering (Brookhaven Instruments Corporation), while the viscosity was determined by a rotating viscometer (Shanghai, China) at 28 °C. The determination of the pH value was carried out with a well-calibrated pH meter (Shanghai, China).

2.6. Morphological characterization

The transmission electron microscopy (TEM, JEM-1230, JEOL Co., Tokyo, Japan) was applied to examine the morphology of the nanoparticles, as well as the possible crystals of CyA formed out of the particles. The samples of S-CyA-S100 NP used were preserved at 25 or 4 °C for 0 and 21 months, respectively.

2.7. Surface analysis

The surface analysis was conducted by X-ray photoelectron spectroscopy (XPS Axis Ultra, Kratos Co., UK) (Ruxandra et al., 2001; Dai et al., 2004). If 100% of CyA is at or near the

surface of the particles, the elemental composition of Nitrogen (N (%)) will be 12.9%. Thus, the percentage of CyA at or near the surface of the nanoparticles (surface % CyA) could be calculated according to following equation:

$$\text{Surface \% CyA} = \text{N (\%)} \times 100/12.9.$$

The samples of S-CyA-S100 NP used were stored at 25 or 4 °C for 0 and 30 months, respectively.

2.8. Bioavailability and pharmacokinetic study after oral administration

The studies on bioavailability and pharmacokinetics have been carried out for samples of S-CyA-S100 NP stored at 25 or 4 °C for 0, 12 and 18 months, respectively. During the tests, all the rats were fasted overnight. After the single oral dose (15 mg/kg) of Neoral® or S-CyA-S100 NP was given by gavage to rats from 08:00 to 09:00 a.m., the fast continued for further 4 h and the rats were allowed for free access to water. At predetermined time intervals (0.5, 1, 2, 3, 5, 7, 9, 12 and 24 h), blood samples were drawn from the ocular vein into heparinized tubes and stored at −20 °C until determination.

CyA concentrations in blood were estimated by a reversed-phase HPLC method as Wang et al. (2004) reported.

The pharmacokinetic parameters were evaluated by noncompartmental methods. The zero-order moment area under the blood concentration–time curve ($AUC_{0–24}$) and the first-order moment mean residence time ($MRT_{0–24}$) were calculated by the trapezoidal rule. The maximal blood concentration (C_{max}) and the time of maximal blood concentration (T_{max}) were obtained directly by observation. The differences among pharmacokinetic parameters in various test groups were estimated by multiple comparison tests with a software named SPSS. Because C_{max} and $AUC_{0–24}$ were suitable for logarithm Gaussian distribution, we conducted multiple comparison tests with $\ln C_{max}$ and $\ln AUC_{0–24}$.

3. Results and discussion

3.1. Preparation of suspended CyA Eudragit® S100 nanoparticle colloids

The CyA-S100 NP preparation had a particle size of 43 nm, and it increased to about 60 nm after Xanthan gum was added into the nanoparticle colloid. The increase in particle size might be due to the coating effect of the polymer (Xanthan gum) on the surface of nanoparticles.

As Dai et al. (2004) reported, most kinds of Eudragit® materials can be used to prepare CyA nanoparticles, including Eudragit® E100, Eudragit® L100, Eudragit® L100-55 and Eudragit® S100. But Eudragit® S100 CyA nanoparticles demonstrate the highest bioavailability among all kinds of Eudragit® CyA nanoparticles. Therefore, we selected Eudragit® S100 to prepare CyA nanoparticles and conducted further studies.

To improve the long-term stability of the nanoparticles, we have tried to lyophilize the nanoparticles to get a solid formulation. Just after the lyophilization, the nanoparticles could disperse very well in water, with little change in particle size. But after the accelerating tests (40 °C, illumination of 4500 lx or RH 92.5%), the nanoparticles could not disperse well in the same medium. The particle sizes were more than 120 nm, and some of the solid sample even could not reconstitute at all (Dai et al., 2005). This probably resulted from the low glass transition temperature (T_g) of the Eudragit®. It was demonstrated via DSC determination that the T_g of Eudragit® S100 was only 43.7 °C. The similar result was observed in CyA PLA nanoparticle preparation. Therefore, we decided to do further studies only on suspended nanoparticles.

3.2. Morphological observation

The TEM photographs of S-CyA-S100 NP stored at 25 or 4 °C for 0 and 21 months were shown in Fig. 1. It could be noticed from the photographs that there were no obvious changes in shape and particle sizes during the storage. Also there were no crystals of the drug observed.

3.3. XPS analysis

The surface analysis results by XPS were described in Table 1. The presence of CyA at or near the surface was about 10%, and there were no obvious differences between the initial formulation and those stored for 30 months at 25 or 4 °C.

3.4. CyA content, particle size, pH and viscosity of the suspended nanoparticle colloids

The changes of the CyA concentration, mean particle size, pH value and viscosity of the suspended nanoparticle colloids were shown in Fig. 2. Generally, the characteristics of the nanoparticle formulation were rather stable. It was demonstrated that during the long-term storage period of 18 months, the content of CyA (Fig. 2A), particle size (Fig. 2B) and viscosity (Fig. 2C) changed very little, while the pH value (Fig. 2D) increased slowly from the value of 5.5 to that of more than 6.0. We also observed that the speed of pH increased at 25 °C was a little faster than that at 4 °C.

To explain the possible reasons of the increase in pH value of the formulation, we analyzed the chemical structures of Eudragit® S100 and Xanthan gum. There are carboxy groups and ester bonds within the structure of Eudragit® S100 as shown in Fig. 3A, accordingly the possibility of the raise in pH value was little, even if the polymer degradated during the period of storage. Xanthan gum is a polysaccharide product of genetic engineering, and its structure is rather complex (Fig. 3B). One possibility is that the hydrolysis of metal ions caused the rising of the pH value, judging from the structure of Xanthan gum. The pH increase of Xanthan gum solution was proved by the accelerating tests, and the results showed that the pH of Xanthan gum solution rised after storage at 40 or 60 °C. The higher concentration of Xanthan gum or the higher the test temperature, the more increases in pH values (data not shown).

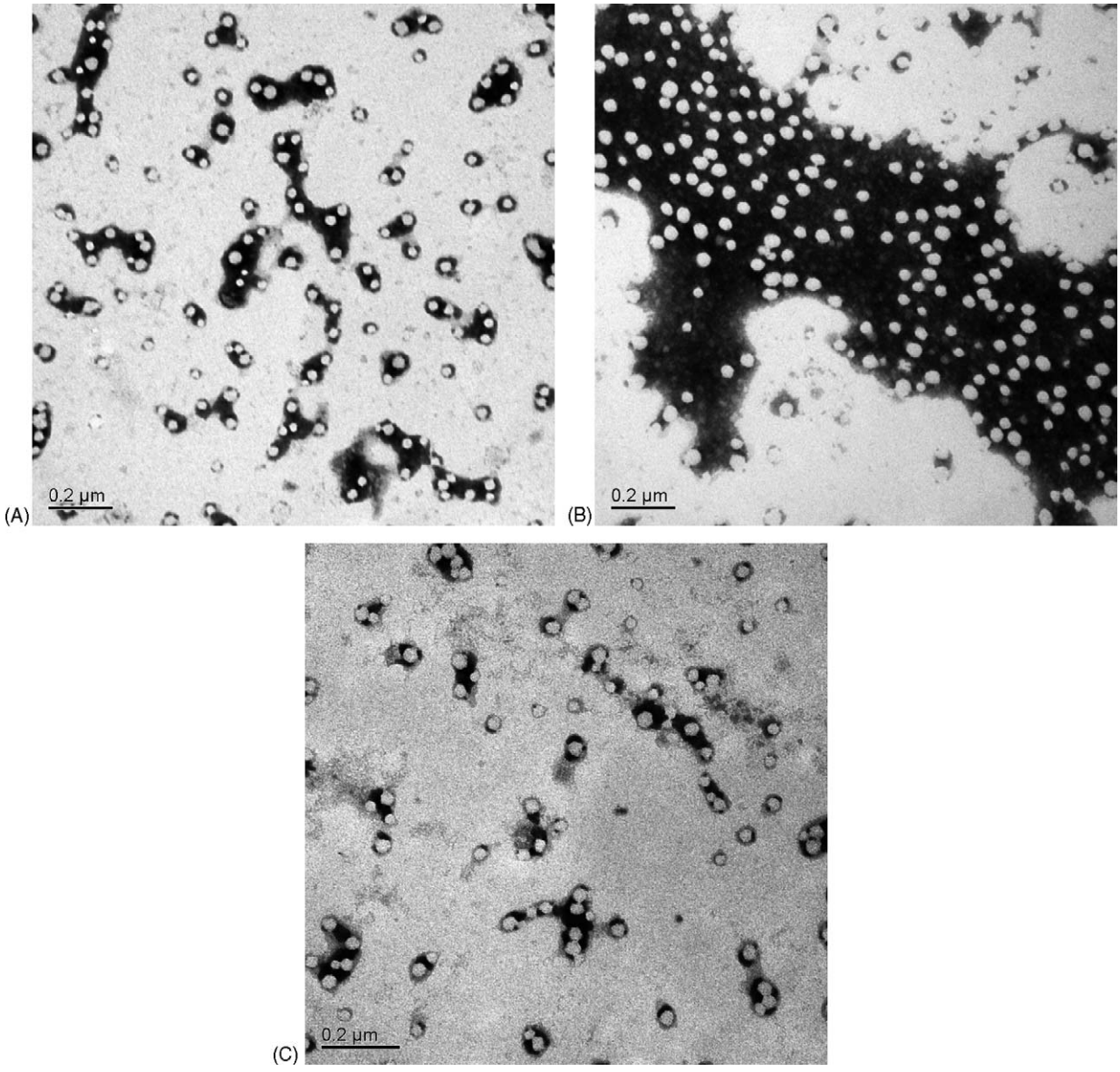


Fig. 1. Transmission electron microscope photographs of Xanthan gum suspended CyA Eudragit® S100 nanoparticles initial (A), 4 °C preserved for 21 months (B), and 25 °C preserved for 21 months (C).

3.5. Bioavailability and pharmacokinetics of the suspended nanoparticle colloids

The mean CyA concentrations in the blood after oral administration of a single dose of S-CyA-S100 NP stored at 4 or 25 °C for 0, 12 and 18 months were illustrated in Fig. 4 (A) for 4 °C and (B) for 25 °C, and Neoral® was taken as a reference. The pharmacokinetic parameters such as C_{max}, T_{max}, $AUC_{0–24}$, mean residence time ($MRT_{0–24}$) and relative bioavailability (F_r) were given in Table 2.

The results suggested that some of the pharmacokinetic parameters of S-CyA-S100 NP were different from those of

Table 1
Atomic composition of the Xanthan gum suspended CyA Eudragit® S100 nanoparticles at or close to the particle surface

Sample	Elemental ratio (%)			CyA at the surface (%)
	C	N	O	
CyA	73.0	12.9	14.1	
S-CyA-S100 NP initial	69.8	1.48	28.1	11.5
S-CyA-S100 NP 30 months at 4 °C	72.0	1.35	26.4	10.5
S-CyA-S100 NP 30 months at 25 °C	69.6	1.20	28.6	9.3

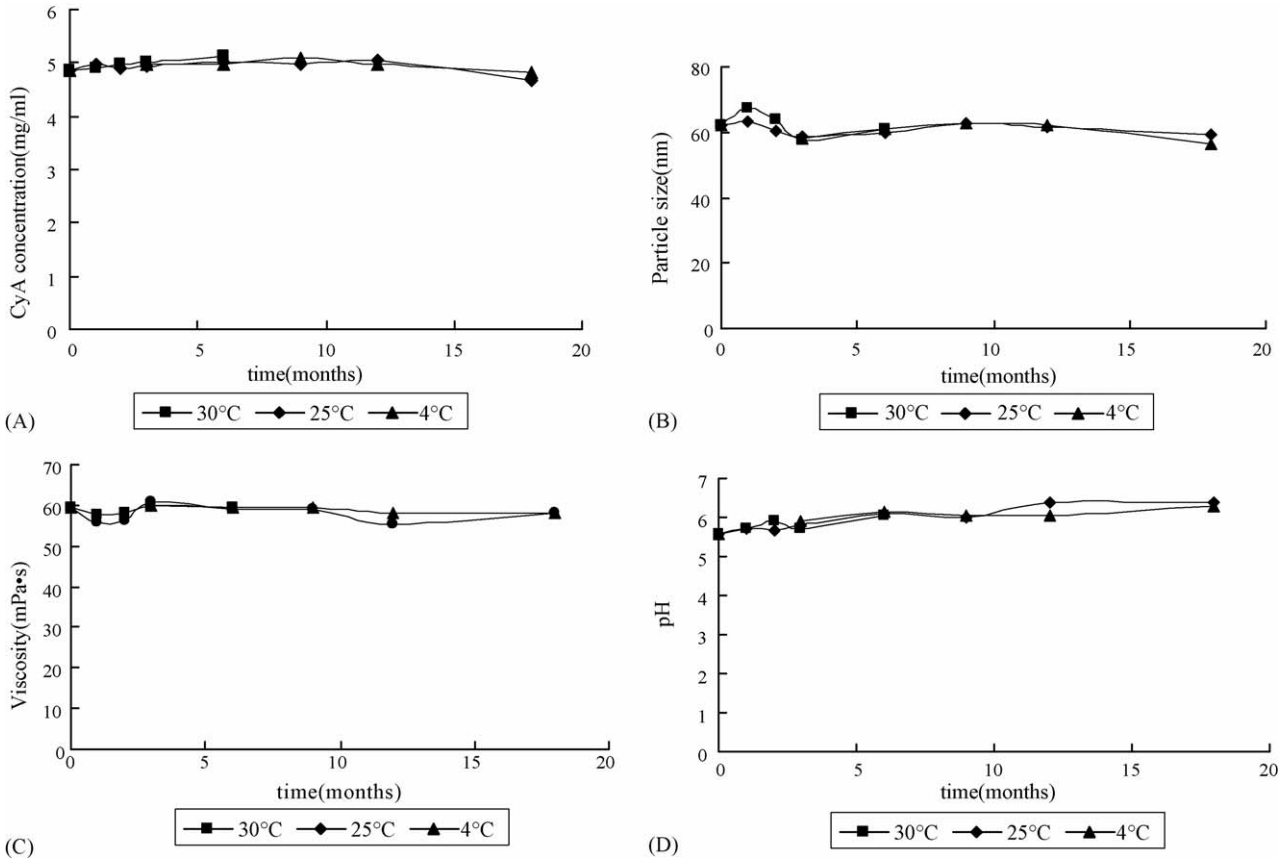


Fig. 2. Variations of the characteristics of Xanthan gum suspended CyA Eudragit® S100 nanoparticles over 18-month storage at different temperature ($n = 3$). (A) CyA concentration, (B) particle size, (C) viscosity, and (D) pH.

Neoral®. All formulations of nanoparticles showed higher C_{max} and higher $AUC_{0–24}$ values than those of reference. The relative bioavailability of S-CyA-S100 NP initial compared with Neoral® was 162.8%. The increase of the relative bioavailability of CyA after oral administration of the nanoparticle colloids might be due to the following reasons: the nanoparticles carrier protected CyA from degradation by GI juice and pH change; CyA molecules highly dispersed in the nanoparticle matrix; nanoparticles increased the bioadhesion of CyA to the GI mucosa.

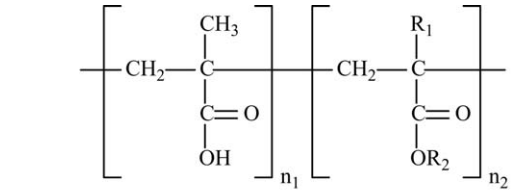
When we compared the pharmacokinetic parameters among the S-CyA-S100 NP preserved for 0, 12 and 18 months at 4 °C, we could see that the C_{max} at 12 and 18 months were 76.1% ($P < 0.01$) and 75.2% ($P < 0.01$), respectively, comparing with that of the initial. The results indicated that the C_{max} at 12 and 18 months were both lower than that of the initial, while there was no obvious difference between the C_{max} at 12 and 18 months. We also noticed that the $AUC_{0–24}$ at 12 and 18 months were 88.2% ($P > 0.05$) and 74.1% ($P < 0.01$), respectively, compared with that of the initial. The results indicated that the $AUC_{0–24}$ was also decreased during the shelf time.

Similar phenomena were observed from the studies of S-CyA-S100 NP stored at 25 °C. The C_{max} and $AUC_{0–24}$ values at 12 and 18 months were both lower than that of the initial.

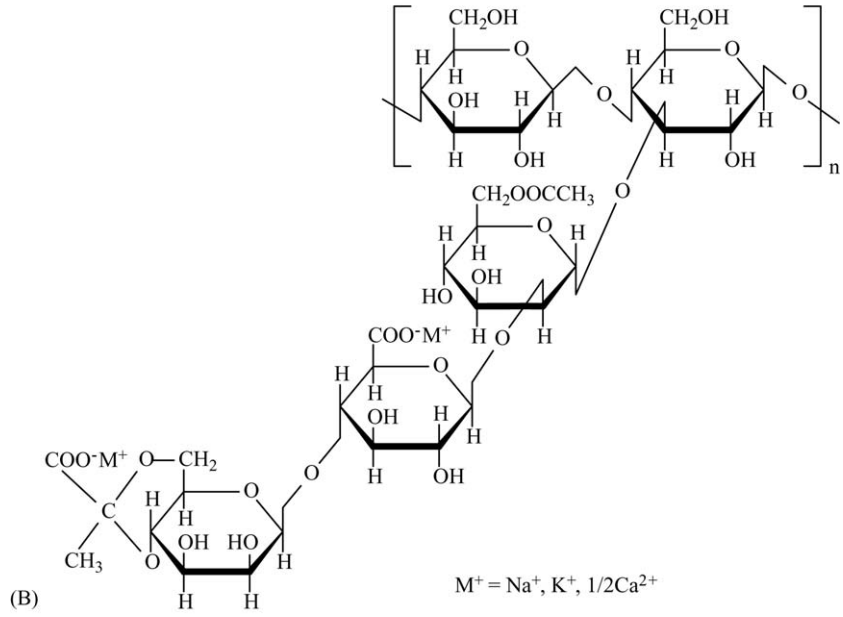
Table 2
Pharmacokinetic parameters of cyclosporine A after oral administration of Neoral® microemulsion and Xanthan gum suspended CyA Eudragit® S100 nanoparticles to rats; mean ± S.E. ($n = 5–8$)

Parameter	Neoral®	Xanthan gum suspended CyA Eudragit® S100 nanoparticles				
		Initial	Storage for 12 months at 4 °C	Storage for 12 months at 25 °C	Storage for 18 months at 4 °C	Storage for 18 months at 25 °C
C_{max} (ng/ml)	2107.0 ± 149.0	$4269.0 \pm 262.2^{**}$	$3249.1 \pm 170.8^{**,\#}$	$3558.5 \pm 267.0^{**}$	$3211.6 \pm 169.7^{**,\#}$	$3094.6 \pm 296.3^{**,\#}$
T_{max} (h)	2.50 ± 0.46	2.62 ± 0.65	2.20 ± 0.37	2.40 ± 0.22	2.33 ± 0.20	2.67 ± 0.49
$AUC_{0–24}$ (ng/ml h)	26341.3 ± 1681.8	$42872.1 \pm 2620.6^{**}$	$37806.3 \pm 1709.3^{**}$	$39296.5 \pm 3207.3^{**}$	$31789.6 \pm 2346.1^{*,\#\#}$	$34521.4 \pm 3650.7^{**,\#}$
MRT (h)	18.8 ± 0.42	$16.6 \pm 0.46^{**}$	17.5 ± 0.54	$16.9 \pm 0.22^{*}$	$16.3 \pm 0.53^{**}$	$17.1 \pm 0.49^{*}$
$F_{r(Test/Neoral®)}$ (%)	–	162.8	143.5	149.2	120.7	131.0

F_r: relative bioavailability = $(AUC_{0–24(Test)} \times 100)/(AUC_{024(Neoral®)})$; $^{*}P \le 0.05$, $^{**}P \le 0.01$ vs. Neoral®; $^{\#}P \le 0.05$,$^{\#\#}P \le 0.01$ vs. Xanthan gum suspended CyA Eudragit® S100 nanoparticles initial. AUC and MRT were calculated by trapezoidal rule; T_{max} and C_{max} were obtained directly by observation.



(A) R_1: $-CH_3$; R_2: $-CH_3$; n_1/n_2=1:2



(B) $M^+ = Na^+, K^+, 1/2Ca^{2+}$

Fig. 3. Chemical structure of Eudragit® S100 (A) and Xanthan gum (B).

There was no significant difference in C_{max} and $AUC_{0–24}$ values between 12 and 18 months. The C_{max} of S-CyA-S100 NP at 12 and 18 months at 25 °C were 83.4% ($P < 0.05$) and 72.5% ($P < 0.01$) that of the initial, while the $AUC_{0–24}$ at 12 and 18 months were 91.6% ($P > 0.05$) and 80.5% ($P < 0.05$) that of the initial. There was no significant difference in $AUC_{0–24}$ values between the initial and 12 months.

As for the T_{max}, there were no significant difference among all the formulations, while the MRT for all tested samples were within the range of 16–19 h.

It is quite interesting to notice that during the shelf time, the CyA concentration, particle size and viscosity did not change obviously, suggesting that CyA and the nanoparticle systems were generally rather stable. However, the relative bioavailability after oral administration gradually decreased, although the degree of such decrease was not so large and the bioequivalency still existed between the tested nanoparticle formulation and the reference after 1-year storage. It seems reasonable when considering such a thermodynamically unstable system.

It was supposed by careful analysis that the decrease in bioavailability might be resulted from the slightly increase of the pH value. Eudragit® S100 is a pH-sensitive polymer solubilized at pH 7.0. When the pH value of S-CyA-S100 NP colloid increased from 5.59 (the initial) to 6.38 (18 months), the percent of solubilized polymer might increase. According to Dai's report (Dai et al., 2004), during the in vitro release experiment at pH 5.5, CyA released only about 26%, while it released about 38% at pH 6.0 and nearly 100% at pH 6.8, respectively. Since the increase of the pH accelerated the release of CyA from nanoparticles, the protection effect of CyA by Eudragit® S100, the dispersion degree of CyA and also the bioadhesion of CyA to GI mucosa might decrease, which might lead to the decrease of the oral bioavailability.

To elucidate the reason further, we did some extra evaluations. Firstly the surface analysis results by XPS demonstrated that there were no obvious differences between the formulation at the initial and 30 months as shown in Table 1. Secondly there were no crystals of the drug observed for the nanoparticle preparation after preservation for 21 months under TEM as shown in Fig. 1. In other word, there is no evidence of CyA leaching out to the particle surface. Then, we studied the in vitro release of CyA from the nanoparticles with the formulation freshly prepared and stored for about 30 months in gastric juice (stomach simulation solution, pH 2), but little difference (data not showed) was found. It was suggested from the test that the burst effect in release studies was not increased after the nanoparticle colloids stored for long time, hence there was also no evidence to support the fact that CyA leached out to the particle surface.

We also found that the relative bioavailability of the S-CyA-S100 NP at 4 °C was lower than that at 25 °C although there

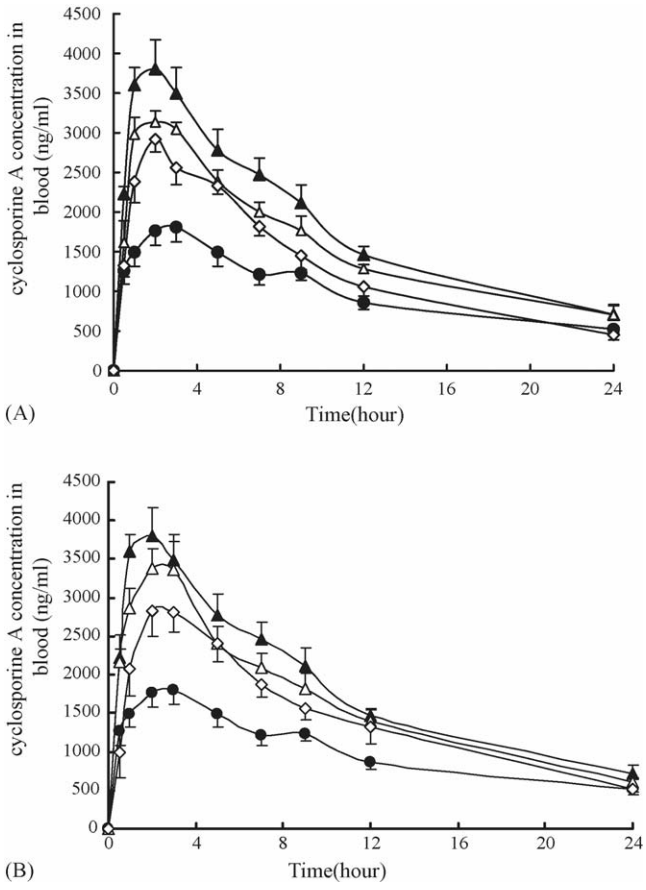


Fig. 4. Mean cyclosporine A concentration in blood following oral administration of Neoral® microemulsion (●), Xanthan gum suspended CyA Eudragit® S100 nanoparticles initial (▲), over 12 months storage (△), and 18 months storage (◊); (A) for 4 °C preservation and (B) for 25 °C preservation; mean ± S.E. (n = 5–8).

were no significant differences in the bioavailability and pharmacokinetic parameters of the formulations preserved at both conditions. This might be due to the difference in viscosity. In our studies we administrated the formulation by gavage just after we drew the preparation from refrigerator where the temperature was about 4 °C. The viscosity of the preparation at 4 °C was about 82 mPa s, which was larger than that at 25 °C (63 mPa s). The larger viscosity might prevent the diffusion of CyA from the nanoparticles system and therefore decrease the absorption of CyA.

4. Conclusion

In summary, this paper demonstrated that CyA, a poorly soluble and poorly absorbable drug, could be easily and reproducibly prepared into the pH-sensitive Eudragit® S100 nanoparticles suspended by Xanthan gum with a particle size about 60 nm. The physical stability of S-CyA-S100 NP was rather stable during the 18-month storage, with no obvious changes found in CyA concentration, particle size and viscosity. Although the AUC_{0-24} decreased during the shelf time, the S-CyA-S100 NP formulation at the initial and 12 months was bioequivalent. The decrease in bioavailability might be due to the pH change of nanoparticle colloid.

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