European Journal of Pharmaceutics and Biopharmaceutics 73 (2009) 247–252

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Improvement of cyclosporine A bioavailability by incorporating ethyl docosahexaenoate in the microemulsion as an oil excipient

Vilasinee Hirunpanich, Hitoshi Sato *

Department of Clinical and Molecular Pharmacokinetics/Pharmacodynamics, Showa University, Tokyo, Japan

ARTICLE INFO

Article history:
Received 29 December 2008
Accepted in revised form 22 June 2009
Available online 26 June 2009

Keywords:
Ethyl docosahexaenoate (DHA-EE)
Microemulsion
Bioavailability
Cyclosporine A

ABSTRACT

The aims of this study were to determine the effect of ethyl docosahexaenoate (DHA-EE) on cyclosporine A (CsA) bioavailability, while also examining the effect of DHA-EE on CsA when DHA-EE was incorporated into a microemulsion formulation as an oil ingredient. The oral co-administration of DHA-EE and CsA increased the blood CsA concentration in a dose-dependent manner, and the AUC and C_{max} both increased by about 2-fold with 100 mg/kg DHA-EE. The microemulsion formulation of CsA consisted of Tween-20, ethanol, water, and DHA-EE (53.3/6.5/35.9/3.3 w/w%) (namely DHA-ME) was transparent and stable with an average particle size of 50 nm, which was similar to that of the control formulation incorporating vitamin E instead of DHA-EE (namely VE-ME). The permeabilities of CsA from DHA-ME, VE-ME and Neoral® formulations across an artificial membrane were not significantly different. The C_{max} and $AUC_{0-\infty}$ of CsA in rats administered DHA-ME significantly increased by approximately 2-fold in comparison to that of VE-ME. The relative oral bioavailability (F_r) of DHA-ME in comparison to Neoral® was determined to be 114%, while the F_r of VE-ME was only 60%. It was, therefore, suggested that the use of DHA-EE as an oil excipient may be promising for the development of a microemulsion formulation of CsA with an improved oral bioavailability.

1. Introduction

Cyclosporine A (CsA) is a potent immunosuppressive agent that has been used to prevent allograft rejection in organ transplantation. It is known that CsA oral bioavailability is low due to its poor absorption, which is related to its low solubility in the gut lumen and the extensive first-pass metabolism in both the liver and gut by cytochrome P-450 3A4 (CYP3A4) and P-glycoprotein (P-gp)-mediated drug efflux [1].

Self-microemulsion emulsifying drug delivery systems (SMEDDS) are the isotropic mixtures of oils, surfactants, and/or co-surfactant and hydrophilic solvent that can produce microemulsions with a particle size less than 100 nm when exposed to an aqueous environment in the gastrointestinal (GI) tract and result in the improvement of the oral bioavailability of highly lipophilic compounds such as CsA, ritonavir and saquinavir [2]. Recently, a novel SMEDDS formulation of CsA, marketed as Neoral®, was developed and is now widely used because of its superior pharmacokinetics properties, i.e., increased CsA oral bioavailability and less variability in comparison to the original CsA formulation, Sandimmune® [3]. However, this formulation contains a high concentration of a surfactant, polyoxyethylated castor oil (Cremophor EL®) (approximately 38 w/w%), which is known to exert some adverse effects, such as hypersensitivity, nephrotoxic and anaphylactoid reactions [4,5]. Thus, extensive efforts have thus been devoted to design and develop a cremophore-free microemulsion formulation [6–8].

Docosahexaenoic acid (DHA) (22:6 ω-3) is a polyunsaturated fatty acid (PUFA) present in fish oil. Various biologically beneficial effects of DHA have been investigated [9]. In addition, previous studies have also reported that DHA inhibited the metabolic reactions catalyzed by the CYP3A enzyme *in vitro*, while also enhancing the oral bioavailability of CsA (dissolved in corn oil), saquinavir and midazolam in rats by inhibiting their pre-systemic intestinal metabolism due to CYP3A but not by affecting P-gp activity [10–13].

The ethyl ester form of DHA (DHA-EE) was recently approved by the US Food and Drug Administration [14] as a composition of drug named Lovaza™ (Reliant Pharmaceuticals, Inc., USA) for the treatment of hypertriglyceridemia. However, the effects of DHA-EE on the oral drug bioavailability of drugs have not yet been clarified. In this study, we therefore determined the effect of DHA-EE on CsA bioavailability. Subsequently, we examined whether DHA-EE is effective as an oral bioavailability enhancer of CsA when incorporated into a microemulsion as an oil ingredient.

* Corresponding author. Department of Clinical and Molecular Pharmacokinetics/Pharmacodynamics, School of Pharmacy, Showa University, Shinagawa-ku, Tokyo 142-8555, Japan. Tel.: +81 3 3784 8611; fax: +81 3 5498 1148.
E-mail address: sato-tky@umin.ac.jp (H. Sato).

0939-6411/$ - see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2009.06.011

2. Materials and methods

2.1. Chemicals

Ethyl docosahexaenoate (DHA-EE), cholesterol, L-α-phosphatidylcholine, L-α-phosphatidylethanol, L-α-phosphatidylserine, and phosphatidylinositol were obtained from Sigma–Aldrich Chemical (St. Louis, MO, USA), and a pre-concentrate CsA microemulsion (Neoral®) was purchased from Novartis Pharmaceutical Company, Tokyo, Japan. The compound 1,7-octadiene was obtained from the Johnson Matthey Company (MA, USA). Ethanol and (±)-α-tocopheral acetate (vitamin E) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Polyoxyethylene sorbitan monolaurate (Tween-20) was purchased from MP Biomedicals, Inc. (Ohio, USA). Saline was obtained from Otsuka Pharmaceutical Factory Inc. (Tokushima, Japan). All other chemicals and solvents were of analytical grades.

2.2. Methods

2.2.1. Preparation of the CsA microemulsion formulations

In order to determine the effect of DHA-EE in a microemulsion formulation of CsA, as a lipid excipient, the type and amount of surfactant, co-solvent and water were kept constant, while two types of oils, DHA-EE vs. vitamin E (as a control), were compared. Ternary phase diagrams, with three corners of a triangle representing three components of system including the surfactant, aqueous and oil ingredients, which consisted of Tween-20, distilled water and DHA-EE or vitamin E, respectively, were constructed to identify the microemulsion regions.

Preliminary experiments to determine the optimal concentration and phase volume of each composition, which produced the most transparent and stable microemulsion, were evaluated. Two CsA microemulsion formulations were formulated by employing the same amounts of Tween-20, ethanol and water, while either DHA-EE or vitamin E was used as an oil ingredient in the formulation of DHA-ME or VE-ME, respectively. The phase volume of the CsA microemulsion compositions were selected as shown in Table 1.

To prepare the above-mentioned microemulsions, CsA was dissolved with ethanol and then mixed with either DHA-EE or vitamin E. In order to prevent the occurrence of an oxidation reaction caused by DHA-EE, 0.5 (w/w%) of vitamin E was added to DHA-ME. Next, the mixtures were mixed with Tween-20, agitated at room temperature for 5 min and followed by the addition of distilled water. The resultant mixtures were gently stirred with a magnetic stirrer for 2 h at room temperature to achieve a state of equilibrium.

2.2.2. Physicochemical properties of CsA microemulsions

2.2.2.1. Visual observations. Each of CsA microemulsion formulation (DHA-ME or VE-ME) was introduced into 10 ml of water in a glass tube at room temperature, and then the contents were gently stirred by shaking. The equilibrated samples were assessed for clarity and transparency by visual inspection.

Table 1
Compositions and phase volume (w/w%) for preparing the cyclosporine A (CsA) microemulsion formulations.

Ingredients	Phase volume (w/w%)	
	DHA-ME	VE-ME
CsA	1.0	1.0
Tween-20	53.3	53.3
Ethanol	6.5	6.5
DHA-EE	2.8	–
Vitamin E	0.5	3.3
Water	35.9	35.9

2.2.2.2. Particle size of CsA microemulsions. The CsA microemulsion formulations were diluted 10-fold by distilled water. The average particle size of each CsA microemulsion formulation was measured in duplicate by using a laser nanoparticle size analyzer (SALD-7100, Shimadzu, Kyoto, Japan).

2.2.2.3. Solubility of CsA in microemulsions. The solubility of the CsA in microemulsions was determined according to a method reported by Jiko et al. [15] with some modifications. Briefly, an excessive amount of CsA was introduced into 2 ml of each microemulsion formulation and the mixture was shaken well for 5 min on a vortex mixture at 25 °C. The samples were centrifuged at 20,000*g* for 20 min. Thereafter, aliquots of supernatant were taken and appropriately diluted with saline before the amount of CsA was quantified by a fluorescent polarization immunoassay (FPIA) as described later.

2.2.3. Permeability of CsA from DHA-ME, VE-ME formulations and Neoral® across the artificial membrane

A diffusion experiment was carried out using polyvinylidene fluoride (PVDF) membranes with a 0.45-μm pore size (Amersham, GE Healthcare, Buckinghamshire, England). After the PVDF membrane was pretreated with methanol for 1 min and was washed with 50-mM phosphate buffer (pH 6.0) for 5 min, it was dried at room temperature for about 1 h and mounted between the donor and acceptor diffusion compartments. The effective area of the membrane was 0.78 cm^2. The membrane was soaked with 15 μl of ice-cold phospholipids solution that contained a mixture of 0.8 w/w% of L-α-phosphatidylcholine, 0.8 w/w% of L-α-phosphatidylethanol, 0.2 w/w% of phosphatidylserine, 0.2 w/w% of phosphatidylinositol, 1 w/w% of cholesterol and 97 w/w% of 1,7-otadiene [16] to mimic enterocytes phospholipids. Two milliliters of 1 mg/ml CsA from DHA-ME, VE-ME or Neoral® dissolved in 50-mM phosphate buffer (pH 6.0) was added to the donor compartment, whereas 1,200 μl of 50 mM phosphate buffer (pH 6.0) was added to the acceptor compartment in which rat plasma (50%) was added to generate a sink condition. After 30, 60, 90 and 120 min, 300 μl of the solution was collected from the acceptor side and an equal volume was added immediately. The diffused amount of CsA in the sample was determined by a fluorescent polarization immunoassay (FPIA) as described later. The rate of passive diffusion of CsA from each microemulsion was calculated from the slope of the diffused amount of CsA vs. time profile.

2.2.4. Pharmacokinetics and bioavailability of CsA in rats

2.2.4.1. Animals and surgery. In vivo experiments were performed using male Wistar rats (Nihon Ikagaku Doubutsu, Saitama, Japan) weighing from 200 to 220 g, according to the guidelines for Animal Experimentation in the Faculty of Pharmaceutical Sciences, Showa University. The rats were acclimatized to environmental control (a temperature-controlled facility with a 12-h light/dark cycle) for at least one week. The rats were fasted for approximately 12 h with water given *ad libitum*. The femoral artery was cannulated with polyethylene tubing (SP-31, Natsume Seisakusho, Tokyo, Japan) to facilitate blood sampling under light ether anesthesia. After the operation, each cannulated rat was kept in a Bolman cage and the experiment was performed following recovery from anesthesia.

2.2.4.2. Effect of DHA-EE on the pharmacokinetics of CsA (dissolved in oil formulation) in rats. Either DHA-EE (25 or 100 mg/kg, dissolved in cornoil) or corn oil (as a control) was orally pre-administered

10 min before 5 mg/kg CsA (dissolved in corn oil) was orally administered into the rat. Blood samples (about 150 μl) were collected from the cannulated femoral artery at 1, 2, 3, 4, 6, 9, 12 and 24 h into plastic tubes containing EDTA, thoroughly mixed, and then stored at 4 °C until analysis. An equivalent volume of saline was replaced through the femoral artery.

2.2.4.3. Pharmacokinetics and bioavailability of CsA administered as DHA-ME, VE-ME and Neoral® formulations in rats. DHA-ME and VE-ME were freshly prepared as mentioned earlier. Each microemulsion (Neoral®, DHA-ME or VE-ME formulation) was dissolved in distilled water to obtain the CsA concentration of 3.5 mg/ml, and then it was administered orally to rats (3.5 mg/kg) using a disposable plastic syringe, followed by the administration of 2 ml of water. Thereafter, blood samples (150 μl) were collected from the cannulated femoral artery into plastic tubes containing EDTA at 0.5, 1, 1.5, 2, 3, 4, 6, 9 and 24 h, thoroughly mixed, and stored at 4 °C until analysis. An equivalent volume of saline was replaced through the femoral artery.

2.2.4.4. Pharmacokinetic analysis. The pharmacokinetic parameters of CsA in rats, i.e., total clearance (CL_{tot}/F), elimination half-life ($T_{1/2}$), the volume of distribution (V_{dss}/F), the maximum blood concentration (C_{max}), time to reach C_{max} (T_{max}), and the areas under the whole blood concentration–time curve from zero to infinity ($AUC_{0-\infty}$) were estimated by a non-compartmental analysis using a computer program, WinNonlin® (version 5.2, Pharsight Corporation, North Carolina, USA). The relative bioavailability (F_r) was calculated using the following equation:

$$F_r(\%) = \frac{AUC_{0-\infty(\text{test})}}{AUC_{0-\infty(\text{Neoral})}} \times 100 \quad (1)$$

where $AUC_{0-\infty(\text{Neoral})}$ is the $AUC_{0-\infty}$ after the oral administration of Neoral®, while $AUC_{0-\infty(\text{test})}$ represents that of DHA-ME or VE-ME formulation.

2.2.5. Quantification of CsA

The CsA concentrations in the samples from the *in vivo* and *in vitro* experiments were assayed by a monoclonal antibody fluorescence polarization immunoassay (FPIA) in a TDxFLx Analyzer® (Abbott Japan Co. Ltd., Tokyo, Japan) using a TDX cyclosporin-SP-Dinapack® kit (Abbott Japan Co. Ltd.), which employs the monoclonal antibody raised against CsA. The assay system was properly calibrated in the range of 50–1500 ng/ml and was routinely evaluated using the quality control samples provided by the manufacturer.

2.2.6. Statistical analysis

The data are expressed as the mean ± standard error of the mean (SEM). Statistical significance was assessed using one-way anova followed by Dunnet's test for the individual differences. The difference was considered to be statistically significant if the probability value was less than 0.05 ($P < 0.05$).

3. Results

3.1. Characteristics and physicochemical properties of the CsA microemulsions

The ternary phase diagrams of a system containing Tween-20, distilled water and DHA-EE for DHA-ME or vitamin E for VE-ME are illustrated in Fig. 1A and B, respectively. The DHA-ME and VE-ME formulations were clear and transparent after being diluted in water. They were physically stable at room temperature, without the occurrence of phase separation, throughout the study.

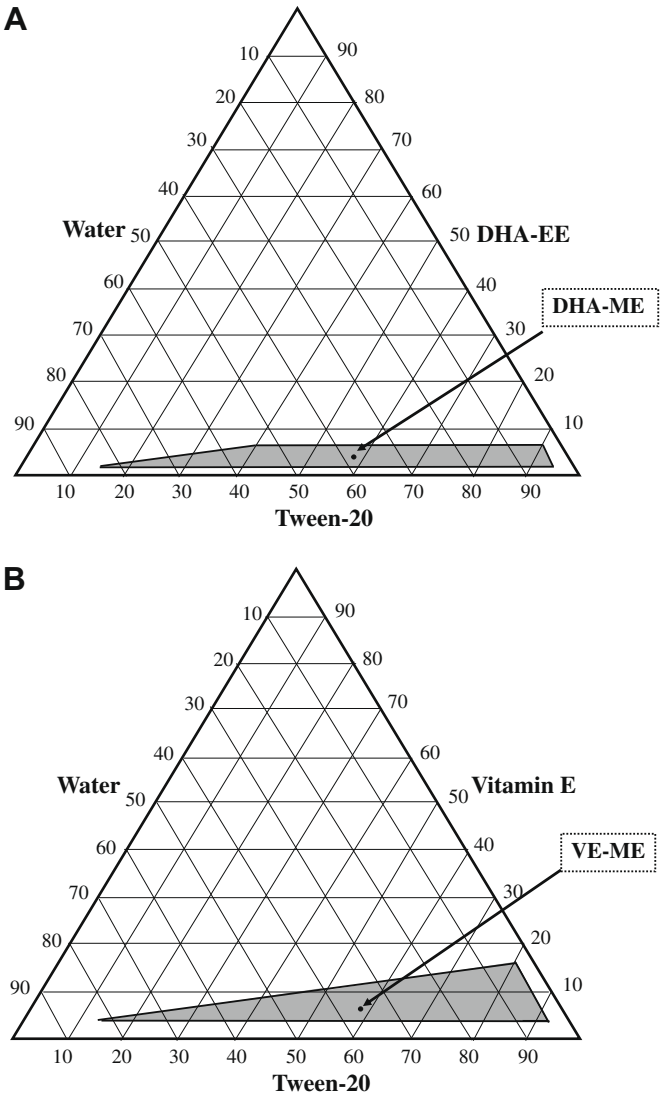

Fig. 1. The ternary phase diagram of the CsA microemulsion system containing Tween-20, distilled water and DHA-EE for DHA-ME (A) or vitamin E for VE-ME (B) as surfactant, water and oil system, respectively. The shaded areas in the phase diagram A and B represent clear and transparent microemulsions. The formulations of DHA-ME and VE-ME microemulsions are indicated in (A) and (B), respectively.

DHA-EE and VE-ME formulations produced comparable mean particle sizes (54.6 ± 13.5 and 52.7 ± 17.7 nm, respectively) that were in the range of a microemulsion (10–150 nm), and the solubilities of CsA from DHA-ME and VE-ME were not significantly different from each other (16.3 ± 4.3 and 12.7 ± 2.8 mg/ml, respectively).

3.2. Permeability of CsA from DHA-ME, VE-ME and Neoral® formulations across the artificial membrane

The rates of permeation of CsA across the artificial membrane (PVDF) from DHA-ME, VE-ME and Neoral® formulations were determined to be 5.8 ± 0.4, 6.0 ± 0.1 and 6.3 ± 1.8 ng/ml/min, respectively; however, the results were not significantly different.

3.3. Effect of DHA-EE on the pharmacokinetics of CsA (dissolved in oil formulation) in the rats

Fig. 2 shows the blood concentrations of the orally administered CsA in rats in combination with or without the oral co-administration of 25 mg/kg or 100 mg/kg DHA-EE. The pharmacokinetic

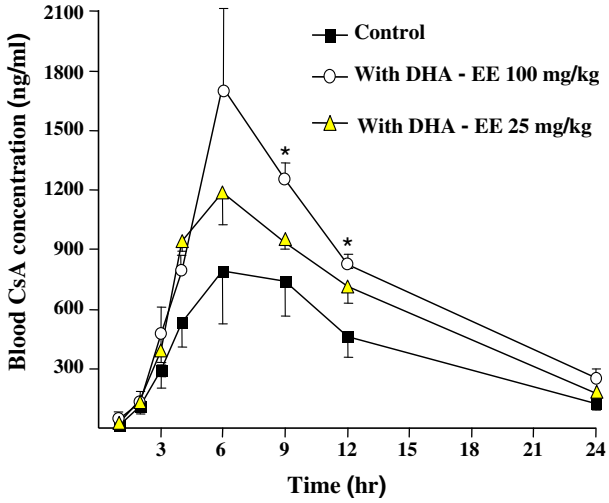

Fig. 2. The effect of the orally administered DHA-EE on blood CsA concentrations after the oral administration of CsA (5 mg/kg dissolved in corn oil) to the rats. The rats were orally administered 5 mg/kg CsA without (control) or with 25 mg/kg or 100 mg/kg DHA-EE. Each symbol with bar represents the mean ± SEM (n = 4).

parameters obtained by a non-compartmental analysis are summarized in Table 2. When CsA was co-administered with DHA-EE, the blood concentrations of CsA increased in a dose-dependent manner with DHA-EE. In the groups of rats administered with 100 mg/kg DHA-EE, the values of AUC_{0-24h} and $AUC_{0-\infty}$ of CsA markedly increased by about 1.4-fold and 1.8-fold, respectively, in comparison to that of the control group ($P < 0.05$). In addition, CL_{tot}/F significantly decreased by approximately 2-fold ($P < 0.05$). The maximum concentration (C_{max}) increased by approximately 2-fold, but the difference was not significant. On the other hand, DHA-EE caused no significant changes in $T_{1/2}$, V_{dss}/F and T_{max}.

3.4. Pharmacokinetics and bioavailability of CsA in rats

Fig. 3 shows the blood concentration–time profiles of CsA after the oral administration of the microemulsions containing CsA, i.e., DHA-ME, VE-ME and Neoral®, in rats. The pharmacokinetic parameters of CsA are presented in Table 3. The C_{max} and $AUC_{0-\infty}$ obtained from DHA-ME were markedly enhanced by about 2-fold in comparison to VE-ME ($P < 0.01$). The CL_{tol}/F of CsA administered as DHA-ME and Neoral® significantly decreased in comparison to VE-ME ($P < 0.01$ and $P < 0.05$, respectively). In addition, no significant difference was seen in the T_{max} of CsA after the oral administrations of DHA-ME and VE-ME, but it was significantly longer than that of Neoral® ($P < 0.05$). However, $T_{1/2}$ was not significantly different among the three groups. The relative oral bioavailability (F_r) of CsA obtained from DHA-ME, in comparison to $AUC_{0-\infty}$ of Neoral®, was calculated to be 114%, which was higher than that of the control group, VE-ME, which showed F_r to be about 60%.

Table 2
Pharmacokinetic parameters of cyclosporine A (CsA) after oral administration in rats.

Parameters	Control (CsA)	100 mg/kg DHA-EE + CsA	25 mg/kg DHA-EE + CsA
$AUC_{0-24\ h}$ (mg/l × h)	9.59 ± 1.88	17.6 ± 1.98*	14.2 ± 1.30
$AUC_{0-\infty}$ (mg/l × h)	10.7 ± 2.16	20.2 ± 2.65*	15.8 ± 1.60
$T_{1/2}$ (h)	6.15 ± 0.42	6.68 ± 0.70	6.17 ± 0.56
CL_{tot}/F (l/h/kg)	0.52 ± 0.09	0.26 ± 0.03*	0.33 ± 0.03
V_{dss}/F (l/kg)	4.51 ± 0.65	2.43 ± 0.19	2.84 ± 0.21
T_{max} (h)	7.00 ± 1.22	7.50 ± 0.87	6.25 ± 1.03
C_{max} (μg/ml)	0.93 ± 0.18	1.82 ± 0.36	1.27 ± 0.11

The rats were orally administered 5 mg/kg of CsA (dissolved in corn oil) without (control) or with either 25 mg/kg or 100 mg/kg DHA-EE. The pharmacokinetic parameters of CsA were calculated as described in the Materials and Methods section. Each value is expressed as the mean ± SEM (n = 4).
* The *asterisks* indicate a statistically significant difference at $P < 0.05$ in comparison to the control group.

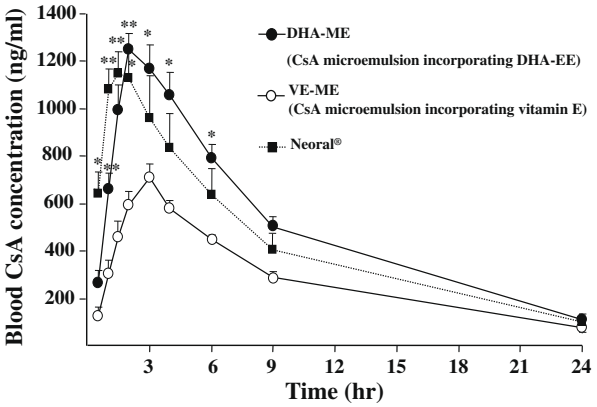

Fig. 3. The blood CsA concentration–time profiles in the rats after the oral administration of 3.5 mg/kg of CsA from DHA-ME, VE-ME and Neoral® formulations. One-way anova followed by Dunnet's test for individual differences was performed, and statistically significant differences $P < 0.05$ and $P < 0.01$ from the control group (VE-ME formulation) are shown by the *asterisks* (∗) and (∗∗), respectively. The symbol and vertical bar represent the mean and standard error of the mean (SEM) (n = 5). In some cases, the SEM fell within the circle.

Table 3
Pharmacokinetic parameters of cyclosporine A (CsA) administered as microemulsions of DHA-ME, VE-ME and Neoral® in rats.

Parameters	VE-ME	DHA-ME	Neoral®
$AUC_{0-\infty}$ (mg/l × h)	6.93 ± 0.33	13.07 ± 0.80**	11.49 ± 1.59*
$T_{1/2}$ (h)	7.05 ± 0.49	6.28 ± 0.76	6.94 ± 0.39
CL_{tot}/F (l/h/kg)	0.509 ± 0.025	0.272 ± 0.175**	0.335 ± 0.057*
V_{dss}/F (l/kg)	5.13 ± 0.25	2.43 ± 0.25**	3.48 ± 0.82
T_{max} (h)	2.70 ± 0.30	2.30 ± 0.30	1.70 ± 0.20*
C_{max} (μg/ml)	0.73 ± 0.08	1.37 ± 0.03**	1.22 ± 0.10**
F_r (%)	60	114	–

F_r (relative bioavailability) $= AUC_{0-\infty(test)}/AUC_{0-\infty(Neoral)} \times 100$

The rats were orally administered 3.5 mg/kg of CsA from DHA-ME, VE-ME and Neoral®. The pharmacokinetic parameters of CsA were calculated as described in the Materials and Methods section. Each value is expressed as the mean ± SEM from five rats.
* The *asterisks* indicate a statistically significant difference at $P < 0.05$, respectively, in comparison to the control group, VE-ME.
** The *asterisks* indicate a statistically significant difference at $P < 0.01$, respectively, in comparison to the control group, VE-ME.

4. Discussion

We previously demonstrated that DHA inhibits the CYP3A activity *in vitro* and enhanced the oral bioavailability of CsA in rats [10]. Moreover, in the present study, we confirmed that the ethyl ester form of DHA (DHA-EE) also significantly enhanced the bioavailability of CsA. This finding led us to develop a novel microemulsion formulation of CsA, incorporating DHA-EE, while hoping that DHA-EE is able to act not only as an oil excipient but also as a bioavailability enhancer for CsA.

According to the biopharmaceutics classification system (BCS), CsA is classified as Class II because its aqueous solubility is very low (27.67 μg/ml at 25 °C) [17]. The correlation of intestinal absorption with solubility is likely to be an important factor for Class II drugs [18,19]. When preparing the microemulsion of CsA, Tween-20, a non-ionic surfactant, was chosen because it was reported to extensively support CsA solubility [20]. Moreover,

Tween-20 is safe and commonly used in pharmaceutical formulations [2]. Therefore, in this study, DHA-ME and VE-ME formulations consisting of Tween-20 dramatically increased the CsA solubility by about 578- and 434-fold, respectively.

Ethanol was used as a co-solvent for the preparation of microemulsions which thus enables the dissolution of CsA in the lipid base [21]. The effects of different lipids in SMEDDS on the oral bioavailability of CsA have previously been studied [22]. Vitamin E, a lipid-soluble vitamin, has been practically used as an oil ingredient in several SMEDDS including Neoral® [21], and there has so far been no report on its effects regarding the CYP 3A and/or P-gp activity [23]; therefore, vitamin E was chosen as a negative control to compare it with the bioavailability-enhancing effect of DHA-EE on CsA in this study.

The structure of the microemulsion was assigned based on the hydrophile–lipophile balance (HLB) of the surfactants. Both (DHA-ME and VE-ME) microemulsion formulations developed in this study contained Tween-20, which has an HLB value of 16.7, thus suggesting that they were considered to be oil in water (O/W) microemulsions [21]. The area of microemulsion determined from the ternary phase diagrams of DHA-ME and VE-ME formulations, as shown in Fig. 1A and B, respectively, were similar, thus indicating that both microemulsion formulations were comparable.

Several physicochemical characteristics of microemulsions have been reported to influence drug bioavailability from SMEDDS such as particle size, solubility and permeability [21]. According to the similar characteristics of DHA-ME and VE-ME with regard to their particle size, solubility and permeability, the difference in the oil ingredients was considered to be the cause of pharmacokinetic differences between these microemulsions. From the *in vivo* pharmacokinetics results, C_{max}, $AUC_{0-\infty}$ and relative bioavailability (F_r) of CsA administered as DHA-ME were markedly higher than those of VE-ME, even though these two microemulsions contained the same excipients except for the oil ingredient. We therefore concluded that DHA-EE may be a crucial factor which can thus affect the differences in CsA absorption obtained from DHA-ME and VE-ME formulations.

The intestinal permeability of CsA has been reported to be limited by intestinal P-gp, which is an efflux pump on the enterocytes membrane [18,19]. The effect of DHA-EE on the P-gp activity was not directly determined in the present study. However, the P-gp activity should not be affected by DHA-EE, based on the fact that DHA did not affect the P-gp activity *in vitro* [10,11,24]. From our *in vivo* study, T_{max} of CsA from rats administered with Neoral® was significantly shorter than that of DHA-ME and VE-ME, thus indicating that the rate of absorption/permeability of CsA from Neoral® was higher than that of DHA-ME and VE-ME. It could be presumed that the superior permeability of CsA from Neoral® was because of the high amount of Cremophore EL®, which has been previously shown to be a strong permeability enhancer for CsA due to P-gp inhibition [18].

The drug bioavailability from SMEDDS can be enhanced by several mechanisms such as reduced enteric metabolism and efflux pump activity [21]. The intestinal first-pass metabolism mediated by CYP3A has been shown to be clinically important for CsA [25]. In this study, it was interesting to note that when DHA-EE was incorporated into the microemulsion formulation, the C_{max} and $AUC_{0-\infty}$ of CsA obtained from DHA-ME significantly increased, while the CL_{tot}/F decreased by about 2-fold in comparison to the control formulation, VE-ME, although their $T_{1/2}$ were almost the same, thus suggesting that DHA-EE did not affect the hepatic metabolism of CsA. These results are in consistent with the finding of an *in vivo* study where we observed the enhancing effect of DHA-EE on the blood concentration of CsA when it was administered as a corn oil formulation and on the increased pharmacokinetic parameters of $AUC_{0-\infty}$ and C_{max} by about 2-fold.

Due to the size similarity among the three microemulsions, the present results cannot definitively confirm the effect of particle size on the CsA release. However, the microemulsion formulation of CsA in a smaller particle size has been reported to show a greater ability to release CsA from the carrier [8,26], thus suggesting a correlation between the particle size and the rate of drug release.

Recently, Aspenström-Fagerlund et al. [27] reported that DHA increased the paracellular absorption of hydrophilic compounds in an experimental model of human absorptive enterocytes. However, the rate of permeation of CsA obtained from DHA-ME was not significantly different from that of the VE-ME and Neoral®, thus suggesting that the membrane penetration of CsA itself may not be influenced by DHA-EE due to the highly lipophilic nature of CsA.

The toxic effects of DHA-EE have not yet been observed. Busnach et al. [28] reported that the use of fish oil as a vehicle for CsA can decrease CsA-induced hypertension, decrease CsA-induced nephrotoxicity and enhance the immunosuppressive effect of CsA. Moreover, DHA has been shown to protect the intestinal mucosa from damage by such drugs as methotrexate *in vivo* [29]. These observations indicate that DHA can, therefore, be a good candidate to be used as a lipid ingredient in drug delivery systems to promote the oral absorption of CsA and to decrease the toxicity of CsA.

Although DHA-EE employed as an oil ingredient in the microemulsion exhibited an enhancing effect on the bioavailability of CsA, its relative bioavailability (F_r) in comparison to Neoral® was close to unity. This result may be due to the fact that Neoral® already contains a P-gp inhibitor, Cremophore EL®. However, various toxicities of Cremophore EL® may limit its clinical use. Since the safety of DHA-EE has already been confirmed as a food supplement and as a pharmaceutical product and its beneficial effects on the CsA side effects have been reported, we therefore developed a novel microemulsion formulation incorporating DHA-EE as a bioavailability-enhancing oil ingredient.

A number of studies have so far been conducted to increase the poor absorption of water-insoluble drugs using the co-administration of P-gp and/or CYP3A inhibitors. However, the co-administration of pharmacologically active drugs (e.g., ketoconazole) as a bioavailability enhancer may not be easily justified in clinical settings. Moreover, variable lag-times associated with the intake of multiple formulations by patients may also cause a variable rate/extent of drug absorption. Therefore, the use of DHA-EE in the microemulsion formulations with a water-insoluble drug can be recognized as a 'combined' medicine with no lag-time of administration.

Because of the characteristics of microemulsion, the DHA-ME formulation exhibited a superior performance, i.e., the rapid absorption of CsA where the T_{max} of DHA-ME is shorter than that of the co-administrations of CsA with DHA-EE 25 and 100 mg/kg by about three times (Tables 2 and 3). In contrast, $AUC_{0-\infty}$ and CL_{tot}/F were comparable between the DHA-ME formulation and co-administration of CsA and 100 mg/kg DHA-EE. The amount of DHA-EE necessary to increase the blood CsA levels, however, was much less with the microemulsion than with the co-administration by approximately 10 times, thus indicating that the DHA-EE effect would be more pronounced when DHA-EE and CsA co-existed in the microemulsion formulation and thereby exhibited a synchronized exposure to the intestinal epithelial cells.

5. Conclusions

The present investigation developed a novel microemulsion formulation of DHA-EE as an oil ingredient and demonstrated that DHA-ME exhibited a higher bioavailability of CsA in comparison to the control microemulsion, possibly due to the inhibition of pre-systemic gut metabolism. Since DHA exhibited inhibitory

effects on CYP3A activity *in vitro* and *in vivo* for CYP3A substrates, i.e., CsA, testosterone, midazolam, saquinavir [10–13], it was, therefore, suggested that DHA-EE might be a potentially useful general bioavailability enhancer agent for CYP3A substrates when they are employed either as a separate additive or as an excipient of microemulsions.

Acknowledgment

This work was supported by a Hitec Research Grant provided by Showa University, Tokyo, Japan.

References

[1] U. Christians, Transport proteins and intestinal metabolism: P-glycoprotein and cytochrome P4503A, Ther. Drug Monit. 26 (2004) 104–106.
[2] R.N. Gursoy, S. Benita, Self-emulsifying drug delivery systems (SEDDS) for improved oral delivery of lipophilic drugs, Biomed. Pharmacother. 58 (2004) 173–182.
[3] K. Midtvedt, Therapeutic drug monitoring of cyclosporine, Transplant. Proc. 36 (2004) 430S–433S.
[4] D.R. Luke, B.L. Kasiske, G.R. Matzke, W.M. Awni, W.F. Keane, Effect of cyclosporin on the isolated perfused rat kidney, Transplantation 43 (1987) 795–799.
[5] T. Cavanak, H. Sucker, Formulation of dosage forms, Prog. Allergy 38 (1986) 65–72.
[6] D.W. Kim, S.Y. Kim, H.K. Kim, S.W. Kim, S.W. Shin, J.S. Kim, K. Park, M.Y. Lee, D.S. Heo, Multicenter phase II trial of Genexol-PM, a novel Cremophor-free, polymeric micelle formulation of paclitaxel, with cisplatin in patients with advanced non-small-cell lung cancer, Ann. Oncol. 18 (2007) 2009–2014.
[7] A.O. Nornoo, D.W. Osborne, D.S. Chow, Cremophor-free intravenous microemulsion for paclitaxel I: formulation, cytotoxicity and hemolysis, Int. J. Pharm. 349 (2008) 108–116.
[8] X.Q. Wang, J.D. Dai, Z. Chen, T. Zhang, G.M. Xia, T. Nagai, Q. Zhang, Bioavailability and pharmacokinetics of cyclosporine A-loaded pH-sensitive nanoparticles for oral administration, J. Control Release 97 (2004) 421–429.
[9] W. Stillwell, S.R. Wassall, Docosahexaenoic acid: membrane properties of a unique fatty acid, Chem. Phys. Lipids 126 (2003) 1–27.
[10] V. Hirunpanich, J. Katagi, B. Sethabouppha, H. Sato, Demonstration of docosahexaenoic acid as a bioavailability enhancer for CYP3A substrates: in vitro and in vivo evidence using cyclosporin in rats, Drug Metab. Dispos. 34 (2006) 305–310.
[11] V. Hirunpanich, K. Murakoso, H. Sato, Inhibitory effect of docosahexaenoic acid (DHA-EE) on the intestinal metabolism of midazolam: in vitro and in vivo studies in rats, Int. J. Pharm. 351 (2008) 133–143.
[12] V. Hirunpanich, H. Sato, Docosahexaenoic acid (DHA-EE) inhibits saquinavir metabolism in vitro and enhances its bioavailability in rats, J. Pharm. Pharmacol. 58 (2006) 651–658.
[13] H.T. Yao, Y.W. Chang, S.J. Lan, C.T. Chen, J.T. Hsu, T.K. Yeh, The inhibitory effect of polyunsaturated fatty acids on human CYP enzymes, Life Sci. 79 (2006) 2432–2440.
[14] FDA Announcement on Qualified Health Claims for Omega-3 Fatty Acids, November 10, 2004, <http://www.fda.gov>.
[15] M. Jiko, I. Yano, H. Wakasugi, H. Saito, K. Inui, Evaluation of pharmacokinetic interaction between cyclosporin A and probucol in rats, Pharm. Res. 19 (2002) 1362–1367.
[16] H. Araya, M. Tomita, M. Hayashi, The novel formulation design of self-emulsifying drug delivery systems (SEDDS) type O/W microemulsion III: the permeation mechanism of a poorly water soluble drug entrapped O/W microemulsion in rat isolated intestinal membrane by the Using chamber method, Drug Metab. Pharmacokinet. 21 (2006) 45–53.
[17] G. Ismailos, C. Reppas, J.B. Dressman, P. Macheras, Unusual solubility behaviour of cyclosporin A in aqueous media, J. Pharm. Pharmacol. 43 (1991) 287–289.
[18] Y.Y. Chiu, K. Higaki, B.L. Neudeck, J.L. Barnett, L.S. Welage, G.L. Amidon, Human jejunal permeability of cyclosporin A: influence of surfactants on P-glycoprotein efflux in Caco-2 cells, Pharm. Res. 20 (2003) 749–756.
[19] E.M. Persson, A. Nordgren, P. Forsell, L. Knutson, C. Ohgren, S. Forssén, H. Lennernäs, B. Abrahamsson, Improved understanding of the effect of food on drug absorption and bioavailability for lipophilic compounds using an intestinal pig perfusion model, Eur. J. Pharm. Sci. 34 (2008) 22–29.
[20] Y. Ran, L. Zhao, Q. Xu, S.H. Yalkowsky, Solubilization of cyclosporin A, AAPS PharmSciTech 2 (2001) E2.
[21] A.S. Narang, D. Delmarre, D. Gao, Stable drug encapsulation in micelles and microemulsion, Int. J. Pharm. 345 (2007) 9–25.
[22] J.M. Odeberg, P. Kaufmann, K.G. Kroon, P. Höglund, Lipid drug delivery and rational formulation design for lipophilic drugs with low oral bioavailability, applied to cyclosporine, Eur. J. Pharm. Sci. 20 (2003) 375–382.
[23] L. Chan, L.M. Humma, C.A. Schriever, L.A. Fahsingbauer, C.P. Dominguez, C.L. Baum, Vitamin E formulation affects digoxin absorption by inhibiting P-glycoprotein (P-GP) in humans, Clin. Pharmacol. Ther. 75 (2004) 95.
[24] Q.Y. Liu, B.K. Tan, Effects of cis-unsaturated fatty acids on doxorubicin sensitivity in P388/DOX resistant and P388 parental cell lines, Life Sci. 67 (2000) 1207–1218.
[25] C.Y. Wu, L.Z. Benet, M.F. Hebert, S.K. Gupta, M. Rowland, D.Y. Gomez, V.J. Wacher, Differentiation of absorption and first-pass gut and hepatic metabolism in humans: studies with cyclosporine, Clin. Pharmacol. Ther. 58 (1995) 492–497.
[26] M.H. El-Shabouri, Positively charged nanoparticles for improving the oral bioavailability of cyclosporin-A, Int. J. Pharm. 249 (2002) 101–108.
[27] B. Aspenström-Fagerlund, L. Ring, P. Aspenström, J. Tallkvist, N.G. Ilbäck, A.W. Glynn, Oleic acid and docosahexaenoic acid cause an increase in the paracellular absorption of hydrophilic compounds in an experimental model of human absorptive enterocytes, Toxicology 237 (2007) 12–23.
[28] G. Busnach, E. Stragliotto, E. Minetti, A. Perego, B. Brando, M.L. Broggi, G.C. Nefrologia, D.T.T. Renale, O.N.C. Granda, Effect of n-3 polyunsaturated fatty acids on cyclosporine pharmacokinetics in kidney graft recipients: a randomized placebo-controlled study, J. Nephro. 11 (1998) 87–93.
[29] T. Horie, M. Nakamura, Y. Masubuchi, Docosahexenoic acid exhibits a potent protection of small intestine from methotrexate-induced damage in mice, Life Sci. 62 (1998) 1333–1338.