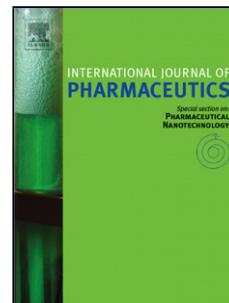


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**Development and evaluation of a tacrolimus cream formulation
using a binary solvent system**

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ABSTRACT

We developed an oil/water-type tacrolimus (FK506) cream formulation as an alternative to Protopic ointment for atopic dermatitis treatment. We determined the effects of solvents used in topical preparations on FK506 solubility and stability, and evaluated FK506 transdermal absorption into rat skin from solutions, emulsions, and creams. Screening indicated that diethyl sebacate (DES), isopropyl myristate (IPM), propylene glycol (PG), and oleyl alcohol (OA) were adequate FK506 solvents. When FK506 solutions prepared using these solvents were transdermally administered, AUC₀₋₂₄ values for DES and IPM were higher than or similar to that for 0.1% Protopic ointment. The AUC₀₋₂₄ values for PG and OA were low, so these solvents did not enhance absorption. The residual ratios of FK506 in DES and IPM solutions after incubation at 70 °C for 9 d were 95.6% and 88.6%, respectively, so DES and IPM were chosen for emulsion preparation. When the emulsions were transdermally administered, the IPM emulsion AUC₀₋₂₄ values increased 4.6-fold; DES emulsions did not show high transdermal absorption, but showed sustained characteristics. A cream formulation prepared by mixture of IPM and DES also showed high absorption and transdermal absorption increased with increasing IPM ratio. We developed an FK506 cream formulation with a controllable transdermal absorption rate by manipulating the IPM:DES ratio.

Keywords: Tacrolimus, Cream, Surfactant, Emulsion, Binary solvent

41 *Abbreviations:* AD, atopic dermatitis; AUC, area under plasma-concentration–time curve; AUC_{0-24} ,
 42 area under plasma-concentration–time curve from time 0 to 24 h of measurable concentration;
 43 BB-5, POE(5) behenyl ether; BB-10, POE(10) behenyl ether; BC-2, POE(2) cetyl ether; BC-5.5,
 44 POE(5.5) cetyl ether; BC-7, POE(7) cetyl ether; BC-10TX, POE(10) oleyl ether; BO-7, POE(7)
 45 oleyl ether; BS-4, POE(4) stearyl ether; CC, cetyl caprylate; C_{max} , maximum plasma concentration;
 46 DES, diethyl sebacate; DIA, diisopropyl adipate; DO, decyl oleate; FK506, tacrolimus; HCO-10,
 47 POE(10) hydrogenated castor oil; HCO-20, POE(20) hydrogenated castor oil; HCO-60, POE(60)
 48 hydrogenated castor oil; HPLC, high-performance liquid chromatography; HL, hexyl laurate; HLB,
 49 hydrophilic–lipophilic balance; IPC, isopropyl caprylate; IPD, isopropyl decanoate; IPM, isopropyl
 50 myristate; IPP, isopropyl palmitate; PG, propylene glycol; POE, polyoxyethylene; OA, oleyl
 51 alcohol; OD, 2-octyldodecanol; OO, oleyl oleate; T_{max} , maximum drug concentration time.

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54 1. Introduction

55 Atopic dermatitis (AD) is a potentially debilitating, chronic skin disorder; it has a
56 significant impact on the human population worldwide. Conventional therapy for AD involves the
57 use of topical corticosteroids; however, steroid-associated side effects have limited their use (Alaiti
58 et al., 1998; Reitamo et al., 2002; Rothe and Grant-Kels, 1996). Tacrolimus (FK506), a
59 23-membered macrolide lactone (molecular weight, 803.5 Da) novel immunosuppressant, is
60 therefore of great interest. FK506 suppresses inflammation by targeting T lymphocytes (Nakagawa
61 et al., 1994), resulting in inhibition of the enzyme calcineurin phosphatase, thereby modifying
62 T-cell function (Fruman et al., 1992). This immunosuppressive activity has been reported to be
63 30–100-times higher than that of cyclosporine in vitro, and 10–20-times higher in vivo (Honbo et
64 al., 1987). An ointment formulation of FK506 is available on the international market, as Protopic[®],
65 for the treatment of AD.

66 Although it is effective, the characteristic greasy and messy nature of the ointment vehicle
67 leads to undesirable stickiness and unpleasant feelings by patients when applied to the skin (Drake
68 et al., 1996; Ference and Last, 2009). The development of a new topical dosage formulation, such
69 as a cream, that overcame these disadvantages would improve the quality of life for patients. In
70 addition, the Food and Drug Administration's medication guide for Protopic[®] (FDA, 2011)
71 describes possible side effects such as skin irritation (stinging, burning, or itching) and other side
72 effects at the application site, which are mainly associated with the drug molecule. The
73 conventional vehicle does not ensure adequate topical delivery of the drug to the target site.

74 Furthermore, FK506 exhibits formulation-related issues such as poor solubility
75 ($4\text{--}12\text{ }\mu\text{g mL}^{-1}$) as a result of its physicochemical properties such as high molecular weight,
76 macrolide structure, and lipophilicity ($\log P$, 6.09; Bos, 2003; Hane et al., 1992; Tamura et al.,
77 2002). Compounds with molecular weights higher than 500 Da have difficulty penetrating skin
78 (Bos and Meinardi, 2000). It is therefore a challenge to overcome the low penetration rate of
79 FK506 through the skin, while restoring the skin barrier properties and, at the same time, achieving
80 site-specific delivery to enhance therapeutic efficacy with minimum toxic effects.

81 The aim of the present study was to develop an FK506 cream formulation to overcome the
82 above-mentioned disadvantages. First, we screened several types of solvents that are widely used in
83 the development of topical preparations, and examined their effects on the solubility and stability of
84 FK506. We then evaluated the transdermal absorption of FK506 into rat skin from solutions,
85 emulsions, and cream formulations.

86

86 2. Materials and methods

87 2.1. Materials

88 FK506 was manufactured by the Fujisawa Pharmaceuticals Co., Ltd. (currently Astellas
89 Pharma Inc., Tokyo, Japan). Diisopropyl adipate (DIA), isopropyl myristate (IPM), and isopropyl
90 palmitate (IPP) were obtained from Croda Japan KK (Tokyo, Japan). Oleyl alcohol (OA), decyl
91 oleate (DO), and oleyl oleate (OO) were obtained from the NOF Corporation (Tokyo, Japan).
92 2-Octyldodecanol (OD), hexyl laurate (HL), cetyl caprylate (CC) were obtained from Henkel Japan
93 Ltd. (Tokyo, Japan). Diethyl sebacate (DES) and all surfactants were obtained from the Nikko
94 Chemicals Co., Ltd. (Tokyo, Japan). Propylene glycol (PG), isopropyl caprylate (IPC), and
95 isopropyl decanoate (IPD) were obtained from the Tokyo Chemical Industry Co., Ltd. (Tokyo,
96 Japan). Carbopol 940 was obtained from the Lubrizol Corporation (Cleveland, OH, USA). All of
97 the reagents used were of the highest grade available from commercial sources.

98

99 2.2. Determination of apparent FK506 solubility

100 An excess of FK506 was added to each solvent (2 mL) at room temperature, and the
101 mixtures were agitated for 1 week. An aliquot of the supernatant was withdrawn and immediately
102 filtered through a membrane filter (0.22 μm , Millipore SLGV 013 SL). The filtered solution
103 (500 μL) was volumetrically diluted with a mixture of tetrahydrofuran (9 mL) and an internal
104 standard solution (1 mL, 0.15 mg mL^{-1} *n*-heptyl *p*-hydroxybenzoate in ethanol solution), and 20 μL
105 of the diluted sample solution were injected into a high-performance liquid chromatography

(HPLC) system to determine the FK506 concentration. The HPLC system consisted of a pump (model 510), a WISP automatic sampler (model 712), and an ultraviolet detector (model 481) from Waters (Milford, MA, USA), and a TSKgel ODS-80TM column (150×4.6 mm, $5\mu\text{m}$) from the Tosoh Co., Ltd. (Tokyo, Japan). The mobile phase consisted of a mixture of water, tetrahydrofuran, and 2-propanol (volume ratio 5:2:2), and the flow rate was 0.6 mL min^{-1} . The wavelength of the detector was set at 220 nm.

2.3. *In vivo* transdermal absorption tests

All of the procedures used in the *in vivo* study were conducted in accordance with the guidelines approved by the Institutional Animal Care and Ethical Committee of the Astellas Pharma Inc.. The abdominal hair of male Sprague–Dawley rats (7 weeks) was removed using an electrical shaver and a hair removal cream (EBA cream; Mitsubishi Tanabe Pharma Corporation, Osaka, Japan). Next day, the skin condition of the rats was checked, and rats with good skin condition, without any scabs, were selected for *in vivo* experiments. The selected rats were fixed, using a restraining device, in the face-up position, without anesthesia, and $50 \mu\text{L}$ of 0.1% w/w FK506 solution were applied to the abdominal region ($2.5 \text{ cm} \times 4.0 \text{ cm}$). At specified times, 0.3 mL of blood were collected from the subclavian vein and stored at -20°C until used. The FK506 plasma concentration was analyzed by enzyme immunoassay using mouse anti-FK506 monoclonal antibodies (FKmAb) and FK506-conjugated peroxidase (FK-POD), as described in a previous study (Kobayashi et al., 1991). Briefly, the stored blood was centrifuged, deproteinized with

methanol, and centrifuged again. The supernatant plasma was dissolved in FK-POD solution and added to a microtiter plate well, which was previously coated with FKmAb, to determine competitive binding of FK-506 and FK-POD with FKmAb. The POD activity was measured using *o*-phenylenediamine and hydrogen peroxide as cosubstrates. The reaction was stopped by addition of sulfuric acid (2 mol L⁻¹ in water), and the optical density (490 nm) was measured using a microplate reader (CS-9300PC; Shimadzu, Kyoto, Japan). The FK506 content was determined by comparison with a standard curve. An FK506 calibration curve was prepared in each assay in the concentration range 0–40 ng mL⁻¹. The correlation coefficient was always more than 0.99, and the limit of quantification was 0.1 ng mL⁻¹.

Pharmacokinetic parameters were calculated by model-independent analysis as described previously (Iwasaki et al., 1995). Maximum plasma concentration ($C_{\max}$), maximum drug concentration time ($T_{\max}$) and area under plasma-concentration–time curve from time 0 to 24 h of measurable concentration (AUC_{0-24}) were estimated.

2.4. Stability study of FK506 solutions

FK506 solutions (0.1% w/w) in ampoules, under nitrogen, were incubated at 70 °C for 9 d or at 50 °C for three months. The residual amounts of FK506 in the solutions were determined by HPLC.

2.5. Emulsifying index tests

Each solvent (1.46 mL) and 0.25 g of surfactant (0.25 g) were placed in a 10 mL glass tube at 65 °C; 3.5 mL of deionized water at 65 °C were added and the mixture was agitated to induce emulsification. The obtained emulsified solutions were then centrifuged at 2500 rpm for 20 min; the emulsifying index was defined as the ratio of the height of the milky solution to that of the total solution.

2.6. Preparation of 0.1% w/w FK506 emulsions

Mixtures of solvent (25 g) and surfactant (5 g) were incubated at 75 °C, and then mixed with 0.1 g of FK506. The mixtures were homogenized with purified water (69.9 g), using a T. K. Auto Homo Mixer (AM-HV-M; Primix Corporation, Osaka, Japan), at 5000 rpm for 10 min to prepare FK506 emulsions.

2.7. Preparation of 0.1% w/w FK506 cream

Cream formulations were prepared by the semi-solidification method using carbopol 940. Carbopol 940 (2.5 g) was soaked in 85.6 mL of purified water at room temperature, and 3.0 mL of sodium hydroxide (1 mol L⁻¹ in water) were then added to obtain a carbopol gel. FK506 (0.25 g) and a surfactant (12.5 g) were dissolved in a cream-based solvent (62.5 g) at 75 °C. Purified water (83.7 g) at 75 °C was then added to the solvent and the mixture was emulsified. The obtained emulsion was mixed with carbopol gel and the mixture was gently stirred using a rubber spatula. The viscosity of the emulsion increased as a result of carbopol soaking, and the FK506 cream was

166 obtained.

167

168 *2.8. pH measurement*

169 pH values in cream were measured by semiconductor sensor using pHBOY-P2

170 (Shindengen Electric Manufacturing Co. Ltd., Tokyo, Japan).

171

172 *2.9. Statistics*

173 Statistical analyses were performed using Student's t-test; a probability value of $p < 0.05$

174 was considered to indicate statistical significance.

175

3. Results and discussion

3.1. Screening to determine optimal solvent for preparing oil/water cream formulation

In order to prepare oil/water (O/W) cream formulations, solvent screening was necessary. The optimal solvent should have the following characteristics: i) it has previously been used as a clinical topical additive; ii) it can dissolve FK506 to a concentration as high as that of 0.1% Protopic ointment; iii) it forms FK506 solutions with long-term stability; and iv) it gives transdermal absorption similar or equal to that of Protopic ointment. In the present study, based on information in the Japanese Pharmaceutical Excipients Directory (JPED) 1996 guidelines (International Pharmaceutical Excipients Council Japan, 1996) and the Inactive Ingredients Guide (IIG; FDA, 2013) on their use as additives for clinical topical products, 11 solvents were chosen (Table 1); the FK506 solubility, absorptivity in rats, and long-term stability in solution were examined.

3.1.1. FK506 solubility

The FK506 solubility in various solvents was determined at 25 °C. The solubility of FK506 was highest in PG; the value was 19% (Table 1). The order of the FK506 solubilities was $PG > DIA > DES > OA > OD > IPM > IPP = HL$ (Table 1). If the upper limit of the solvent ratio is set at 50% when preparing a 0.1% FK506 O/W-type cream formulation, an FK506 solubility of more than 0.2% is necessary, suggesting that the solvents up to IPP and HL, in which FK506 showed solubilities of 0.2%, are possible candidates. IPP and HL are both listed as official additives

in JPED, and IPP is listed as an official additive in compendiums such as the United States Pharmacopeia/National Formulary. PG, DIA, DES, OA, OD, IPM, and IPP were therefore chosen as candidates for dissolving FK506.

3.1.2. Transdermal absorption of FK506 in solvent by rat epidermis

Prior to examination of FK506 absorption by rat epidermis, we determined whether hair removal using EBA cream was necessary. Fig. 1 shows the time courses for FK506 in rat plasma following transdermal administration of 0.1% Protopic ointment to rat epidermis, with or without treatment with EBA cream. The pharmacokinetic parameters are summarized in Table 2. FK506 was highly absorbed and provided $C_{\max}$ values of 1.3 ng mL^{-1} in the rat treated with EBA cream. In contrast, FK506 was slowly and slightly absorbed by the rat epidermis that was not treated with EBA cream; the $C_{\max}$ value was 0.4 ng mL^{-1} , which was lower than that for the epidermis treated with EBA cream. The AUC_{0-24} values for the rat epidermis with and without EBA cream were 22.9 and 5.8 ng h mL^{-1} , respectively, indicating that 3.9-times more FK506 was absorbed by the rat epidermis with EBA cream than by that without EBA cream. This difference might be explained by deterioration of the barrier function of rat skin, caused by the chemical depilator in the EBA cream, resulting in an increase in FK506 absorption. Generally, the stratum corneum of the skin of AD patients has a depressed cutaneous barrier function (Yoshiike et al., 1993). It is easy to evaluate the screening of transdermal absorption of FK506 under depressed cutaneous barrier conditions if FK506 is highly absorbed. Furthermore, the $C_{\max}$ values were too low, e.g., 0.4 ng mL^{-1} , when the

formulation was applied to rat epidermis untreated with EBA cream; this value is close to the detection limit. Accordingly, EBA cream was used in subsequent experiments. The pharmacokinetic parameters of 0.1% Protopic ointment with EBA cream were used as positive controls in further studies.

Fig. 2 shows the time courses for FK506 in rat plasma following transdermal administration of 0.1% FK506 solutions prepared using seven different solvents, as described above, to rat epidermis treated with EBA cream. The pharmacokinetic parameters are summarized in Table 3. The AUC_{0-24} values for the DES, IPM, and DIA solutions were 31.2, 26.9, and 22.9 ng h mL⁻¹, respectively, indicating higher or almost similar values than that for 0.1% Protopic ointment (22.9 ng h mL⁻¹). In addition, compared with the AUC_{0-24} values for IPM and OD, which gave 0.5% and 0.6% FK506 solubilities (Table 1), the value for OD was 0.9 ng h mL⁻¹ and FK506 was not absorbed at all. The value for IPM was 26.9 ng h mL⁻¹, and 28-times more FK506 was absorbed. In addition, FK506 in OA, which gave FK506 solubility of 2.7%, was not absorbed at all. The FK506 solubilities in DES and DIA were 5% and 6%, respectively, and 100-times and 73-times more FK506, respectively, were absorbed. There was no correlation between the AUC_{0-24} value and molecular weight or solubility, as shown in Fig. 3(a) and (b). Classification of the solvents as fatty acid esters or alcohols showed that the use of fatty acid esters as solvents tended to produce better transdermal absorption than the use of alcohols did. Ozawa et al. (1988) and Wato et al. (2012) have reported that the percutaneous absorption of some drug molecules was enhanced by some fatty alcohols and fatty acid esters. Similar results were obtained with FK506 for

transdermal absorption with fatty acid esters; fatty acid esters were considered to be essential components of the solvents.

In addition, because the fatty acid esters IPM (C14) and IPP (C16) exhibited high transdermal absorption properties, we investigated the transdermal absorption properties of IPC and IPD (C8 and C10 isopropyl esters, respectively), which have different fatty acid chain lengths from IPP and IPM. Aungst et al. (1986) reported that maximum skin penetration enhancement was observed with C12 saturated hydrophobic groups, so isopropyl esters were screened. The results showed that the AUC_{0-24} for C10 IPD was $22.4 \text{ ng h mL}^{-1}$, almost the same as the AUC_{0-24} for IPM, and that C8 IPC had a lower AUC_{0-24} of 6.5 ng h mL^{-1} and a relatively short $T_{\max}$ value of 3 h (Table 3). The transdermal absorption properties up to 1 h after application tended to increase more rapidly as the fatty acid chain length decreased (Fig. 4), but it can be seen that more rapid elimination from rat plasma also tended to occur as the fatty acid chain length decreased. With regard to methods for using external preparations, because usage methods in which one or two applications per day are repeated are commonplace, it was thought that immediate release preparations such as IPC and preparations with sustained absorption over periods of 24 h or longer, such as IPP, were not needed. Among the fatty acid esters with isopropyl groups, it was therefore thought that those with fatty acid chains of length 10–14 carbon atoms, such as IPD and IPM, would be useful in the development of external preparations. However, because usage statistics for IPD are not given in the JPED or in the FDA's IIG, IPM was selected as a suitable solvent (base) for transdermal preparations of FK506.

3.1.3. Stability of FK506 in solvents

Solutions were prepared by dissolving 0.1% FK506 in various oil-phase bases and evaluated in terms of the proportion (%) of FK506 remaining under elevated temperature conditions (Table 4). The proportions remaining for DES (70 °C, 9 d), DIA (50 °C, 3 months), IPM (70 °C, 9 d), and PG (50 °C, 3 months) were 80% or higher. However, for OD (70 °C, 9 d), IPD (70 °C, 9 d), and IPC (70 °C, 9 d), the proportions remaining were 58.7%, 50.4%, and 1.3%, indicating significant decomposition of FK506. DES, DIA, IPM, and PG exhibited high stabilities.

3.1.4. Solvent (base) selection

DES, DIA, and IPM are candidates for cream bases, in view of their use in pharmaceutical products, and fundamental research on the solubilities, transdermal absorption properties, and solution stabilities of FK506 in these solvents. IPM was thought to be a strong candidate because it exhibits a high AUC up to 24 h and a low reduction in concentration in blood between 8 h and 24 h, showing sustained presence in plasma. In addition, because it has occasionally been reported that DIA causes skin irritation, it was thought that DES, which causes relatively low skin irritation (International Pharmaceutical Excipients Council Japan, Safety Data), was suitable. Based on the above screening results, IPM and DES were selected as cream bases and subjected to additional investigations in creams.

3.2. Transdermal absorption from emulsions in rats

3.2.1. Emulsion formulation

When considering improvements in formulations by carrying out cream formulation studies, having fewer formulated ingredients facilitates investigations. In order to be able to produce a cream using only one type of surfactant, surfactants that exhibit excellent emulsification and dispersion of IPM or DES were screened by investigating their emulsifying indices (Table 5). The surfactants selected were polyoxyethylene (POE) alkyl esters, because of their range of physicochemical properties and the expectation that they would act as enhancers (Walters et al., 1980, 1988). The screening results for the emulsification and dispersion of IPM showed that HCO-10, BC-2, BS-4, BC-5.5, BO-7, and BC-7, which achieved emulsifying indices of 100, were suitable candidates as surfactants for emulsifying IPM. The emulsified product of BC-2 formed a gel and was therefore eliminated from the screening; studies of transdermal absorption in rats from IPM emulsions obtained using the surfactants were carried out. In addition, the surfactants BB-5 and BB-10 exhibited excellent emulsification and dispersion of DES, achieving emulsifying indices of 100, so studies of transdermal absorption in rats from DES emulsions obtained using these two surfactants were carried out.

3.2.2. Transdermal absorption from emulsions in rats

The results of the investigations into transdermal absorption from emulsions showed that in the case of IPM emulsions, absorption of emulsions obtained using BC-5.5 was the most

enhanced, but that in the case of emulsions obtained using BS-4 or HCO-10, the AUC_{0-24} values were approximately 0.80-fold and 0.78-fold those of the respective solvents, meaning that absorption from the emulsion was reduced (Fig. 5a and Table 6). DES emulsions obtained using two surfactants exhibited significantly reduced absorption (Fig. 5b and Table 6). It is thought that surfactants generally facilitate absorption (Barry, 1987), but in the case of DES emulsions, the surfactants greatly inhibit movement of drugs into the skin.

3.2.3. Surfactant selection

Four surfactants (POE alkyl ethers) were used to prepare IPM emulsions; all four surfactants had POE chain lengths of between 4 and 7. In terms of absorption facilitation, it was assumed that the differences among the alkyl groups, not among the POE chain lengths, would affect the absorption properties. The alkyl group in BC-5.5 is a cetyl group, and BC-5.5 exhibited the highest transdermal absorption. It was therefore thought that cetyl groups are advantageous in terms of transdermal absorption. Four cetyl ether surfactants with different POE chain lengths, between 2 and 10 (BC-2, BC-5.5, BC-7, and BC-10TX), were emulsified, and the transdermal absorption from IPM was then investigated in order to optimize the POE chain length. In addition, in order to eliminate, as far as possible, changes in transdermal absorption properties caused by different dosage form, creams of emulsions obtained by gelling the outer aqueous phase with an aqueous polymer were evaluated with respect to transdermal absorption properties in rats (Table 7). The results showed that the absorptions achieved by BC-7 and BC-5.5 were higher than those

achieved by the other two surfactants (average values of $69.3 \text{ ng h mL}^{-1}$ and $61.1 \text{ ng h mL}^{-1}$,
 respectively), and that a POE chain length of approximately 5.5 to 7 produced the highest AUC_{0-24} .
 Moreover, when the dosage form was changed from an emulsion to a cream, BC-5.5 had an
 AUC_{0-24} value approximately 0.58-times lower than that of the emulsion, but the BC-5.5 cream still
 exhibited an AUC_{0-24} value that was 2.7-times higher than that of an ointment. There were no
 significant differences between the AUC_{0-24} values for an emulsion and a cream in the case of
 BC-7, but the $T_{\max}$ was prolonged for the cream. A prolonged $T_{\max}$ was also seen with BC-5.5, and
 it was thought that the fluidity of FK506 in the base was reduced, as a result of the preparation
 becoming semi-solid when it was changed from an emulsion to a cream. It was therefore assumed
 that the prolonging of $T_{\max}$ was caused by inhibition of FK506 release from the base. In addition,
 with regard to the prolonged $T_{\max}$, caused by reduced fluidity in the base, it is thought that the
 AUC_{0-24} for the BC-5.5 cream is lower than that for the emulsion because of a delay in absorption
 in the initial 24 h, which is reflected in the AUC_{0-24} value. Moreover, the BC-7 and BC-5.5 cream
 preparations exhibited high AUCs, but it was found that BC-7 continued to exhibit transdermal
 absorption at 24 h (Fig. 6). Because external preparations are applied at least once or twice per day,
 sustained absorption for 24 h or longer is not essential, and it is therefore concluded that the
 optimal POE cetyl ether surfactant has a POE chain length of 5.5 (BC-5.5).

3.3. Controlling transdermal absorption from cream containing two solvents in rats

It was thought that transdermal absorption of FK506 from creams could be controlled if

the characteristics of IPM creams, which exhibited the best transdermal absorption, and those of DES creams, which did not exhibit transdermal absorption, were used, *i.e.*, if a mixed solvent of IPM and DES was used. The transdermal absorption properties from creams with solvents blended in various proportions were therefore evaluated in rats. The surfactant used was BC-5.5, which was confirmed to facilitate absorption in IPM creams. As shown in Fig. 7 and Table 8, transdermal absorption from DES creams was observed in rats when BC-5.5 was used, and the results showed that BC-5.5 is a surfactant that acts as a transdermal absorption facilitator. Furthermore, when a mixture of IPM and DES was used, the AUC_{0-24} value increased as the proportion of IPM increased; an IPM:DES ratio of 75:25 gave an AUC_{0-24} value that was approximately 5.4-times higher than that obtained with a cream containing only DES, and approximately 1.4-times higher than that obtained with a cream containing only IPM. The T_{max} value of the DES cream was 24 h, which suggested that absorption continued beyond 24 h (see Fig. 7). It is therefore thought that a mixed IPM/DES cream is a preparation in which the sustained absorption properties of a DES cream are imparted to an IPM cream, which has a T_{max} value of 13.3 h, that is, a mixed IPM/DES cream functions as a cream that maintains the high C_{max} value of an IPM cream, while having an extended T_{max} value. These results suggest that by preparing a cream in which the solvents IPM and DES are mixed in specific proportions, it is possible to obtain an FK506 cream with a specific AUC_{0-24} value. This in turn suggests that it is possible to develop an external preparation with a controlled therapeutic range.

3.4. Stability of FK506 and pH values in cream containing two solvents

The accelerated and long-term stability of FK506 in creams prepared by two solvents system are shown in Table 9. The proportion (%) of FK506 remaining in all formulations were 85% or higher under both incubating conditions, except for IPM:DES=100:0 (70°C, 9 d), and the FK506 remaining was increased with an increase of proportion of DES, indicating that DES might be involved in the high stability of cream. In addition, as for the pH values, no significant changes were observed in all formulation. Taken together, FK506 was stable in these cream formulations. In the near future, further studies using Franz diffusion cells *in vitro* or determining the accumulation of FK506 in rat skin *in vivo* should be required to develop novel FK506 cream formulation.

4. Conclusion

Screening of solvents that had previously been used as clinical topical additives showed that the best solvents for FK506 were DIA, DES, IPM, and IPP among esters, and PG, OA, and OD among alcohols. When FK506 solutions prepared using these solvents were percutaneously administered, the AUC₀₋₂₄ values for DES, IPM, and DIA were 31.2, 26.9, and 22.9 ng h mL⁻¹, respectively, indicating higher or similar values than that for 0.1% Protopic ointment (22.9 ng h mL⁻¹). In contrast, the AUC₀₋₂₄ values for PG, OA, and OD were quite low, indicating that these solvents did not enhance absorption. Solvent selection was considered in terms of FK506 solubility, transdermal absorption in solution, FK506 stability under incubating conditions, and the

risk of skin irritation. DES and IPM were chosen for preparing emulsions. When the emulsions were percutaneously administrated, IPM emulsions showed AUC_{0-24} values that were 4.6-times higher than those of solutions; however, DES emulsions did not show high transdermal absorptions. To develop a cream formulation, surfactants were screened for transdermal absorption. BC-5.5 facilitated FK506 transdermal absorption. The cream formulation showed high absorption when BC-5.5 was used as the surfactant. When the mixing ratio of IPM and DES was changed, high transdermal absorption was observed as the IPM content increased. In conclusion, we have successfully developed an FK506 cream formulation with a controllable transdermal absorption rate through manipulation of the IPM:DES ratio.

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420 (–)-1*R*,9*S*,12*S*,13*R*,14*S*,17*R*,18*E*,21*S*,23*S*,24*R*,25*S*,27*R*)-17-allyl-1,14-dihydroxy-12-[(*E*)-2
421 -[(1*R*,3*R*,4*R*)-4-hydroxy-3-methoxycyclohexyl]-1-methylvinyl]-23,25-dimethoxy-13,19,21,
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- 459

459 **Table captions**

460 **Table 1.** Molecular weights of selected solvents and FK506 solubilities in these solvents ($n = 2$).

461 **Table 2.** Transdermal absorption of 0.1% Protopic ointment from rat skin, with or without EBA
462 cream (mean $\pm$ standard error, $n = 3$).

463 **Table 3.** Transdermal absorption of FK506 solution from rat skin (mean $\pm$ standard error, $n = 3$).

464 **Table 4.** FK506 stability in solution under incubating conditions ($n = 2$).

465 **Table 5.** Physicochemical properties of selected surfactants and emulsifying indices for IPM and
466 DES.

467 **Table 6.** Transdermal absorption of FK506 emulsion from rat skin (mean $\pm$ standard error, $n = 3$).

468 **Table 7.** Transdermal absorption of FK506 cream from rat skin (mean $\pm$ standard error, $n = 3$).

469 **Table 8.** Transdermal absorption of FK506 cream with IPM/DES binary solvent system from rat
470 skin (mean $\pm$ standard error, $n = 3$).

471 **Table 9.** FK506 stability in cream and pH values of cream using IPM/DES binary solvent system
472 under various incubating conditions ($n=2$).

473 **Table 1**

Solvent		Molecular weight (Da)	FK506 solubility (%, w/w)
Alcohols	PG	342.56	19
	OA	268.48	2.7
	OD	298.55	0.6
Esters	DIA	230.30	6.0
	DES	258.35	5.0
	IPM	270.45	0.5
	IPP	298.50	0.2
	HL	284.48	0.2
	CC	368.64	0.1
	DO	422.73	0.1
	OO	532.92	<0.05

474

475 **Table 2**

Pretreatment	$C_{\max}$ (ng mL ⁻¹)	$T_{\max}$ (h)	AUC ₀₋₂₄ (ng h mL ⁻¹)
With EBA cream	1.3 ± 0.4	7.0 ± 1.0	22.9 ± 5.4
Without EBA cream	0.4 ± 0.1	24.0	5.8 ± 2.1 ^a

476 ^a $p < 0.05$.

Table 3

Solvent		$C_{\max}$ (ng mL ⁻¹)	$T_{\max}$ (h)	AUC ₀₋₂₄ (ng h mL ⁻¹)
Alcohols	PG	0.3 ± 0.03	6.0 ± 1.0	2.1 ± 0.1 ^a
	OA	0.04 ± 0.04	24.0	0.3 ± 0.3 ^a
	OD	0.2 ± 0.1	12.5 ± 11.5	0.9 ± 0.7 ^a
Esters	DIA	1.6 ± 0.1	3.7 ± 0.7	22.9 ± 0.7
	DES	1.8 ± 0.6	8.0	31.2 ± 9.2
	IPM	1.7 ± 0.5	17.7 ± 6.3	26.9 ± 7.1
	IPP	0.9 ± 0.2	24.0	13.1 ± 1.2
	IPC	0.4	3.0	6.5 ± 0.1 ^a
	IPD	1.6 ± 0.8	7.0 ± 1.0	22.4 ± 10.0

^a $p < 0.05$, from 0.1% Protopic ointment, with EBA cream treatment.

Table 4

Solvent	Storage conditions	FK506 remaining after incubation
PG	50 °C, 3 months	83.6%
OD	70 °C, 9 d	58.7%
DIA	50 °C, 3 months	95.7%
DES	70 °C, 9 d	95.6%
IPM	70 °C, 9 d	88.6%
IPC	70 °C, 9 d	1.3%
IPD	70 °C, 9 d	50.4%

481 **Table 5**

Surfactant	Abbreviation	Hydrophilic– lipophilic balance	Emulsifying index (%)	
			IPM	DES
POE(5) hydrogenated castor oil	HCO-5	6	3	26
POE(10) hydrogenated castor oil	HCO-10	6.5	100	26
POE(5) behenyl ether	BB-5	7	32	100
POE(2) oleyl ether	BO-2	7.5	34	68
POE(2) cetyl ether	BC-2	8	100	29
POE(2) stearyl ether	BS-2	8	39	66
POE(4) stearyl ether	BS-4	9	100	87
POE(2) lauryl ether	BL-2	9.5	39	29
POE(10) behenyl ether	BB-10	10	18	100
POE(5.5) cetyl ether	BC-5.5	10.5	100	47
POE(7) oleyl ether	BO-7	10.5	100	32
POE(20) hydrogenated castor oil	HCO-20	10.5	90	26
POE(30) hydrogenated castor oil	HCO-30	11	66	47
POE(4.2) lauryl ether	BL-4.2	11.5	39	29
POE(7) cetyl ether	BC-7	11.5	100	26
POE(40) hydrogenated castor oil	HCO-40	12.5	79	79
POE(10) cetyl ether	BC-10TX	13.5	24	26
POE(50) hydrogenated castor oil	HCO-50	13.5	47	79
POE(10) oleyl ether	BO-10TX	14	26	24
POE(60) hydrogenated castor oil	HCO-60	14	34	89
POE(9) lauryl ether	BL-9EX	14.5	34	24
POE(80) hydrogenated castor oil	HCO-80	15	29	53
POE(15) cetyl ether	BC-15TX	15.5	26	26
POE(15) oleyl ether	BO-15TX	16	29	21
POE(100) hydrogenated castor oil	HCO-100	16.5	36	32
POE(20) cetyl ether	BC-20TX	17	26	26
POE(20) oleyl ether	BO-20	17	21	24
POE(23) cetyl ether	BC-23	18	26	26
POE(20) stearyl ether	BS-20	18	21	24
POE(50) oleyl ether	BO-50	18	39	24
POE(25) cetyl ether	BC-25TX	18.5	26	26
POE(21) lauryl ether	BL-21	19	39	24
POE(25) lauryl ether	BL-25	19.5	39	24
POE(30) cetyl ether	BC-30TX	19.5	24	24
POE(40) cetyl ether	BC-40TX	20	26	24

Table 6

Oil phase in emulsion	$C_{\max}$ (ng mL ⁻¹)	$T_{\max}$ (h)	AUC ₀₋₂₄ (ng h mL ⁻¹)
IPM/HCO-10	1.4 ± 0.4	24.0	21.0 ± 4.1
IPM/BS-4	1.2 ± 0.2	18.7 ± 5.3	21.5 ± 3.4
IPM/BC-5.5	6.3 ± 0.6	8.0	105.9 ± 9.6 ^a
IPM/BO-7	3.1 ± 1.0	13.3 ± 5.3	58.6 ± 19.1
IPM/BC-7	3.2 ± 0.5	24.0	53.8 ± 12.6
DES/BB-5	n.d. ^b	n.d. ^b	n.d. ^b
DES/BB-10	0.05 ± 0.05	5.0	0.1 ± 0.1 ^a

^a $p < 0.05$, from 0.1% Protopic ointment, with EBA cream treatment.

^bnot determined.

Table 7

Oil phase in cream	$C_{\max}$ (ng mL ⁻¹)	$T_{\max}$ (h)	AUC ₀₋₂₄ (ng h mL ⁻¹)
IPM/BC-2	1.2 ± 0.6	24.0	16.8 ± 9.0
IPM/BC-5.5	3.1 ± 0.6	13.3 ± 5.3	61.1 ± 12.2 ^a
IPM/BC-7	4.6 ± 1.0	24.0	69.3 ± 9.2 ^a
IPM/BC-10TX	3.0 ± 0.6	24.0	34.8 ± 6.7

^a $p < 0.05$, from 0.1% Protopic ointment, with EBA cream treatment.

Table 8

Oil phase in cream	$C_{\max}$ (ng mL ⁻¹)	$T_{\max}$ (h)	AUC ₀₋₂₄ (ng h mL ⁻¹)
IPM:DES = 100:0	3.1 ± 0.6	13.3 ± 5.3	61.1 ± 12.2 ^a
IPM:DES = 75:25	5.5 ± 0.6	24.0	87.4 ± 4.7 ^a
IPM:DES = 50:50	3.5 ± 0.5	18.7 ± 5.3	57.6 ± 3.0 ^a
IPM:DES = 27:75	3.1 ± 0.3	24.0	35.2 ± 5.4
IPM:DES = 0:100	1.5	24.0	16.1 ± 0.6

^a $p < 0.05$, from 0.1% Protopic ointment, with EBA cream treatment.

493 **Table 9**

494

Oil phase in cream	FK506 remaining after incubation		pH value		
	70 °C, 9 d	40 °C, 1 month	Initial	70 °C, 9 d	40 °C, 1 month
IPM:DES = 100:0	75.7%	85.9%	3.8	4.0	4.0
IPM:DES = 75:25	88.3%	92.9%	4.2	4.1	4.1
IPM:DES = 50:50	85.5%	94.4%	4.3	4.2	4.1
IPM:DES = 27:75	94.0%	94.9%	4.3	4.3	4.1
IPM:DES = 0:100	91.7%	102.8%	4.2	3.9	4.2

Figure captions

Fig. 1. Time course of plasma concentration after administration to rat skin of 0.1% Protopic ointment.

Open symbols represent treated with EBA cream, and closed symbols represent treated without EBA cream. Each value represents the mean $\pm$ standard error of three experimental trials.

Fig. 2. Time course of plasma concentration after administration to rat skin of 0.1% FK506 solution.

Closed symbols indicate fatty acid esters and open symbols indicate fatty alcohols.

IPM: isopropyl myristate, DES: diethyl sebacate, DIA: diisopropyl adipate, IPP: isopropyl palmitate, PG: propylene glycol, OA: oleyl alcohol, OD; 2-octyldodecanol. Each value represents the mean $\pm$ standard error of three experimental trials.

Fig. 3. Relationship between AUC₀₋₂₄ and solubility (a) or molecular weight (b).

Fatty acid esters and fatty alcohols are represented by closed and open symbols, respectively.

Fig. 4. Time course of plasma concentration after administration to rat skin of FK506 solution prepared with isopropyl esters.

IPM: isopropyl myristate, IPD: isopropyl decanoate, IPC: isopropyl caprylate, IPP: isopropyl palmitate. Each value represents the mean $\pm$ standard error of three experimental trials.

514

515 **Fig. 5. Time course of plasma concentration after administration to rat skin of FK506**516 **emulsion and cream in IPM system (a) and DES system (b).**

517 IPM: isopropyl myristate, DES: diethyl sebacate, BC-5.5: POE(5.5) cetyl ether, BC-7: POE(7)

518 cetyl ether, BS-4: POE(4) stearyl ether, BO-7: POE(7) oleyl ether, HCO-10: POE(10)

519 hydrogenated castor oil, BB-5: POE(5) behenyl ether, BB-10: POE(10) behenyl ether. Each value

520 represents the mean $\pm$ standard error of three experimental trials.

521

522 **Fig. 6. Time course of plasma concentration after administration to rat skin of FK506 cream**523 **prepared with POE cetyl esters.**

524 IPM: isopropyl myristate; BC-2: POE(2) cetyl ether; BC-5.5: POE(5.5) cetyl ether; BC-7: POE(7)

525 cetyl ether; BC-10TX: POE(10) cetyl ether. Each value represents the mean $\pm$ standard error of

526 three experimental trials.

527

528 **Fig. 7. Time course of plasma concentration after administration to rat skin of FK506 cream**529 **with various IPM:DES ratios.**530 IPM: isopropyl myristate, DES: diethyl sebacate. Each value represents the mean $\pm$ standard error

531 of three experimental trials.

532

Graphical Abstract

Yamanaka *et al.*

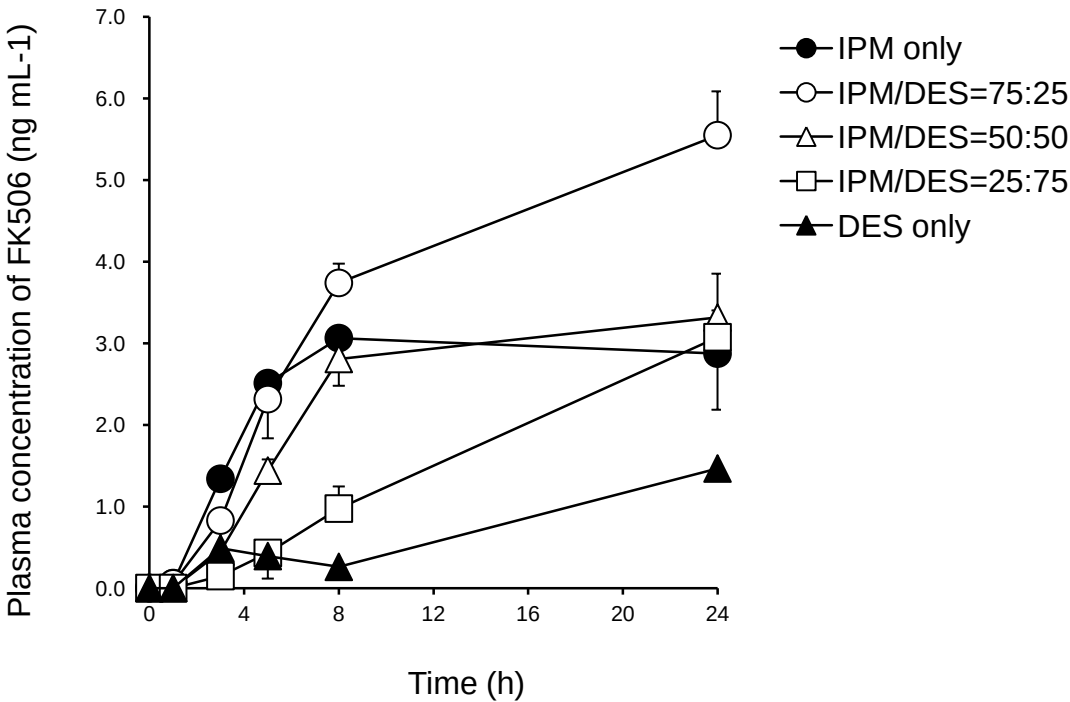


Fig. 1.

Yamanaka *et al.*

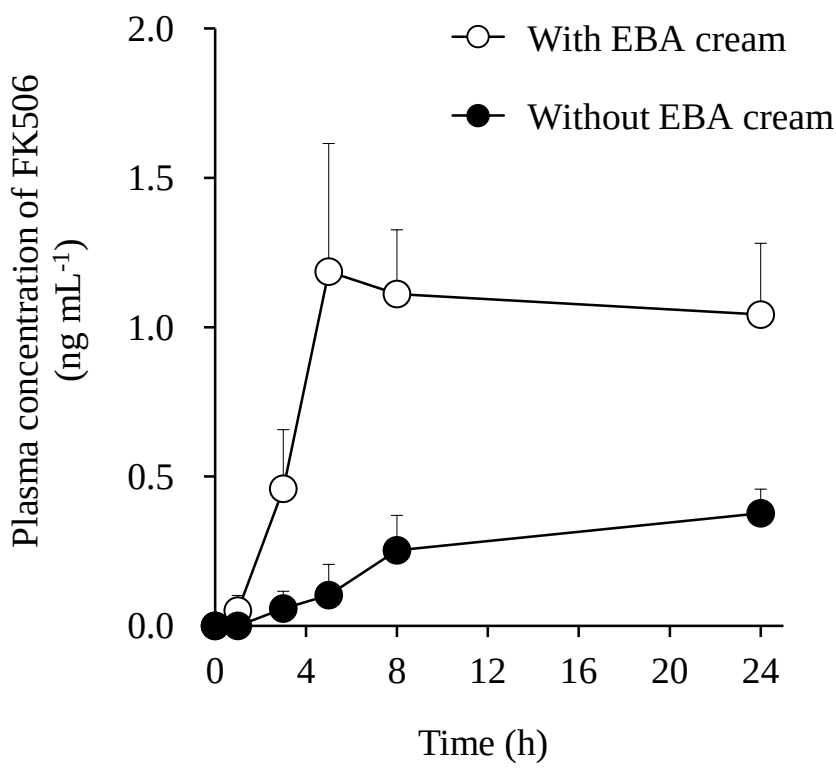


Fig. 2.

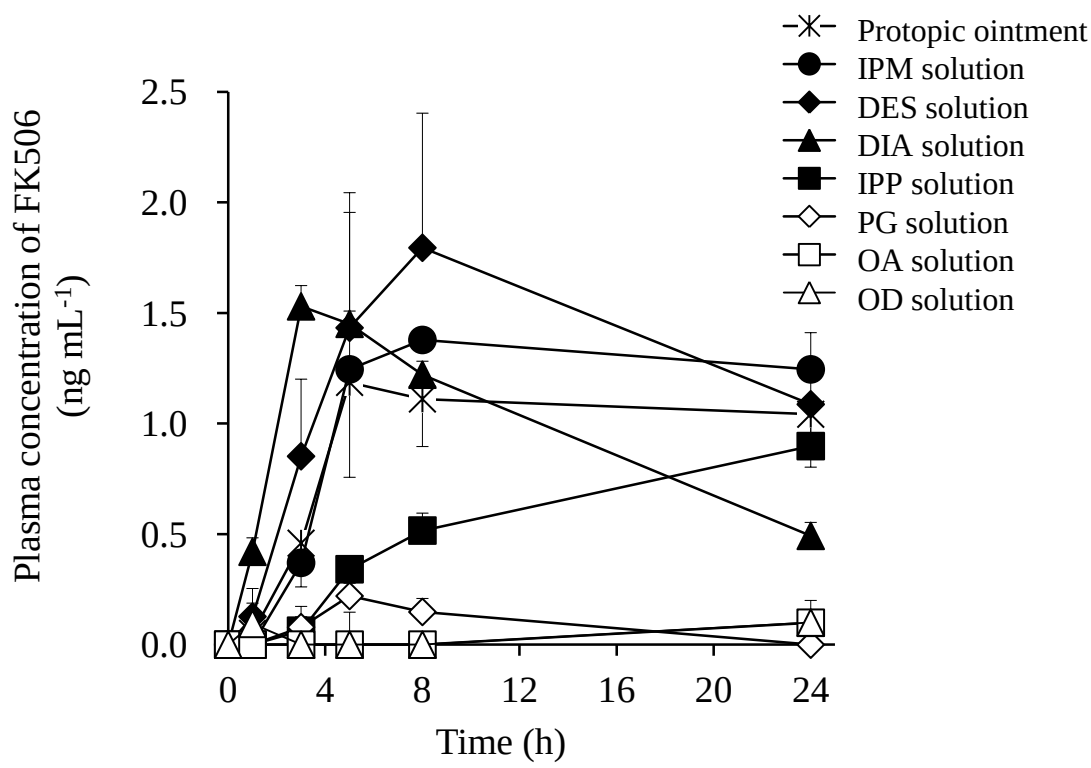


Fig. 3.

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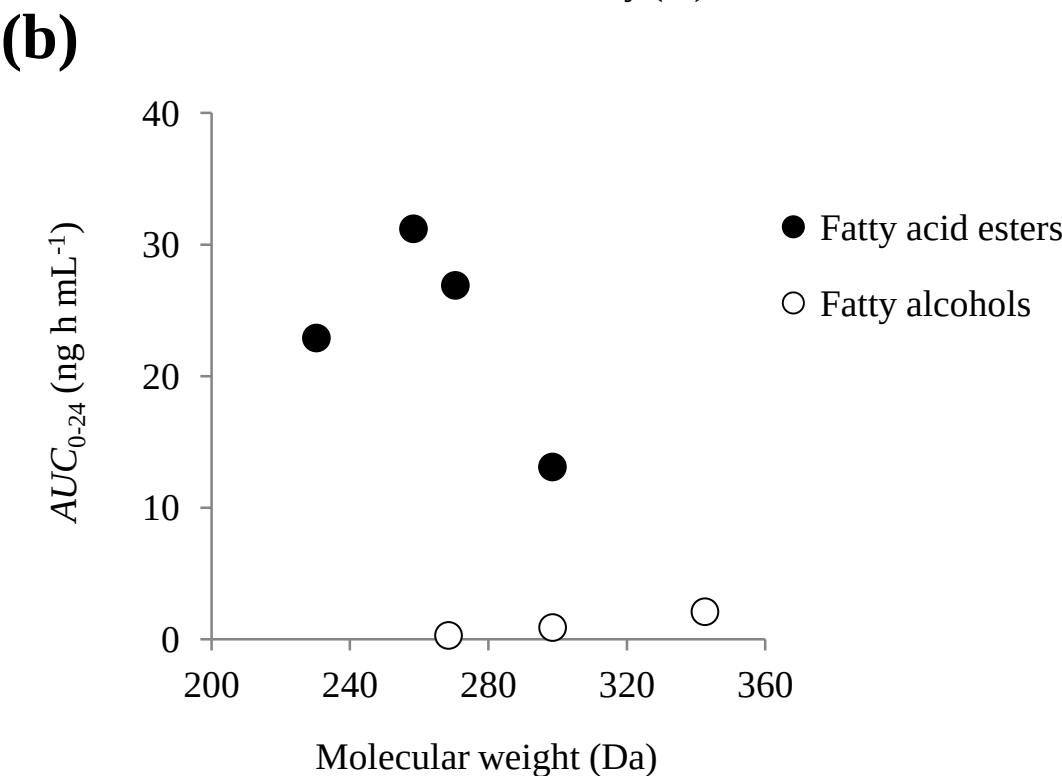
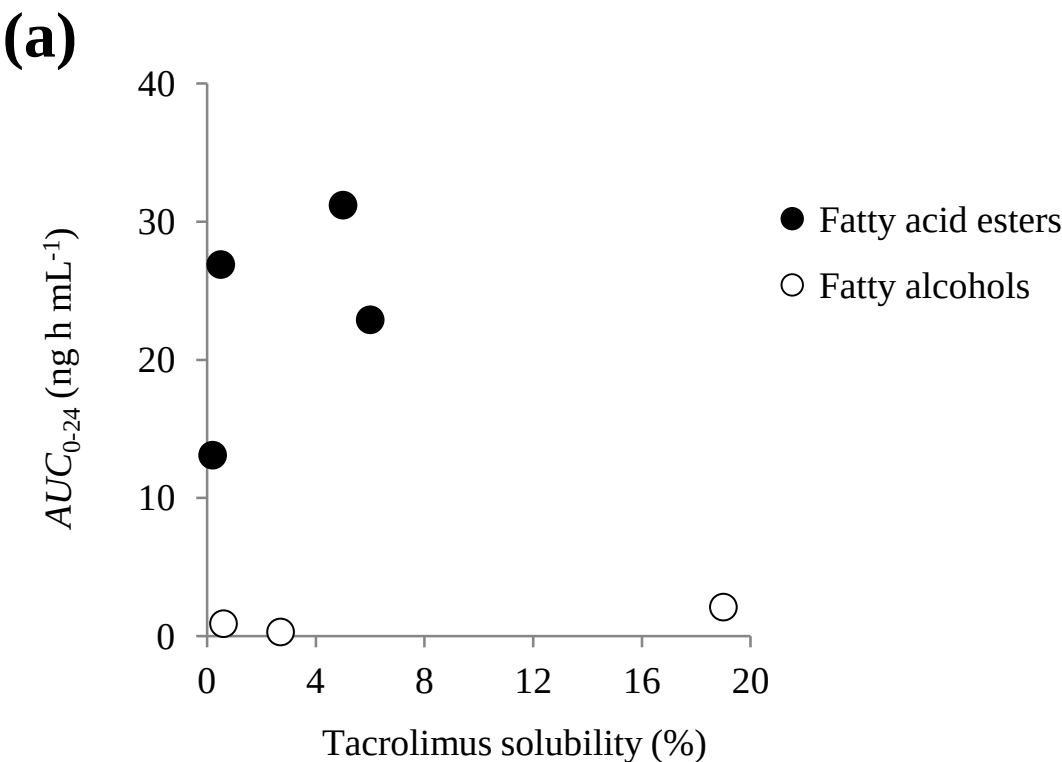


Fig. 4.

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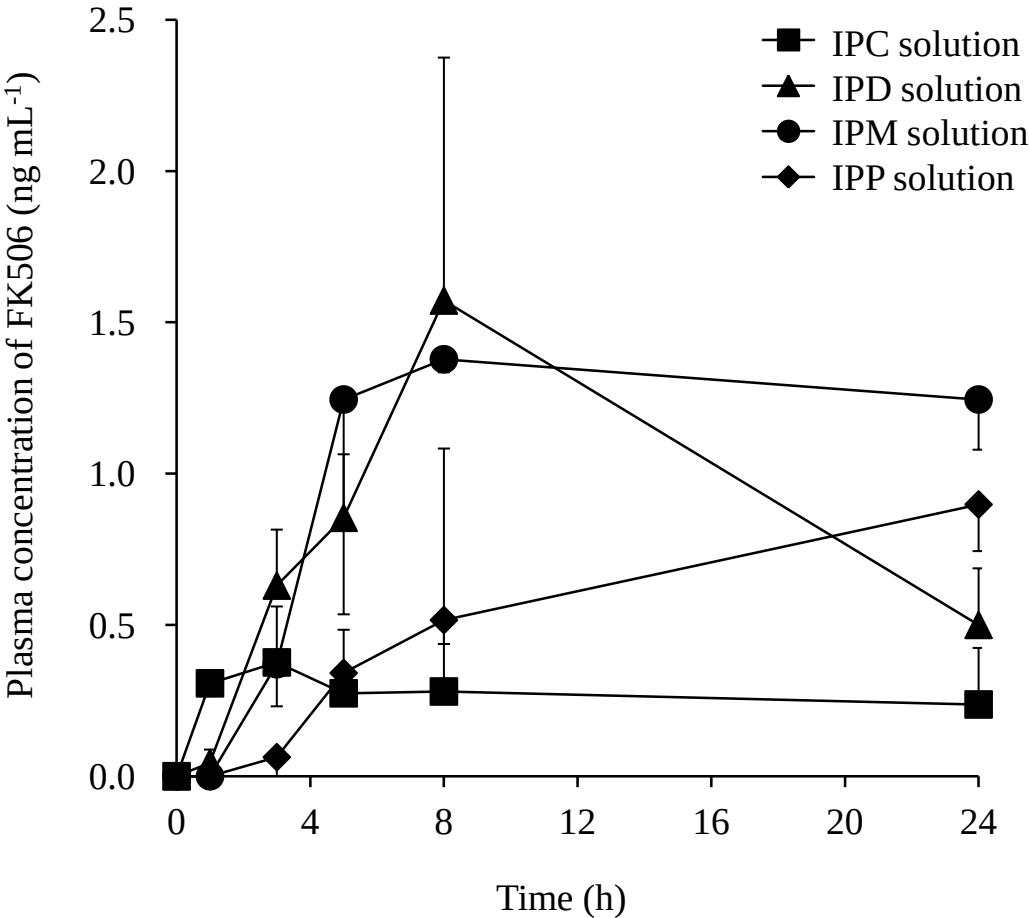


Fig. 5.

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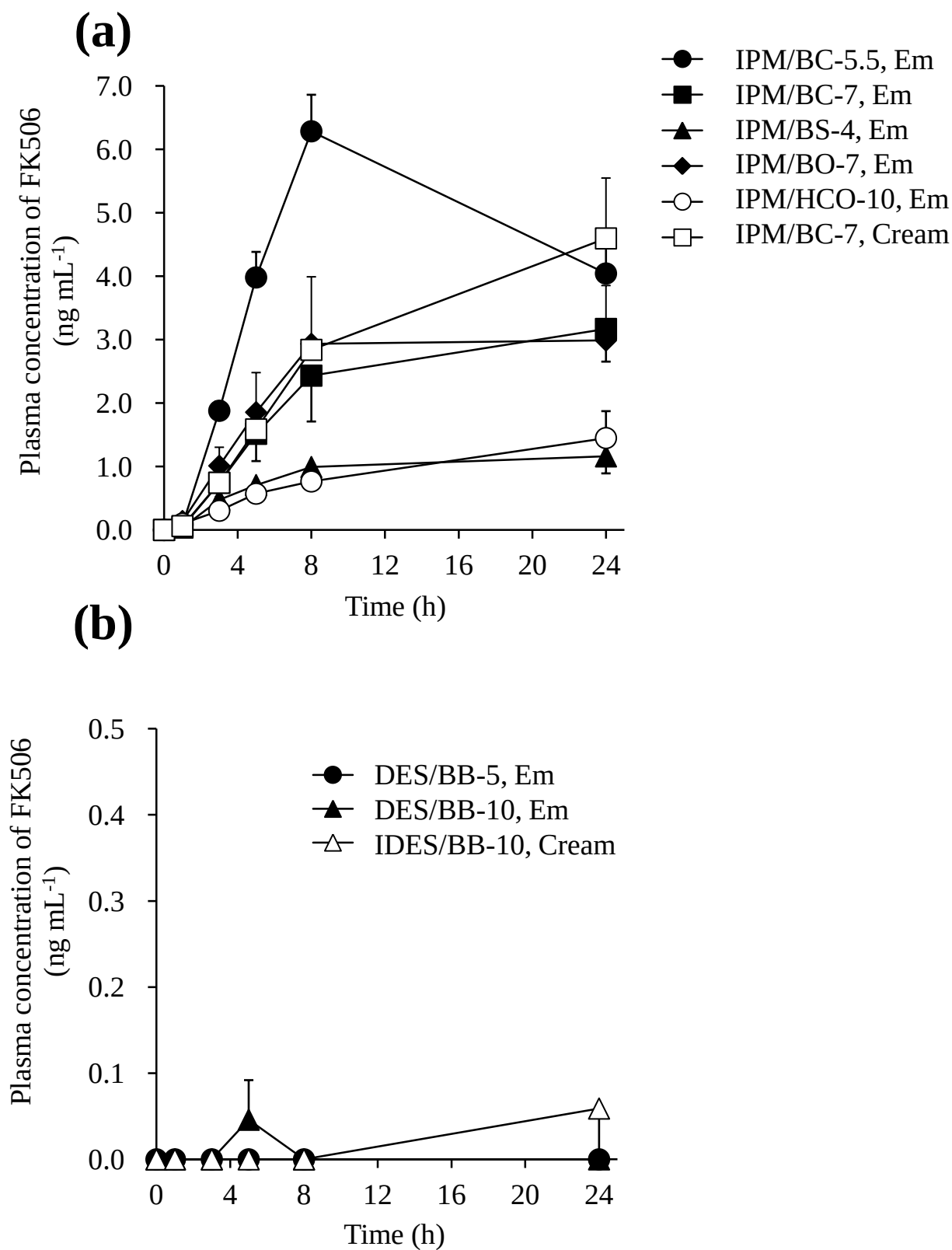


Fig. 6.

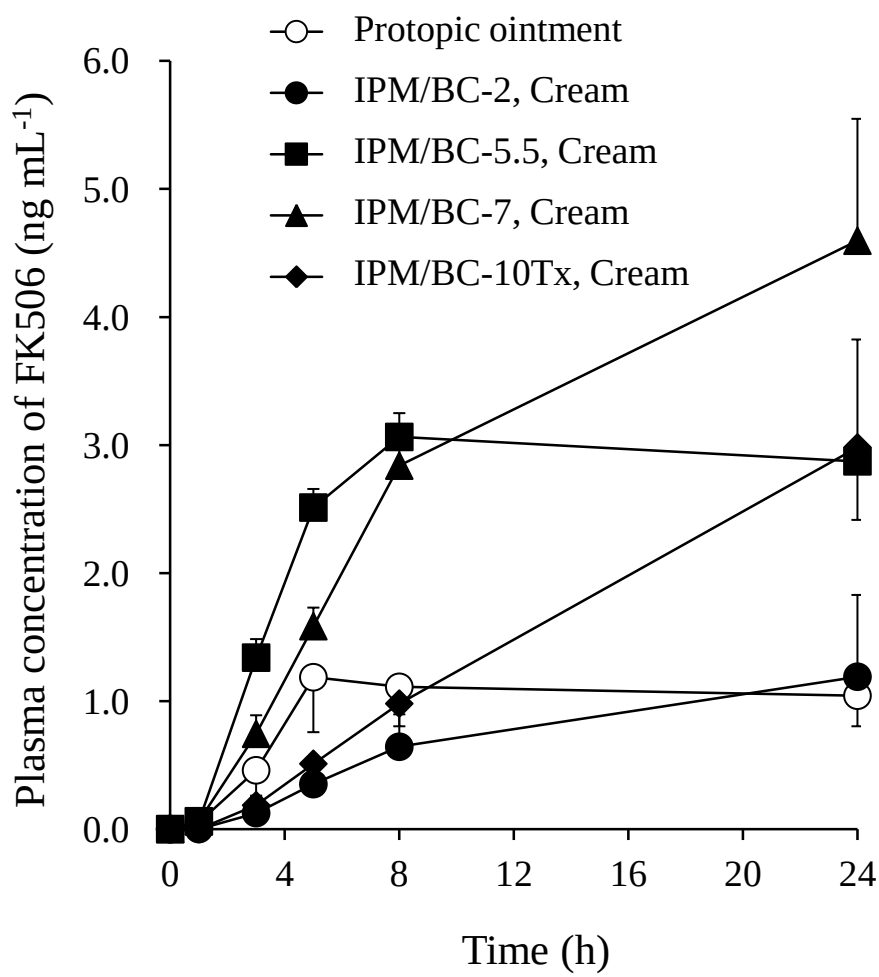


Fig. 7.

