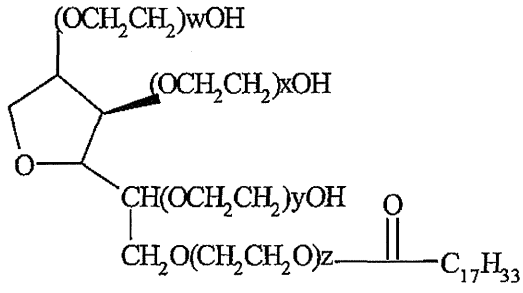

WhereW+X+Y+Z=20

Fig. 1 Molecular Structure of Polysorbate80 (PS80)

SDS試薬が市販品の中に見受けられ，溶出試験に適用した場合，溶出率を上昇させることを明らかにした[3]．また，SDS試薬を溶解したときのpHの違いが，溶出性に影響を及ぼすことも報告している[4]．

本稿では，SDSと同様，溶出試験に界面活性剤として使用されるPS80試薬の品質に着目した．比較的高濃度のPS80を使用する試験が設定されているナブメトン酸錠，リボフラビン酪酸錠及びアリルエストレノール錠を対象とし，溶出性に影響を及ぼすPS80試薬の品質について検討を行った．

実験方法

1. 試験製剤

ナブメトン酸400 mg錠（レリフェン錠400 mg），リボフラビン酪酸40 mg錠（ハイボン錠40 mg）及びアリルエストレノール25 mg錠（パーセリン錠25 mg）を試験製剤として用いた．

2. PS80試薬

市販のPS80試薬（販売メーカー：6社，10種類）を試験に使用した．

3. 標準物質

ナブメトン酸は，SIGMA-ALDRICH社製の分析用試薬を使用した．酪酸リボフラビン（生化学用）及びアリルエストレノール（薬理研究用）は，和光純薬工業株式会社製の試薬を使用した．

4. pHの測定

第16改正日本薬局方（日局）に従い，PS80試薬の5.0 w/v%水溶液についてpHを測定した[2]．pHメーターは，株式会社堀場製作所製のHORIBA F-52を使用した．

5. 酸価，けん化価及びヨウ素価の測定

日局に従い[2]，PS80試薬の油脂試験（酸価，けん化価及びヨウ素価）を行った．

6. HPLCによる分析

HPLCによる分析は，野村らの方法に従った[5]．PS80試薬約1 gを秤量し，メタノール4 mLを加えた後，10分間振り混ぜ，試料溶液とした．HPLCの分析条件は以下の通りである．

装置：株式会社島津製作所製LC-10AD$_{VP}$シリーズ
検出器：示差屈折率検出器（RID-10A）
移動相：メタノール
カラム：L-column ODS（5 μm，4.6 mm × 250 mm）
カラム温度：40°C
注入量：10 μL
流速：1.0 mL/min

7. フーリエ変換赤外分光光度計（FT-IR）による分析

塩化ナトリウム板を用いた液膜法により，各PS80試薬の赤外吸収スペクトルを測定した．試薬約0.1 mLを窓板（塩化ナトリウム）にはさみ，日本分光株式会社製のフーリエ変換赤外分光光度計（FT-IR/4100型）を使用し，測定を行った．得られたPS80試薬の赤外吸収スペクトルについて，装置付属の構造解析ソフト（Know-ItAll, BIO-RAD社製）を使用し，部分構造の解析を行った．なお，FT-IRの測定条件は，以下の通りである．分解能：4 cm^{-1}，積算回数：16回，測定波長：700 cm^{-1} ～ 4000 cm^{-1}

8. 溶出試験

日本薬局方外医薬品規格第三部（局外規第三部）に従い[6~8]，溶出試験を実施した．溶出試験機は，株式会社大日本精機社製の全自動型（RT-3std）を使用した．また，溶出挙動を比較する際の試験液採取は，品質再評価で定められた時間[9]で行い，溶出率は全てn=6の平均値とした．なお，各製剤の試験で使用されるPS80試薬の濃度は，以下の通りである．

ナブメトン酸400 mg錠：3.0 w/v%，
リボフラビン酪酸40 mg錠：1.5 w/v%，
アリルエストレノール25 mg錠：1.0 w/v%，

結果及び考察

1. PS80試薬の色調

局外規第三部の溶出試験で使用されるPS80試薬には，日局「ポリソルベート80」の規格が適用される．その

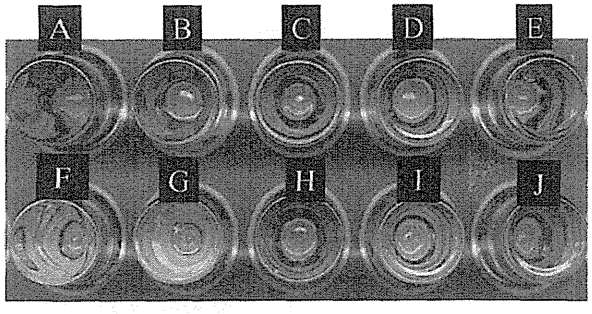

Fig. 2　Description of Commercial PS80 Sample

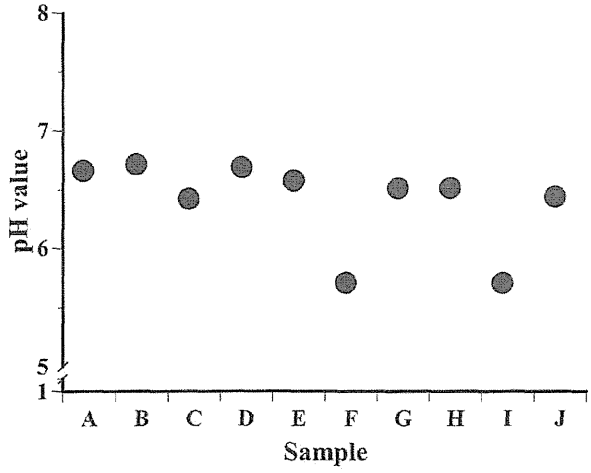

Fig. 3　The pH Value in Aqueous Solution of Commercial PS80 (5 w/v%)

性状の項には，各条品の適否を示すものではないが，色調に関する記述があり，「本品は無色～だいだい黄色の粘稠性のある液」とされている[2]．

本稿で使用した各 PS80 試薬の色調は全て，性状の項に記載された範囲内であった．しかし，PS80 試薬により色合いは大きく異なっていた（Fig. 2）．PS80-A は，最も「だいだい黄色」に近い色調であり，PS80-F 及び PS80-I は，ほぼ「無色」であった．

2.　PS80 試薬の pH

PS80 試薬の 5.0 w/v%水溶液を調製し，pH を測定した（Fig. 3）．日局「ポリソルベート 80」の性状の項には，各条品の適否を示すものではないが，5.0 w/v%水溶液の pH が規定されている[2]．PS80 試薬の pH は，5.73～6.73 の範囲であり，日局「ポリソルベート 80」の性状の項の液性（pH 5.5 ～ pH 7.5）を満たしていた．試薬による pH の差は，ほとんど認められなかったが，PS80-F 及び PS80-I が若干低い値を示した．

著者らは先に，SDS 試薬について，同様の検討を行っており，試薬により pH が大きく異なること，溶解した時の pH の違いが溶出性に影響を及ぼすことを明らかにしている[4]．

3.　酸価，けん化価及びヨウ素価

日局「ポリソルベート 80」の規格のうち，品質への影響が大きいと考えられる酸価，けん化価及びヨウ素価を測定し，各試薬間で比較した．

各 PS80 試薬の試験結果は全て，日局の規格に適合していた（Table 1）．各 PS80 試薬のけん化価及びヨウ素価は，ほぼ同一の値を示したが，酸価は，試薬により大きく異なっていた．PS80-F の酸価は，日局の規格上限値（2.0 以下）付近であり，PS80-A の約 5 倍の値を示した．

酸価は，油脂中の遊離脂肪酸含量を示しており，通常，精製が進むに従い低下する[10]．各 PS80 試薬の色調との関係では，目視的に精製度が高いと考えられる，ほぼ「無色」の PS80-F が最も高い値（1.88）を示し，「だいだい黄色」に近い PS80-A が最も低い値（0.38）を示した．

Table 1　Examination Result by the Official Method (Japanese Pharmacopoeia)

	Fat and Fatty Oils Test		
	Acid Value	Sponification Value	Iodine Value
Quality Standards	Not more than 2.0	45－55	19－24
A	0.38±0.01	50.0±0.6	22.8±0.1
B	0.69±0.01	50.3±0.4	20.3±0.2
C	0.83±0.02	53.0±0.6	20.0±0.1
D	0.62±0.01	53.3±0.1	20.1±0.1
E	0.74±0.01	48.0±0.9	21.3±0.3
F	1.88±0.01	50.9±0.7	21.6±0.5
G	1.30±0.02	49.2±0.9	22.2±0.2
H	1.26±0.01	50.5±0.4	21.8±0.4
I	1.41±0.02	46.5±1.2	21.8±0.1
J	1.70±0.02	45.9±0.6	23.0±0.4

Mean±SD, N=3

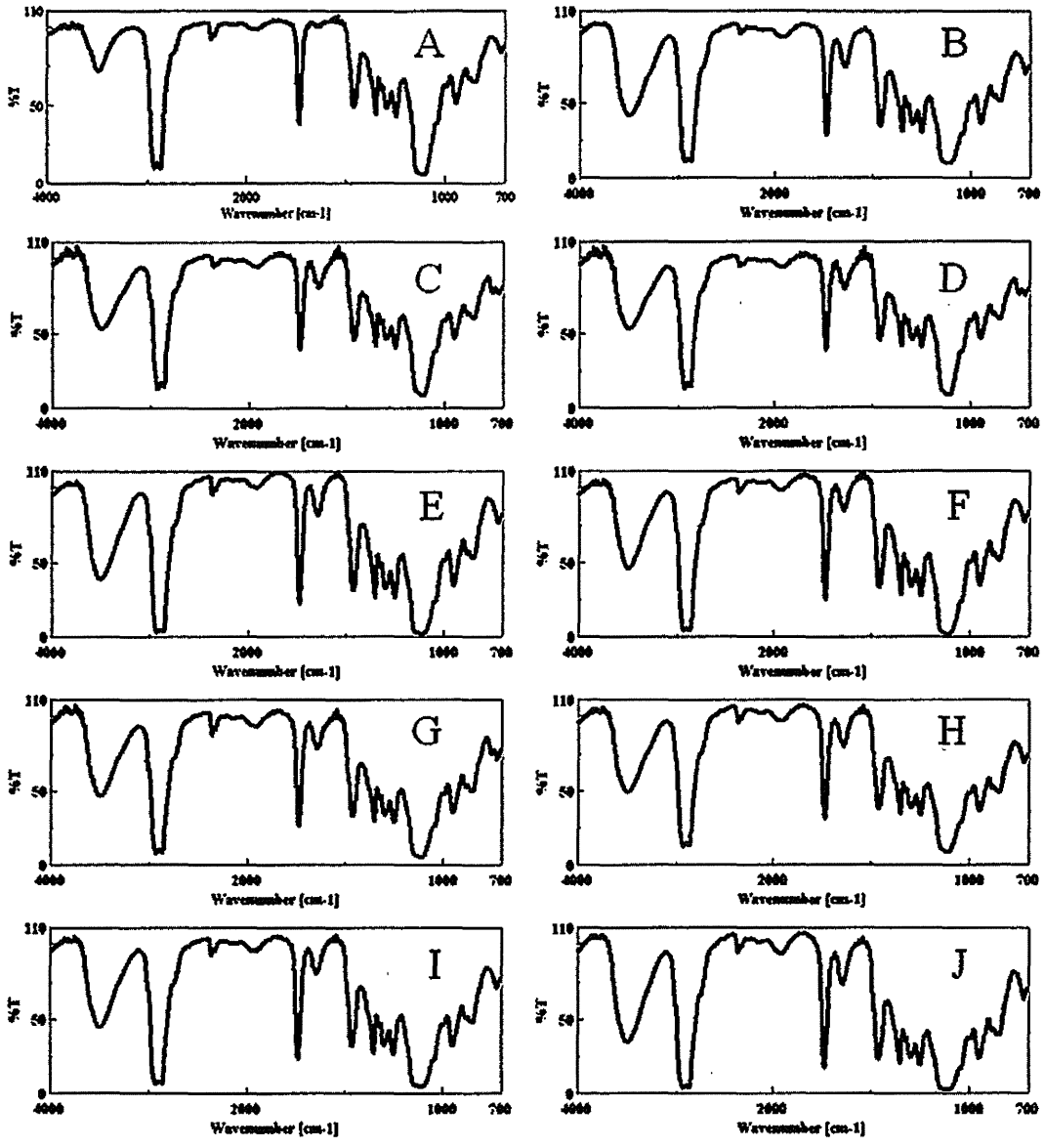

Fig. 4　Infrared Absorption Spectra of Commercial PS80 Sample

4. FT-IR による分析

PS80 試薬を FT-IR により分析し，赤外吸収スペクトルを比較した（Fig. 4）．指紋領域（1500 cm^{-1} 以下）の比較では，全ての PS80 試薬のスペクトルが，同一であった．しかし，1500 cm^{-1} ～ 4000 cm^{-1} の領域では，PS80-A のスペクトルが，他とは異なっていた．PS80-A のスペクトルでは，1640 cm^{-1} 付近の吸収帯がほとんど認められず，3400 cm^{-1} 付近の吸収帯も弱かった．装置付属の構造解析ソフト等による解析結果から，3400 cm^{-1} 付近の幅広い吸収帯は，水素結合した水酸基の伸縮振動によるものであることが推測された[11, 12]．また，1640 cm^{-1} 付近の吸収帯が，ほとんど認められなかった理由は，試薬間における水分含量の差によるものである可能性がある．すなわち，日局収載の「グリセリン」と「濃グリセリン」の参照赤外吸収スペクトルを比較してみると，水分含量が多いグリセリンのスペクトルでは，1650 cm^{-1} 付近にブロードな吸収帯が認められるのに対し，濃グリセリンのスペクトルでは認められない[13]．更に，水酸基の伸縮振動によるものであることが推測される 3400 cm^{-1} 付近の吸収帯が PS80-A のスペクトルでは弱いことを考慮すると，1640 cm^{-1} 付近の吸収帯は，H-O-H の変角振動によるものであることが示唆される．

この様に，市販の PS80 試薬の赤外吸収スペクトルには，水分含量の差に基づく若干の相違が認められるものの，品質上の問題は確認できなかった．

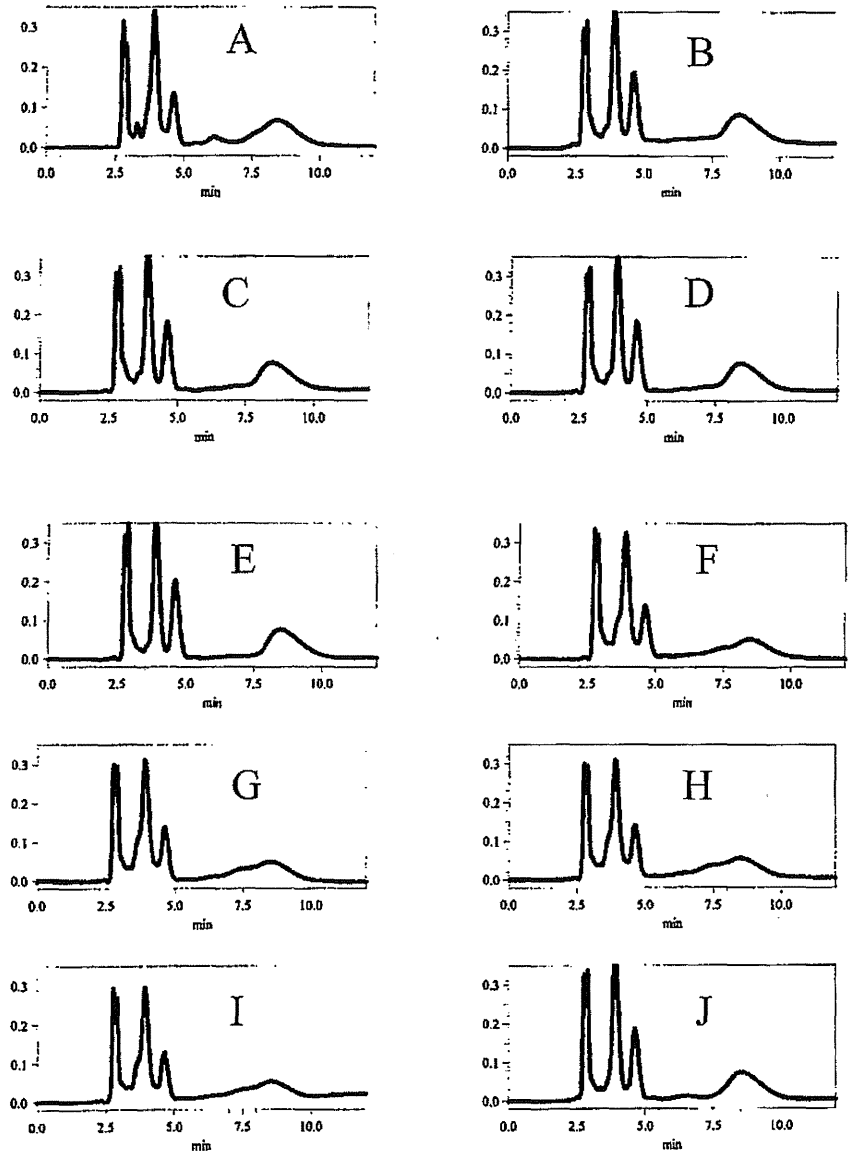

Fig. 5　Chromatographic Patterns of Commercial PS80 Sample

5. HPLC による分析及び品質の違いが及ぼす溶出挙動への影響

各 PS80 試薬を HPLC により分析し，クロマトグラムを比較した（Fig. 5）．全ての PS80 試薬のクロマトグラムから，複数のピークが確認された．PS80 は，ソルビタンとオレイン酸を反応させて生成するソルビタン脂肪酸エステルにオキシエチレン基を付加重合させて合成される[14]．理論的には，ソルビタン 1 分子にオレイン酸が 1 分子とオキシエチレン基が 20 個付加重合した化合物とされているが（Fig. 1），実際には様々な分子種から構成される複雑な混合物であることが報告されている[15, 16]．

各 PS80 試薬のクロマトグラムは，ピーク形状等の比較から，3 種類に分類された．PS80-A のクロマトグラムでは，他と比較し，より多くのピークが確認された．また，ピーク形状の比較から，PS80-B，C，D，E 及び J のクロマトグラムが類似しており，PS80-F，G，H 及び I のパターンがほぼ同一であった．

以上の検討結果を踏まえ，理化学的な品質が異なる PS80-A，E，F を使用した溶出試験を実施し，溶出挙動を比較した（Fig. 6）．試験製剤として，ナブメトン酸錠，リボフラビン酪酸錠及びアリルエストレノール錠を対象とした．これらの製剤は全て，局外規第三部に溶出試験が収載されており，比較的高濃度の PS80 試薬を使用する製剤である．

PS80-A，E，F を用いた場合の溶出挙動は類似しており，3 種類の試験製剤とも，使用する試薬による溶出

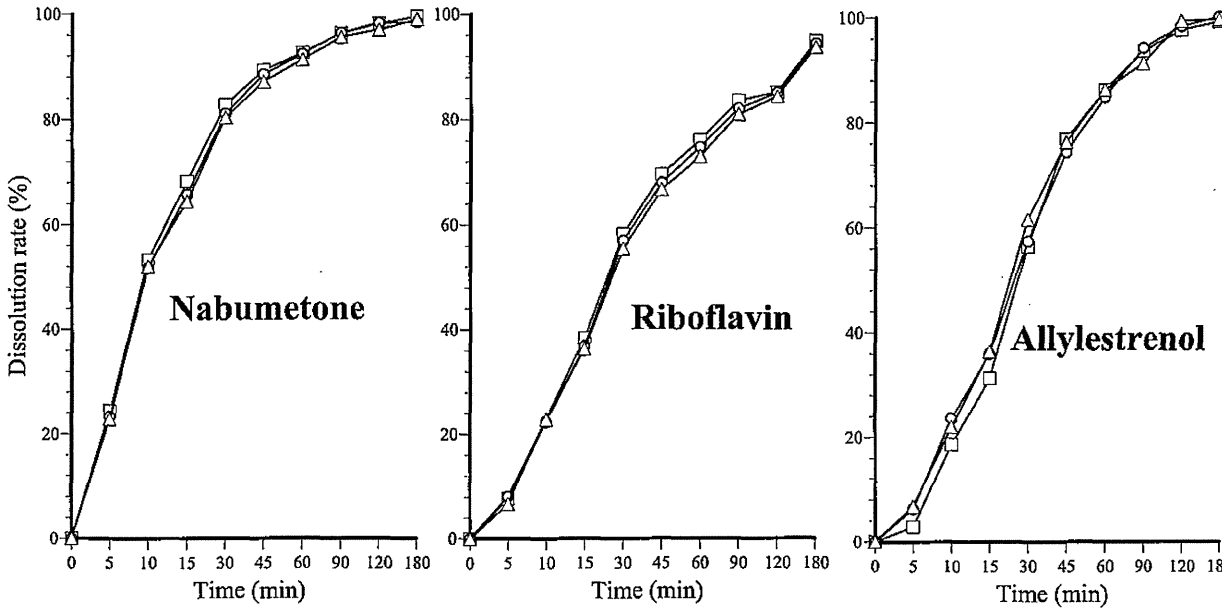

Fig. 6 Dissolution Behavior of Nabumetone, Riboflavin and Allylestrenol Tablet
□ ; Polysorbate80 (PS80)-A, ○ :PS80-E, △ :PS80-F

性の差違は確認できなかった．この様に，本稿において確認されたPS80試薬の品質の相違は，溶出挙動に影響を及ぼすものではなかった．

結　論

本稿では，溶出性に影響を及ぼすPS80試薬の理化学的品質について検討を行った．市販のPS80試薬は，色調，油脂試験，HPLCのクロマトグラム及び赤外吸収スペクトル等が同一ではなかった．しかし，ナブメトン酸錠，リボフラビン酪酸錠及びアリルエストレノール錠を対象とした試験では，試薬による溶出挙動の差は特に認められず，品質上問題となるものではなかった．

謝　辞

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Alterations in the Detergent-Induced Membrane Permeability and Solubilization of Saturated Phosphatidylcholine/Cholesterol Liposomes: Effects of Poly(ethylene glycol)-Conjugated Lipid

Hiroko Shibata,* Haruna Saito, Chikako Yomota, Toru Kawanishi, and Haruhiro Okuda

National Institute of Health Sciences; 1–18–1 Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan.
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We have investigated the effects of two bile salts, chenodeoxycholate (CDC) and ursodeoxycholate (UDC), and a widely used detergent, Triton X-100 ($T_{X\text{-}100}$), on normal and poly(ethylene glycol)-modified liposomes (PEGylated liposomes). We tested various lipid compositions, including hydrogenated soybean phosphatidylcholine/cholesterol/PEG-conjugated lipid (HSPC/PEG-lipid). Alterations in permeability were determined by the rate of drug release from the liposomes and solubilization was assessed by measuring the particle size of liposomes. In addition, we attempted to observe interactions between the detergents and lipid bilayers by using surface plasmon resonance (SPR). CDC induced drug release from liposomes in a dose-dependent manner, and the PEGylated liposomes tended to be susceptible to CDC. While UDC did not strongly induce drug release from liposomes, UDC exhibited a similar tendency with CDC. In case of $T_{X\text{-}100}$, there were significant differences in the percentage of released drug between normal and PEGylated liposomes, and the percentage of $T_{X\text{-}100}$-induced drug release further increased with an increased ratio of PEG-lipid. SPR analysis revealed that the lipid bilayer including PEG-lipid was selectively solubilized by $T_{X\text{-}100}$, correlating with the drug release data. These results suggest that the effect of detergents on the lipid bilayer of liposomes depends on both the kind of detergent and the lipid composition, including the presence or absence of PEG-lipid. Moreover, the effects of $T_{X\text{-}100}$ on the lipid bilayers of the PEGylated liposomes significantly differed from those on the lipid bilayers of the normal liposomes.

Key words liposome; release; detergent

Many commercial liposomal products have been developed,[1] and DOXIL®, which is the antitumor agent doxorubicin (DXR) encapsulated in a poly(ethylene glycol)-modified (PEGylated) or so-called "stealth" liposome,[2] has been approved in Japan. Drug release is one of the most important formulation properties for quality assessment of liposomal products.[3] *In vitro* drug-release tests would be very useful for assessing lot-to-lot variability.[4,5] However, it is difficult to develop the *in vitro* release testing which can completely mimic *in vivo* release profile of liposomal products, since the *in vivo* behavior of the liposomal product is quite complicated.[6,7] Additionally, it takes some dozens of days to obtain sufficient drug-release under normal conditions, such as suspension in buffered saline or human serum/plasma at 37°C. On another front, the property of lipid bilayer such as a physical state will be one of critical parameters related to drug release from liposomes. Therefore, the investigation of *in vitro* release under multiple conditions has been proposed.[8] For examples, one is under physiological conditions, and the other is under physical/chemical stress conditions to evaluate the property of lipid bilayers. Thus, by using detergent as a chemical stress, the measurement of drug release associated with detergent-induced destabilization of lipid membranes is one potential method to evaluate the property of liposome membranes and to shorten the testing time.

The solubilization and reconstitution of lipid bilayers induced by the addition of detergents has attracted attention from a biological and physicochemical point of view. As a result, many studies have examined the corresponding mechanisms and intermediate processes involved in the process by using liposomes as artificial lipid bilayers.[9–13] The effects of bile salts on cell membranes and liposomes have also been studied because bile salts are typical intravital detergents. In particular, deoxycholate, chenodeoxycholate (CDC), and ursodeoxycholate (UDC), which are common in bile and serum, have been compared for their abilities to permeabilize and solubilize membranes of egg phosphatidylcholine (EPC) liposomes.[10,13] Triton X-100 ($T_{X\text{-}100}$) is another commonly used detergent. While the membrane solubilization by $T_{X\text{-}100}$ and its mechanism have already been examined physicochemically,[9,11] additional studies have recently investigated the effects of temperature and variable carbon chain length on membrane solubilization.[14,15] In the above studies, the liposomes were mainly composed of a single phosphatidylcholine.

In contrast, liposomal products for systemic administration are mainly composed of saturated lipids or synthetic lipids, including hydrogenated soybean phosphatidylcholine (HSPC), distearoylphosphatidylcholine (DSPC), and distearoylphosphatidylglycerol (DSPG), while unsaturated lipids are sometimes used in liposomal products for topical applications. The increased proportion of cholesterol in the lipid composition and the addition of poly(ethylene glycol)-conjugated lipid (PEG-lipid) are strategies often applied to enhance the *in vivo* stability of liposomal products.[16,17] Detergents have been used as a positive control or as additives in the test medium for the *in vitro* test to evaluate drug release from liposomes consisting of the above mentioned lipid components.[18] But, the solubilizing and permeabilizing effects of detergents on lipid bilayers made of saturated phospholipids and cholesterol on liposome encapsulated drug substances have yet to be fully investigated. Further, to the best of our knowledge, no previous studies have investigated the effect of detergents on PEGylated lipid bilayers.

Before applying detergents to the *in vitro* drug release

The authors declare no conflict of interest.

* To whom correspondence should be addressed. e-mail: h-shibata@nihs.go.jp

testing of liposomal products, it is important to collect basic information on detergent-induced permeabilization, solubilization, and drug release from liposomes that are similar in lipid composition to commercially available products. In this study, we examined the detergent-induced permeabilization and solubilization of lipid bilayers by using drug-encapsulated liposomes consisting of HSPC and cholesterol, with or without PEG-lipid, to assess whether the effects of detergents are altered by PEG-lipid. In addition, we used CDC, UDC, and $T_{X\text{-}100}$ as detergents, because they have previously been used to study lipid-detergent interactions.

Experimental

Materials Two bile salts, sodium chenodeoxycholate and sodium ursodeoxycholate, were obtained from Calbiochem (Merck KGaA, Darmstadt, Germany) and Tokyo Chemical Industry (Tokyo, Japan), respectively. $T_{X\text{-}100}$ was purchased from Sigma-Aldrich Japan (Tokyo, Japan). The phospholipids, hydrogenated soybean phosphatidylcholine (HSPC; C16 : 0 approx. 10%, C18:0 approx. 90%) and (*N*-(carbonyl-methoxypolyethylene glycol 2000)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE-PEG2000), were purchased from NOF Corporation (Tokyo, Japan). Cholesterol (Chol) was of analytical grade (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Adriacin® injection 10 (Kyowa Hakko Kirin Co., Ltd., Tokyo, Japan), a doxorubicin hydrochloride (DXR) injection, was purchased from a general sales agency for drugs. PD-10 desalting columns were purchased from GE Healthcare Japan (Tokyo, Japan).

Liposome Preparation Liposomes, liposomes-entrapped DXR, and empty liposomes were prepared by modified ethanol injection method.[19] DXR was encapsulated into liposomes by remote loading using an ammonium sulfate gradient.[20] Briefly, all lipids (200 μmol) were dissolved in about 5 mL of ethanol in different compositions: SL1, HSPC/Chol/DSPE-PEG2000 (79/16/5 mol/mol); SL2, HSPC/Chol/DSPE-PEG2000 (55/40/5 mol/mol); L1, HSPC/Chol (5/1 mol/mol); and L2, HSPC/Chol (6/4 mol/mol). The ethanol was removed with a rotary evaporator leaving behind about 1 mL of the ethanol solution. Next, 8 mL of 300 mM ammonium sulfate (for liposomes-entrapped DXR) or saline (for empty liposomes) was added to the ethanol solution. Liposomes formed spontaneously after further evaporation of the residual ethanol. After 5 freeze-thaw cycles, liposomes were extruded through a series of polycarbonate filters (Nucleopore, CA, U.S.A.) with pore sizes ranging from 0.4 to 0.1 μm. The mean diameter of extruded liposomes was in the range of 100–150 nm. Following extrusion, liposomes were ultracentrifuged at 80000 rpm for 45 min at 4°C, and suspended in normal saline. Phospholipid concentration was determined by colorimetric assay using Phospholipids C Test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan). For encapsulation of DXR, DXR was added to the ammonium sulfate-containing liposomes at a DXR/liposome ratio of 0.2 : 1 (w/w), and the liposomes were incubated for 1 h at 55°C. The liposome-encapsulated DXR was exchanged by eluting through a PD-10 desalting column equilibrated with normal saline.

Determination of DXR Release The release of DXR from liposomes was studied using a fluorescence-dequenching assay, according to the method described by Ishida *et al.*[21] with some modifications. CDC, UDC, and $T_{X\text{-}100}$ were dissolved in 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer (pH 7.5) at indicated concentrations. Liposomes were incubated in various buffer conditions at a final lipid concentration of 40 nM (DXR, 2 μg/mL) at 42°C for 30 min; these intervals and temperature values were chosen as the minimum period of time and heating needed to achieve a constant level of DXR release. The DXR release in an aliquot of the incubation mixture was determined by the increase in sample fluorescence at 470/590 nm (emission/excitation) by using a fluorescence spectrophotometer (JASCO, Tokyo, Japan). The percentage of released DXR was calculated using the pre-incubation sample (zero release) and complete lysis sample with isopropanol (100% release). Values were normalized using the standard curve of DXR at various concentrations of CDC, UDC, or $T_{X\text{-}100}$.

Dynamic Light Scattering (DLS) Measurements The particle size distribution and mean diameter of each liposome after incubation were measured using a dynamic light scattering photometer DLS-7000 (Otsuka Electronics Co., Ltd., Osaka, Japan) equipped with a He–Ne laser source (wavelength, 632.8 nm). All DLS measurements were made at a scattering angle of 90°. Data were gathered using a counting period of 100 s. Histogram analysis was performed to calculate the average particle size and standard deviation.

Surface Plasmon Resonance (SPR) Analysis Experiments were performed at 25°C using L1 sensor chip on Biacore 2000 (Biacore®, Uppsala, Sweden) based on previous reports.[22,23] Fifty microliters of 20 mM zwittergent 3–14 detergent, followed by 50 mM HCl in 50% v/v isopropanol, was applied at a flow rate of 100 μL/min to precondition new sensor chips. Empty liposomes (SL1, SL2, L1, L2) were diluted in running buffer HBS-N to 10 mM and captured to saturation (2 min) across isolated flow cells at 5 μL/min which resulted in an increase of resonance units (RU=1 pg/mm^2): SL1, 6000 RU; SL2, 7000 RU; L1, 12000 RU; L2, 15000 RU. The flow rate was switched to 100 μL/min, and fresh lipid surfaces were washed by applying two 30-s pulses of running buffer. The flow rate was then switched to 5 μL/min, and 10 μL of detergent at indicated concentrations was injected for 2 min to monitor the interaction of the detergent with lipid surfaces. Again, the flow rate was switched to 100 μL/min, and 2 consecutive 30-s pulses of 50 mM HCl in 50% v/v isopropanol were applied to regenerate the sensor surface. These procedures, including liposome capture and lipid dissociation induced by detergent, are shown in Fig. 4. The sensor surface was recoated with a fresh liposome solution for the next interaction cycle. The concentration of dissociated lipid was calculated from the difference between the RU of immobilized lipid and that of equilibrium value after injection of detergent.

Results and Discussion

Detergent-induced solubilization and drug release were assessed on 2 PEGylated and 2 normal liposomes: SL1, HSPC/Chol/DSPE-PEG2000 (79/16/5 mol/mol); SL2, HSPC/Chol/DSPE-PEG2000 (55/40/5 mol/mol); L1, HSPC/Chol (5/1 mol/mol); and L2, HSPC/Chol (6/4 mol/mol). DXR, which is encapsulated in the liposomal product DOXIL®, was encapsulated in the above mentioned liposomes.

Alteration of Membrane Permeability and Particle Size The effects of 2 kinds of bile salt, CDC and UDC, were examined in concentration ranges of 0.01–10 mM and 0.02–20 mM,

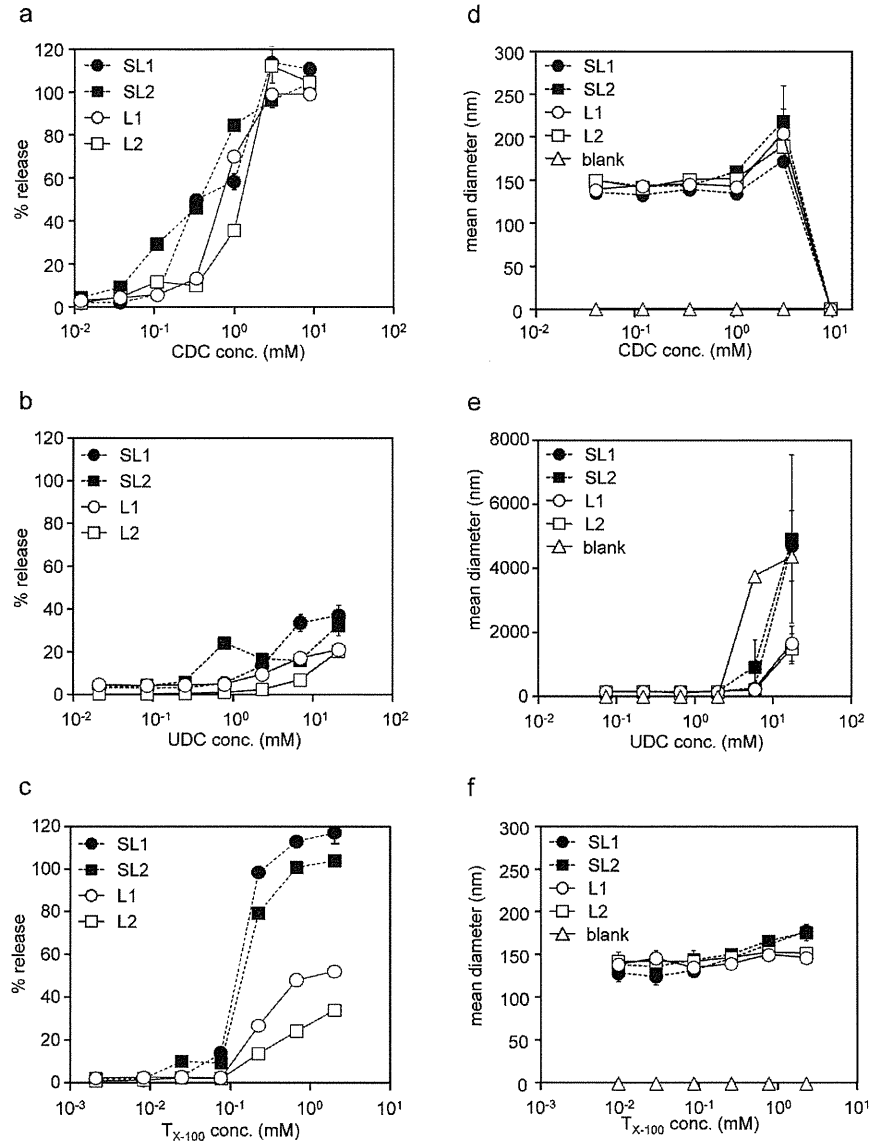

Fig. 1. DXR Release from Liposome Induced by (a) CDC, (b) UDC, or (c) T_{X-100}, and the Influence of (d) CDC, (e) UDC, or (f) T_{X-100} on the Size of Liposomes

Liposomes were incubated with each detergent at the indicated concentration for 30 min at 42°C. Results are means of triplicate analysis±S.D. SL1; HSPC/Chol/DSPE-PEG2000 (79/16/5 mol/mol), L1; HSPC/Chol (5/1 mol/mol). SL2; HSPC/Chol/DSPE-PEG2000 (55/40/5 mol/mol), L2; HSPC/Chol (6/4 mol/mol). The blank used was the solution of each detergent.

respectively. CDC induced DXR release from liposomes in a dose-dependent manner. DXR release was observed below the critical micelle concentration (cmc: approx. 3 mM[24]), and the percentage of released DXR reached almost 100% at 3.3 mM (Fig. 1a). While there were no significant differences in the permeability enhancement of CDC based on lipid composition, the percentage of released DXR from the PEGylated liposomes (SL1 and SL2) tended to be slightly higher than that from normal liposomes. From the particle size analysis of liposomes, an increase in particle size was observed in all liposomes at 3.3 mM CDC, but the intensity of all liposomes in solution was decreased at 10 mM, indicating that the lipid bilayer of each liposome was solubilized, and that they became small particles whose size was below the detection limit (10 nm) of the equipment (Fig. 1d).

In case of UDC, although the rate of release of DXR was low at 20–40%, at even 20 mM over cmc (approx. 12 mM[24]), the rate of release of DXR from the PEGylated liposomes (SL1 and SL2) tended to be slightly higher than that from normal liposomes (Fig. 1b). The particle size was increased at 6.6 mM, reaching more than 1000 nm (Fig. 1e). However, the increase of particle size was observed in the solution of UDC (blank). This could be caused by the phase transition from micelle to liquid crystal phase with the increase of concentration of UDC. The value of each liposome in Fig. 1e showed the particle size of mixture of each liposome and the assembly of UDC molecule, thus we could not measure the effect of UDC on the alteration of particle size of liposome.

These data indicate that CDC altered the permeability of lipid bilayers at concentrations below the cmc and induced drug release from liposomes without complete solubilization of the bilayer. They also indicated that UDC has a small effect

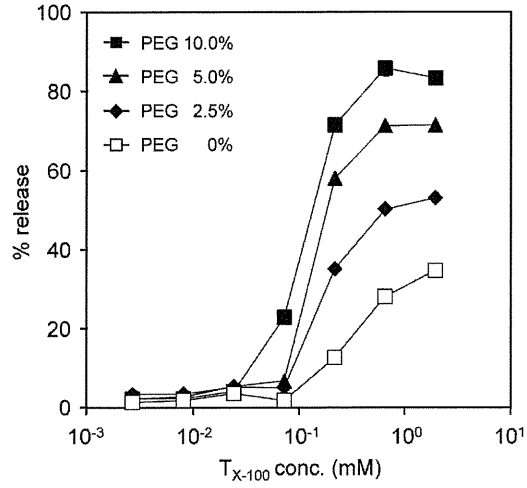

Fig. 2. T_{X-100} Specifically Induced DXR Release from PEGylated Liposomes

Results are means of triplicate analysis±S.D. PEG 0%; HSPC/Chol (6/4 mol/mol), PEG 2.5%; HSPC/Chol/DSPE-PEG2000 (57.5/40/2.5 mol/mol), PEG 5.0%; HSPC/Chol/DSPE-PEG2000 (55/40/5 mol/mol), PEG 10%, HSPC/Chol/DSPE-PEG2000 (55/40/5 mol/mol).

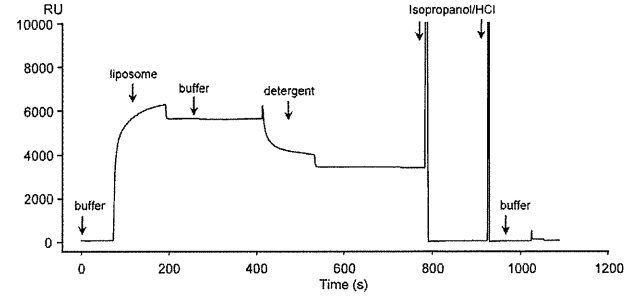

Fig. 3. Sensorgram of Complete Liposome Capture and Detergent-Induced Lipid Dissociation

Empty liposomes are immobilized, and 2 buffer injections are administered to remove any free liposomes and stabilize the surface. After washing, each detergent is injected over the liposome. Two injections of 50 mM HCl in 50% v/v isopropanol regenerate the sensor chip surface. A final injection of buffer removes any residual regeneration solution, thereby preparing the surface for the next assay cycle.

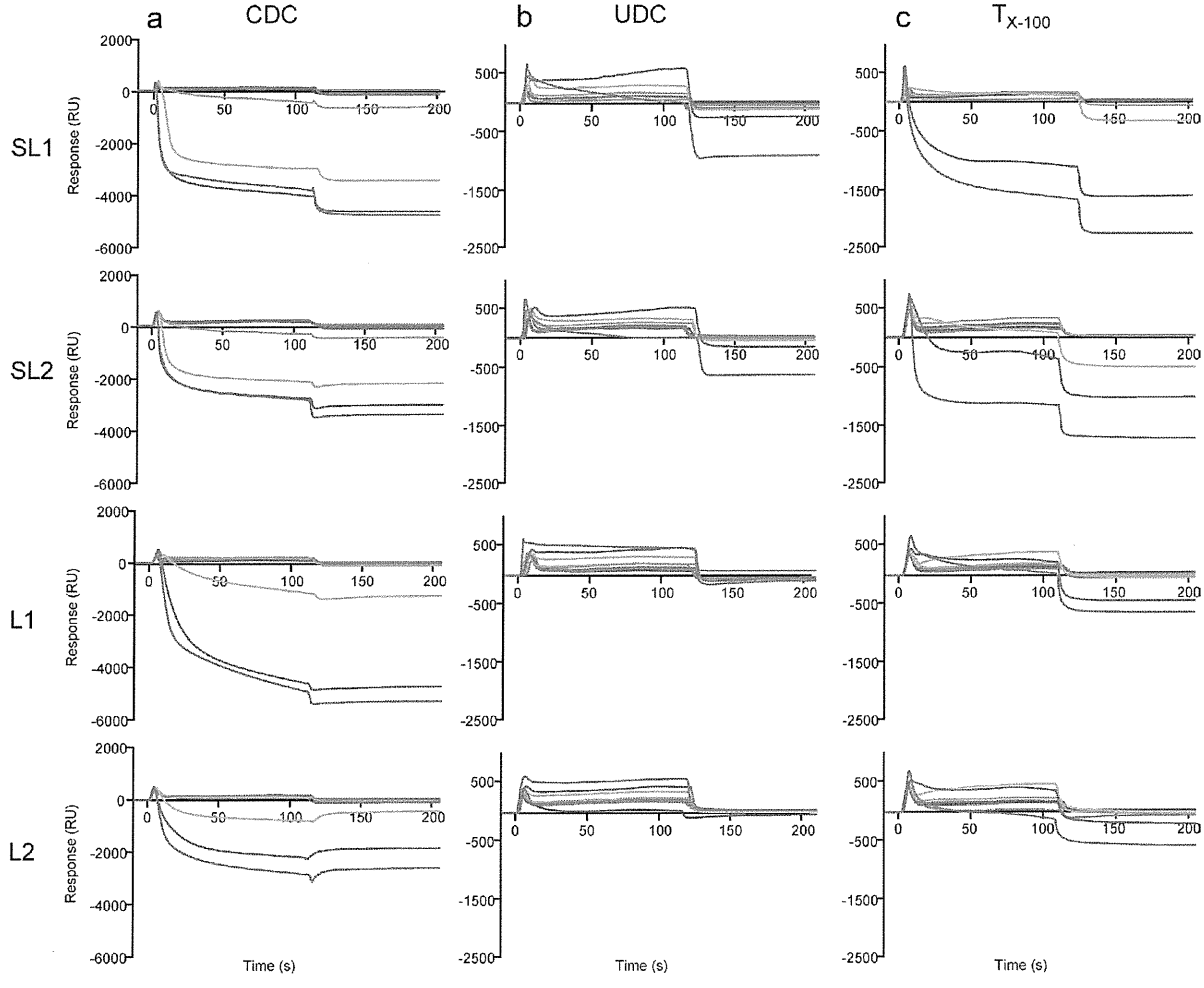

Fig. 4. Responses Obtained for Detergents Interacting with Liposome Surfaces

Each detergent was injected at 0 (—), 0.313 (—), 0.625 (—), 1.25 (—), 2.5 (—), 5.0 (—), and 10 (—) mM over a freshly prepared lipid surface. SL1: HSPC/Chol/DSPE-PEG2000 (79/16/5 mol/mol), L1: HSPC/Chol (5/1 mol/mol), SL2: HSPC/Chol/DSPE-PEG2000 (55/40/5 mol/mol), L2: HSPC/Chol (6/4 mol/mol).

on membrane permeability. These results mostly agreed with those of previous studies in which CDC exhibited a greater disruption of egg phosphatidylcholine (EPC)-liposome lipid bilayers than UDC at concentrations below cmc.[10,13,25] Therefore, it was suggested that, even in the case that the liposomes are not composed of EPC, CDC and UDC can exhibit similar tendencies with those observed in the previous studies,[10,13,25] and the DXR release induced by both bile salts from the PEGylated liposomes tend to be slightly higher than that from normal liposomes.

$T_{X\text{-}100}$ induced DXR-release in a dose-dependent manner, and the increased release was observed from above cmc (approx. 0.2 mM[26]) (Fig. 1c). Interestingly, the percentage of released drug of the PEGylated liposomes SL1 and SL2 were significantly increased compared with the normal liposomes L1 and L2. There were no significant changes in the particle size of the liposomes in response to the concentration of $T_{X\text{-}100}$ (Fig. 1f), but the decreased intensity and increased polydispersity index were observed at 2 mM of $T_{X\text{-}100}$ (data not shown). Thus $T_{X\text{-}100}$ can induce drug release from liposomes without complete solubilization of the bilayer, and mixed micelles and empty liposomes can be existed in the solutions.

There are no reports indicating that $T_{X\text{-}100}$ significantly and selectively enhances membrane permeability in PEGylated bilayers. Thus, to assess the effect of PEG-lipid, we prepared DXR-encapsulated liposomes with various ratios of PEG-lipid (0, 2.5, 5.0, 10 mol% (mol/mol)), and measured $T_{X\text{-}100}$-induced DXR release. The increased ratios of PEG-lipid (*i.e.*, 2.5, 5.0, 10%) caused significant increases in the rate of DXR release (Fig. 2). This result emphasizes that PEG-lipid greatly affects $T_{X\text{-}100}$-induced DXR release from PEGylated liposomes. However, a previous study indicated that giant unilamellar vesicles consisting of dipalmitoyl phosphatidylcholine (DPPC) (C16:0)/Chol with 1% PEG-lipid in the liquid-ordered phase were stable, and that no leakage of encapsulated substance was observed in the presence of high concentrations of $T_{X\text{-}100}$ by using fluorescence microscopy.[27] The difference between these results could be attributed to the fact that in this study, we used HSPC (C16 : 0 approx. 10%, C18:0 approx. 90%) liposomes with higher content of PEG-lipid. It was reported that the increasing the carbon chain length decreased the detergent/lipid ratios causing solubilization.[14]

Interaction Analysis by SPR The different interactions between each detergent and the lipid bilayers were considered one of the factors leading to different effects on membrane permeability and solubilization, as described above. SPR can easily capture lipid vesicles on chips in cases where alkane groups have been introduced to the dextran matrix. Thus, this method, using drug-bilayer interaction analysis, has been applied to predict intestinal permeability of drugs as an alternative to parallel artificial membrane permeability assay (PAMPA).[22,23] We, therefore, attempted to observe the interactions between detergents and lipid bilayers. That is, the more detailed information than DLS, how detergent interfaces or is inserted into the lipid bilayers as well as the process of solubilization such as membrane saturation with detergent and formation of mixed micelle, could be observed. Figure 3 outlines the complete sensorgram of measurement cycle and Fig. 4 shows the responses obtained after the injection of detergent. In the sensorgrams, the increasing RU corresponds to the interface or dispersion of detergent to lipid bilayers during the injection of detergent, and the decreasing RU corresponds to the dissociation of lipids or lipid particles from the sensor chip.

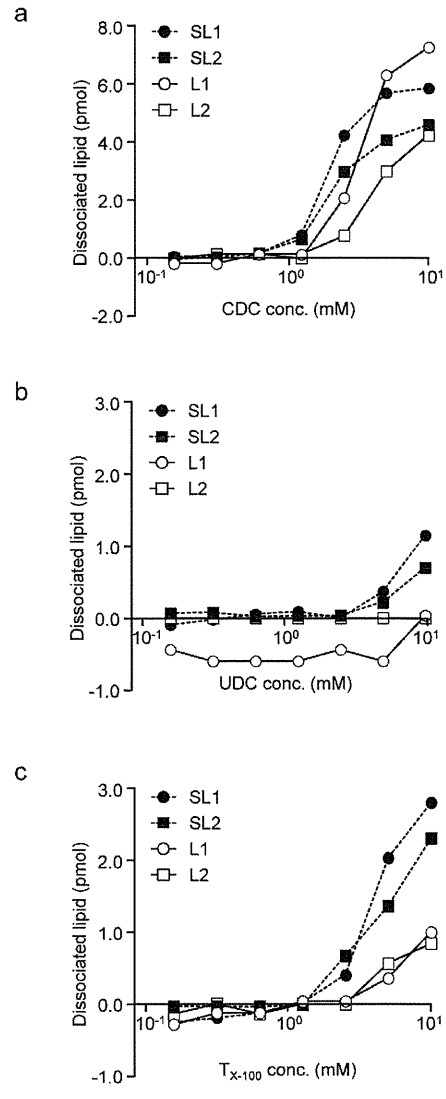

Fig. 5. Effects of Lipid Composition on Detergent-Induced Dissociation of Lipid Bilayers

Dissociated lipid (pmol) was plotted against detergent concentrations. SL1, HSPC/Chol/DSPE-PEG2000 (79/16/5 mol/mol); L1, HSPC/Chol (5/1 mol/mol); SL2, HSPC/Chol/DSPE-PEG2000 (55/40/5 mol/mol); L2, HSPC/Chol (6/4 mol/mol).

In SPR analysis, CDC exhibited the highest solubilizing ability among the 3 detergents tested and significantly induced the dissociation of lipids from 4 kinds of liposomes (Fig. 4a). Binding to the interface and incorporation of UDC into lipid bilayers occurred in a dose-dependent manner (Fig. 4b). In case of $T_{X\text{-}100}$, the binding to the normal liposomes L1 or L2 increased with higher detergent concentrations, and slight dissociation was observed. However, $T_{X\text{-}100}$ caused a significant dose-dependent dissociation in the PEGylated liposomes (Fig. 4c). Figure 5 shows the amount of dissociated lipid, an index of membrane solubilization, as a function of the concentration of each detergent. The amount of dissociated lipid was calculated from the difference between the equilibrium RU after liposome capture and that after the injection of detergent. These results correlated in part with the results for membrane

permeability (Figs. 1a–c), indicating that the lipid membrane of liposomes was partially solubilized and converted to mixed micelles in the presence of detergent, and that DXR-release was significantly increased. Therefore, it was clearly indicated that $T_{X\text{-}100}$ induces DXR release from PEGylated liposomes, because $T_{X\text{-}100}$ can exhibit higher solubilization of PEGylated liposomes *versus* normal liposomes.

The membrane solubilization induced by detergent has been evaluated by turbidity (spectral photometer) and particle size (DLS).[28] The partitioning of detergent into bilayers has been investigated using thermodynamic analyses, including isothermal titration calorimetry, differential scanning calorimeter, and ^{31}P-NMR.[29,30] To assess a more detailed mechanism of membrane solubilization, electron microscopy and dark-field microscopy have been used.[11,12] In the SPR technique used in this study, there are no standard parameters for solubilization compared with the above-indicated physicochemical approaches. However, using SPR, we observed that the detergent was either bound to or partitioned into bilayers. Subsequently, lipid bilayers were solubilized and dissociated from the chip, and it was found that the interactions of detergents with lipid bilayers differ depending on the kind of detergent. While a detailed comparison of the SPR results with the physicochemical approaches is needed, SPR may be a simple and efficient method for monitoring the solubilization and interaction of detergents with lipid bilayers because of its automated measurements.

The liquid-ordered phase of bilayers is known to be formed in the membrane consisted of saturated phospholipid, such as DPPC or distearoyl phosphatidylcholine (DSPC), and Chol. It was reported that DPPC/Chol membranes containing a high ratio of Chol (≥25 mol%) which are in lo phase were stable and resistant to solubilization by $T_{X\text{-}100}$.[15,27] In the case of CDC, it was indicated that the presence of Chol exhibited no significant effect on the induced drug release.[13] Our results mostly agreed with those of previous reports in which normal liposomes containing higher ratio of Chol were much more resistant to solubilization by $T_{X\text{-}100}$, although there were no significant differences in the permeability enhancement and solublization by CDC based on lipid composition. On the other hand, in our results, the significant differences in membrane permeability and solubilization in the presence or absence of PEG-lipid were observed when mixed with $T_{X\text{-}100}$. In general, the solubilizing ability of detergent is higher for solubilizate molecules which have higher polarity. The structure of $T_{X\text{-}100}$ is similar to that of PEG-lipid, which consists of a big hydrophilic group (a flexible polyethylene chain) and carbon chains. Therefore, while the details remain unclear, we hypothesized that PEG-lipid can easily form mixed micelle with $T_{X\text{-}100}$ because of its high polarity and structural similarity with $T_{X\text{-}100}$.

Our ultimate goal was to determine whether detergents could be applicable to *in vitro* drug-release testing of liposomal products. Because drug release from liposomes in normal buffered saline or serum/plasma takes several days, achieving a shortened test time would be a primary consideration in developing new *in vitro* release test. Our study demonstrates that the addition of detergents to test media can shorten the test time, with up to 100% release in 1 h. The lipid composition, especially the content of PEG-lipid, is a critical factor in determining the *in vivo* behavior of liposomal products. From this perspective, testing the enhancement of release with $T_{X\text{-}100}$, which can distinguish lipid compositions, may be a useful tool in developing new test methods. Further, because the purpose of evaluation is to assess quality control for efficacy and safety, biorelevance should also be considered in developing *in vitro* release tests. That is, the correlation of *in vitro* release rates with *in vivo* drug release mechanisms and behaviors of liposomal product may be required. FDA has proposed the investigation of *in vitro* drug leakage under multiple conditions in the draft guidance for generic version of doxorubicin-encapsulated liposomes (DOXIL®).[8] It is considered to appropriately characterize the drug release from liposomal products by combining different testing conditions, such as under biorelevant conditions for a given period of time to confirm the stability of liposomes, and under physicochemical stress to evaluate the physical state of lipid bilayers. Therefore, while detergent-induced drug release may not mimic the *in vivo* behavior of liposomal products, it could be useful for process control or quality control of liposomal products when using a combination of biorelevant methods.

Conclusion

Our results indicate that the differences in the membrane solubilization of PEGylated liposomes compared with normal liposomes vary depending on the detergent. $T_{X\text{-}100}$, for example, induced significant membrane permeabilization and solubilization of PEGylated liposomes compared with normal liposomes, while other detergents did not have this differential effect. Further, on the basis of SPR analysis, we suggest that this difference is due to the higher degree of solubilization exhibited by $T_{X\text{-}100}$ for PEGylated membranes than for normal membranes.

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Comparison of Particle Size and Dispersion State among Commercial Cyclosporine Formulations and Their Effects on Pharmacokinetics in Rats

Hiroko Shibata,* Haruna Saito, Toru Kawanishi, Haruhiro Okuda, and Chikako Yomota

National Institute of Health Sciences; 1–18–1 Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan.
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Generic versions of Neoral, a microemulsion capsule formulation of cyclosporine, have been approved worldwide. However, there are concerns about the quality and efficacy of the generics due to the formulation specificity and differences in inactive ingredients among products. In this study, we measured the physicochemical properties of both the innovator and the generic formulations, and compared their bioavailability in rats. When the capsule contents were dispersed in water, the absorbance (600 nm wavelength) of generic products was higher than that of the innovator. Whereas the dispersion solution of the innovator in Fed State Simulated Intestinal Fluid was nearly clear, that of all the generics became white and turbid. The mean diameter of the microemulsion (or emulsion) formed in water by the generics was 39.7, 57.7, 64.5, and 74.8 nm, all of which were larger than that of the innovator (26.4 nm). Although the T_{max} of the generics tended to be long relative to that of the innovator, there were no significant differences between the innovator and generics with regard to maximum blood concentration (C_{max}) or area under the curve (AUC). These results suggest that the physicochemical differences between the innovator and the generics will not have a significant effect on C_{max} or AUC, which is necessary to ensure bioequivalence.

Key words microemulsion; emulsion; biorelevant medium; innovator; generic

Cyclosporine (CsA) is an immunosuppressive agent,[1,2] and is categorized as a Biopharmaceutics Classification System (BCS) Class II drug with high lipophilicity[3] and low aqueous solubility.[4] One way to improve the aqueous solubility of such drugs is to prepare them as self-emulsifying formulations.[5,6] The first generation of orally administered formulations containing CsA consisted of a corn-oil-based solution encapsulated in soft gelatin (Sandimmune), which is now referred to as a "self-emulsifying drug delivery system" (SEDDS). The oily solutions are emulsified by bile salts, which form mixed micelles in the gastrointestinal fluid, and the CsA in these mixed micelles is then absorbed from the upper intestinal tract.[7] Thus, the absorption of CsA in Sandimmune is susceptible to the effects of bile acid secretion and the ingestion of food, resulting in variability of absorption within individual patients.[8,9]

To address the variability in absorption of Sandimmune, an improved formulation of CsA, Neoral, has been developed. Neoral is a microemulsion pre-concentrate formulation, which has recently been referred to as a "self-microemulsifying drug delivery system" (SMEDDS).[6] After oral administration of Neoral, a microemulsion with stable dispersibility is easily formed in the intake water or gastric fluid, and the drug is quickly absorbed from the upper intestinal tract. Therefore, when compared to Sandimmune, Neoral demonstrates a significantly higher and more consistent absorption profile that is unaffected by bile acid secretion or food consumption.[10–12]

The need to reduce healthcare costs in many countries has led to the production of generic substitutions for original drugs. Four generic versions of Neoral have already been approved in Japan; in other countries, several generics have been marketed, such as Gengraf, Eon, and Cicloral. In the U.S.A., SangCya, which is the liquid generic form of Neoral, was recalled because it is not bioequivalent to Neoral when administered with apple juice.[13] There are reports that in transplant recipients the area under the curve (AUC) and maximum blood concentration (C_{max}) values of the generic tended to be lower than those of the innovator, and that the bioavailability of the innovator decreased when administered after a fat-rich meal, whereas that of the generic increased.[14,15] A recent report at a scientific meeting in Japan also indicated that the physical appearance and particle diameter of generics were different from those of the innovator.[16] In addition, another group reported that after oral administration in rats, the pharmacokinetics of CsA metabolites differed between the innovator and generics.[17]

Thus, as we describe above, there are concerns about the quality and efficacy of generics due to the formulation specificity of the innovator and the narrow therapeutic window of CsA. To our knowledge, there have been no direct comparisons of innovator and generics using both *in vitro* and *in vivo* assessment. Therefore, in this study, we assessed the physicochemical properties of various commercial formulations of CsA when dispersed in solution, such as their physical appearance and particle diameter. We examined the oil-based formulation Sandimmune, the microemulsion formulation Neoral, and 4 generic products of Neoral that are approved by the regulatory agency in Japan. In addition, we compared the pharmacokinetics of CsA and its metabolites for these formulations, and investigated whether or not the differences in physicochemical properties are likely to affect their pharmacokinetics.

Experimental

Materials Six cyclosporine A (CsA) capsule products were purchased from a general sales agency for drugs in Japan and used in this study: Sandimmune® capsule, 50 mg (Product A, Lot No. S0016; Novartis Pharma K.K., Basel,

The authors declare no conflict of interest.

* To whom correspondence should be addressed. e-mail: h-shibata@nihs.go.jp

Switzerland); Neoral® capsule, 50 mg (Product B, Lot No. S1046; Novartis Pharma K.K., Basel, Switzerland); Amadora® capsule, 50 mg (Product C, Lot No. 34006; TOYO CAPSULE Co., Ltd., Shizuoka, Japan); Cicporal® capsule, 50 mg (Product D, Lot No. EC2501; Nichi-Iko Pharmaceutical Co., Ltd., Toyama, Japan); cyclosporine capsule, 50 mg "Mylan" (Product E, Lot No. 0450RH; Mylan Seiyaku, Tokyo, Japan); and cyclosporine capsule, 50 mg "FC" (Product F, Lot No. 9C1; Fuji Capsule Co., Ltd., Shizuoka, Japan). The official CsA reference standard was purchased from the Phramaceutical and Medical Device Regulatory Science Society of Japan. Sodium taurocholate and lecithin were purchased from Wako Pure Chemical Industries (Osaka, Japan). Cyclosporin D (CsD) (ALEXIS® Biochemicals) was purchased from Enzo Life Sciences (Farmingdale, NY, U.S.A.). Rat liver microsomes were obtained from Celsis In Vitro Technologies (Baltimore, MD, U.S.A.). β-Nicotinamide-adenine dinucleotide phosphate (NADP), glucose-6-phosphate 1-dehydrogenase (G-6-PDH), and glucose-6-phosphate (G-6-P) were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan).

Physicochemical Characteristics Eighty percent of the contents of a single capsule was placed in a test tube, and 10 mL of test medium was added. The solution was mixed by gentle inversion until the capsule contents were dispersed homogeneously in the test medium. Using this solution, 5-fold and 25-fold dilutions were prepared in different test tubes.

Preparation of Test Medium The 1st Fluid and 2nd Fluid for the dissolution test were prepared according to the Japanese Pharmacopoiea (JP)16. Fasted State Simulated Intestinal Fluid (FaSSIF) and Fed State Simulated Intestinal Fluid (FeSSIF) were prepared by the modified method reported by Galia *et al.* and Jantratid *et al.*[18,19] FaSSIF was formulated using approximately 900 mL of blank buffer, which was prepared by dissolving sodium chloride (4.01 g), sodium hydrate (1.39 g), and maleic acid (2.22 g) in 900 mL distilled water. The pH was then adjusted to 6.5. Sodium taurocholate (1.613 g) was dissolved in 50 mL of blank buffer, to which lecithin (0.15 g) was added and dissolved with heat and agitation until the solution became clear. The volume was adjusted to 1 L using the remaining blank buffer and distilled water. In the case of FeSSIF, sodium taurocholate (8.07 g) was dissolved in 50 mL of blank buffer (potassium chloride [15.20 g] and acetate [8.65 g] in 900 mL distilled water, pH 5.0), to which lecithin (2.81 g) was added and dissolved with heat and agitation until the solution became clear and yellow. The volume was adjusted to 1 L as with FaSSIF. Mixed micelles were not detected in either FaSSIF or FeSSIF when examined by a dynamic light scattering (DLS) photometer.

Absorbance To assess the degree of turbidity, the absorbance at 600 nm of each capsule sample was measured by a spectrophotometer (UV-2550/2450; Shimadzu, Kyoto, Japan) after mixing the samples with different test media.

Solubility The solubility of CsA in the dispersed solution was measured. The dispersed solution of each capsule sample was filtrated by 0.45 μm filter, and its concentration of CsA was measured by HPLC. The apparatus used for the HPLC system consisted of a constant pump (L-7200, Hitachi High-Technologies Corporation, Tokyo, Japan), a degasser (L-7610, Hitachi), an autoinjector (L-7200, Hitachi), a column oven (L-7300, Hitachi), an UV detector (214 nm) (L-7405, Hitachi), and a system controller (D-7000, Hitachi). The separation was carried out at 70°C on a Inertsil ODS-3 (100×4.0 mm i.d., 5 μm) from GL Science (Tokyo, Japan). The mobile phase consisted of water–tetrahydrofuran (5 : 3.6), and flow rate was 1.0 mL/min. A standard stock solution of CsA was prepared by dissolving 10 mg of CsA in 10 mL of ethanol, and stored at 4°C. A 10 μL aliquot of a sample was injected.

Particle Size Distribution The size distribution and mean diameter of particles in the capsule content samples were measured using a DLS photometer DLS-7000 (Otsuka Electronics Co., Ltd., Osaka, Japan) equipped with an He–Ne laser source (wavelength, 632.8 nm) after mixing the samples with different test media. All DLS measurements were made with a scattering angle of 90°. The neutral density filter was adjusted depending on intensity. Data were gathered with a counting period of 100 s. Histogram analysis was performed to assess the particle size distribution, and cumulant analysis was performed to calculate the mean diameter. The data between different products were statistically analyzed using a one-way analysis of variance followed by Dunnett's test.

The number of large-diameter particles (>0.5 μm) in the solution of the capsule content in 10 mL water was measured by an Accusizer 780A instrument (Particle Sizing Systems, Santa Barbara, CA, U.S.A.). This instrument is based on light extinction (LE) or light scattering (LS) that employs a single-particle optical sizing (SPOS) technique, and was equipped with an automatic dilution system. In this study, the summation mode, which is a combination of LE and LS, was applied. Duplicate measurements were made for each sample at the appropriate time point using the following conditions: data collecting time, 60 s; flow rate, 60 mL/min; injection loop volume, 1.04 mL; syringe volume, 2.5 mL; second dilution factor, 40.

Sample Preparation for Assay A 100 μL aliquot of each blood sample was transferred to a microtube. A 200 μL aliquot of internal standard (IS) solution (8.3 ng/mL of CsD in methanol–0.3 mol/L $ZnSO_4$, 7 : 3 v/v) was added to each tube. Tubes were tapped and vortexed for a few minutes until the pellet was completely dispersed. After centrifugation at 12000 rpm for 5 min, the supernatant was filtered by a centrifugal filter device (Ultrafree-MC, 0.22 μm polyvinylidene difluoride (PVDF); Millipore, Billerica, MA, U.S.A.). After further centrifugation at 10000 rpm for 2 min, the filtered sample was directly applied to the liquid chromatography/mass spectrometry (LC/MS) system.

Due to difficulty in obtaining reference standards of CsA metabolites, the *in vitro* metabolic reaction was performed by following the method for rat liver microsomes, and reactants containing metabolized CsA were used to confirm the LC separation of CsA and its metabolites. First, a reduced nicotinamide adenine dinucleotide phosphate (NADPH) regenerating system (NRS; 1.7 mg/mL NADP, 7.8 mg/mL G-6-P, 6.0 units/mL G-6-PDH in 2% (w/v) $NaHCO_3$) was prepared. A 50 μL aliquot of rat liver microsomes, 5 μL of 500 ng/mL CsA in acetonitrile, and 320 μL of 50 mM Tris buffer were mixed in a microtube, and then pre-incubated at 37°C for 5 min. Next, 125 μL NRS was added and the solution was thoroughly mixed. After incubation at 37°C for 60 min, 500 μL internal standard solution was added to terminate the reaction. After centrifugation at 10000 rpm for 5 min, the supernatant was filtered, as described above, and applied to the LC/MS system.

Assay for Cyclosporine A and Its Metabolites CsA and its metabolites in whole blood were measured by LC/MS

system in accordance with Koseki *et al.*,[20] with some modifications. LC/MS was performed on a Shimadzu LCMS-2010 system that includes a constant pump, column thermostat, degasser, autosampler, and quadrupole mass spectrometer (Shimazdu Corporation, Kyoto, Japan). The HPLC column was a Symmetry C8 (4.6×75 mm, 3.5 μm; Waters, Milford, MA, U.S.A.) with a guard column (Opti-Guard-min C8, 1×15 mm; Optimize Technologies, Oregon City, OR, U.S.A.). LC/MS grade water and methanol were prepared as mobile phase A and B, respectively. The flow rate was set to 0.3 mL/min and the column temperature was 80°C. A linear gradient separation was used, with 72% of mobile phase B from 0 to 1 min, then 72% to 85% of mobile phase B over 5 min, holding for 3 min, and finally 72% of mobile phase B over 6 min. The total run time was 15 min for each injection. A 20 μL aliquot of each prepared sample was injected.

The mass spectrometer was interfaced with an electrospray ionization (ESI) source used in the positive ion mode. The following parameters were retained for optimal detection of all analytes: nitrogen gas flow rate, 1.5 L/min; interface voltage, 4.5 kV; desolvation line voltage and temperature, 20.0 V and 250°C, respectively; block heater, 200°C. For the determination of CsA and its metabolites as well as CsD, the sodium adducts were measured at *m/z* 1210.9 (AM4N), *m/z* 1224.9 (CsA), *m/z* 1238.9 (CsD, IS), and *m/z* 1240.9 (AM1, AM9, AM1c) by using selected ion monitoring (SIM). Retention times for AM1, AM9, AM1c, AM4N, CsA, and CsD were 8.6, 8.9, 9.5, 10.4, 11.4, and 12.2 min, respectively. Quantification of CsA and its metabolites was achieved with a calibration curve of CsA (concentration range, 7.8–500 ng/mL). The limit of detection (signal-to-noise ratio, 3) and quantification (signal-to-noise ratio, 10) of CsA was approximately 1.5 ng/mL and 5 ng/mL, respectively.

Animal Study The animal experiments were outsourced to Charles River Japan and performed in accordance with the Guideline for Animal Experiments of Charles River Japan. Male Sprague-Dawley rats weighing 220–250 g were fasted overnight with free access to water. The content of each CsA capsule product was diluted in distilled water to obtain a CsA concentration of 1.0 mg/mL. The CsA solution equivalent of 3.5 mg/kg CsA was orally administered to rats using a stomach sonde. Next, blood samples (300 μL) were collected from the jugular vein at 0.5, 1, 2, 3, 6, 12, and 24 h using a syringe flushed with 100 mg/mL ethylenediaminetetraacetic acid (EDTA), and stored at −80°C until analysis by LC/MS. The pharmacokinetic parameters of CsA and its metabolites, such as *AUC*, C_{max}, and time to reach C_{max} (T_{max}), were estimated by non-compartmental analysis using WinNonlin (version 5.2; Pharsight Corporation, Sunnyvale, CA, U.S.A.). The data between different products were compared for statistical significance by a Kruskal–Wallis test.

Results

Physicochemical Characteristics Neoral, a microemulsion pre-concentrate formulation, is composed of lipophilic solvent, hydrophilic solvent, surfactant, and drug. Table 1 shows the difference in additive composition of CsA capsule contents among products. First, we removed the contents of each capsule and compared the physical appearances before dispersion. Product A was the oil-based formulation Sandimmune, Product B was the innovator Neoral, and Products C to F were generic formulations. As seen in Fig. 1a, Product A and Product F were yellow, Product B and Product E were slightly yellow, and Product C and Product D were almost clear. Next, we dispersed the contents of 1 capsule in 10 mL of each test medium and compared the physical appearances (Fig. 1b). For the test media, water, 1st and 2nd Fluids for the dissolution test, artificial intestinal juice, Fasted State Simulated Intestinal Fluid (FaSSIF), and Fed State Simulated Intestinal Fluid (FeSSIF) were used. For all capsules the contents were homogeneously dispersed in each test medium except for Product A. The dispersion liquid of SEDDS is typically turbid and inhomogeneous, whereas that of SMEDDS is usually nearly clear. Thus, the dispersion state of the generics was obviously different from that of Product A (SEDDS). When the capsule contents were dispersed in water, Product B and Product E produced a clear and almost clear solution, respectively; Product F produced a white solution; and Product C and D produced bluish milky solutions. Assessment using the other test media indicated the same tendency. On the other hand, when the capsule contents were dispersed in FeSSIF, all generic products produced a white cloudy solution, whereas Product A produced a clear solution.

To quantify the degree of turbidity, we measured the absorbance at 600 nm of the dispersion solutions. The absorbance of the solutions formed by dispersion in water was high and showed the following order: B≈E<C=D<F<<<A. This same tendency was observed for the other test media (Fig. 2). These results correlated with the physical appearance of the solutions, including the significantly higher absorbance of generics dispersed in FeSSIF. In the case of Product F, a slight precipitate was formed in the dispersion solution several hours after initial dispersion (data not shown). The absorbance of Products B through E in each test medium decreased with increasing dilution, and no creaming or precipitation was observed. The solubility of dispersion solution was measured and found that there were no differences between the innovator and the generics with regard to solubility.

The distribution and number of particles of the microemulsion (or emulsion) in the dispersion solution were determined. The contents of 1 capsule were dispersed in 10 mL of water, and the distribution and number of particles (>0.5 μm) were measured by the single-particle optical sizing (SPOS) method. As seen in Fig. 3a, the particle size of the oil-based formulation Product A was significantly larger than that of the other products. Whereas the particle number (>0.5 μm) of products B and E was almost the same, that of Products C and D was 5-fold larger, and that of Product F was 25-fold larger, than that of Product B, which correlates with their physicochemical appearances (Fig. 3b). These results indicated that the particle distribution of generic products in solution is wider than that of the innovator.

The mean diameter and distribution of the particles of the microemulsion (or emulsion) in the dispersion solution formed from the capsule contents were measured by dynamic light scattering (DLS) (Fig. 4, Table 2). The mean diameter of each product in water at a 5-fold dilution was as follows: Product B, 26.4 nm; E, 29.7 nm; C, 74.8 nm; D, 64.5 nm; and F, 79.2 nm. Thus, the mean diameter of the generics tended to be larger than that of the innovator. The same tendency was observed in 1st and 2nd Fluids for the dissolution test, and FaSSIF. When the capsule contents of the generics were dispersed

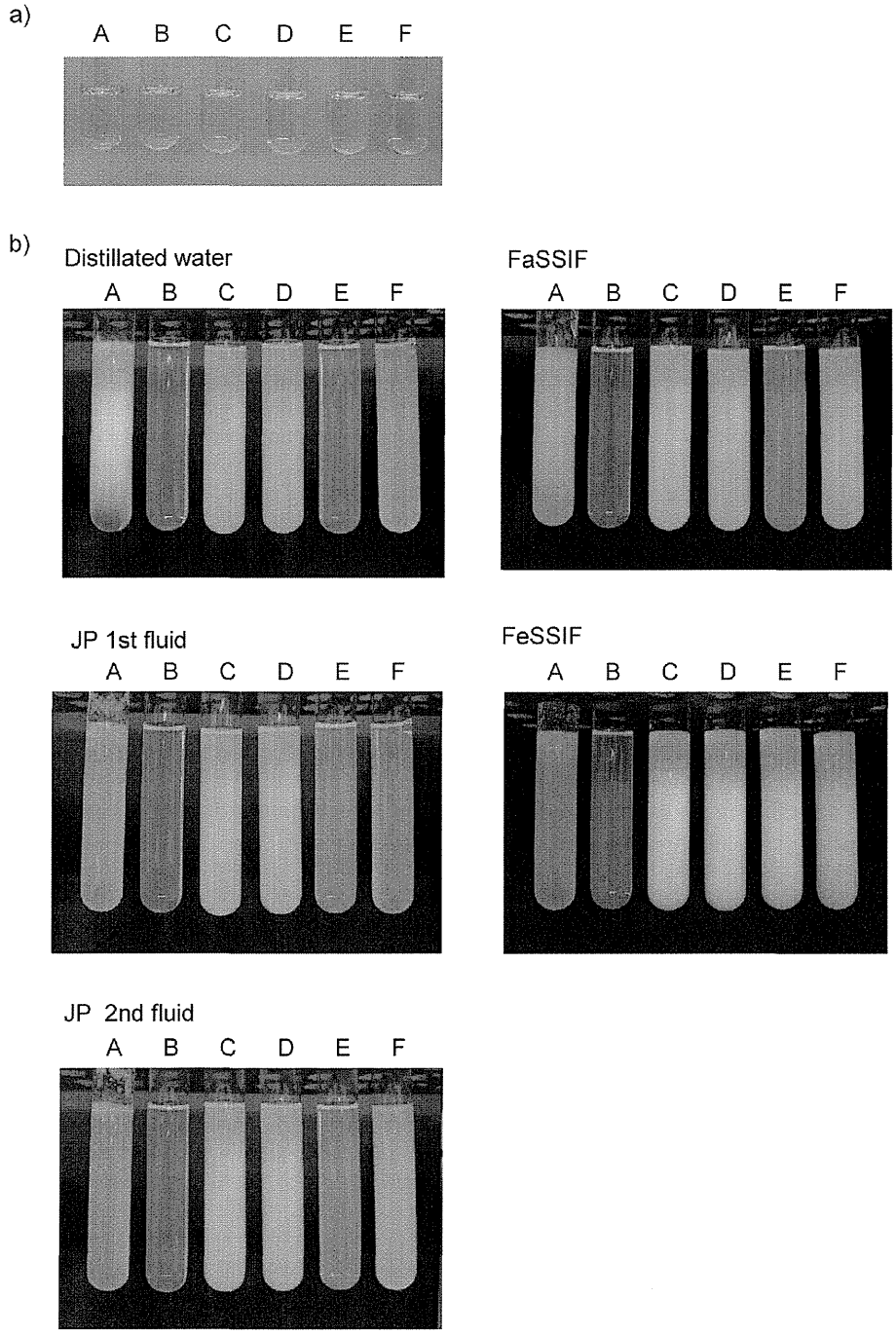

Fig. 1. Physical Appearances of (a) the Contents of Each CsA Capsule and (b) the Solution of the Contents of 1 CsA Capsule in 10 mL Water, 1st Fluid, 2nd Fluid, FaSSIF, and FeSSIF

in FeSSIF, the mean diameter of the generics increased to 100–200 nm. Among the generic products, dispersion of Product F in either the 2nd fluid or FaSSIF increased the mean diameter to 120–200 nm (Table 2), and the particle distribution expanded with increasing dilution (Fig. 4). In addition, when Product F was dispersed in either water or the 1st Fluid, the value of the mean diameter varied widely when compared with those of the other products (Table 2). These results, including those seen in Fig. 3, indicated that the particles in the dispersion solution of Product F could not be homogeneous. On the other hand, the mean diameter of the innovator, Product B, was very small (about 30 nm) in water, and in both 1st and 2nd test Fluids, and there were no changes in the mean diameter or distribution even when its capsule contents were dispersed in either FaSSIF or FeSSIF containing lecithin and taurocholic acid. This indicated that, after oral administration, a microemulsion of Product B is likely to be formed in the gastrointestinal tract. The mean particle diameter and distribution were not determined in the 1-fold and 5-fold dilutions of Product A because of the high scattering intensity. The small mean diameter of the generics in FeSSIF may be caused by multiple scattering.

Table 1. Composition of CsA Capsule Contents

	Product A	Product B	Product C	Product D	Product E	Product F
Solvent	Esterified corn oil	Glycerol esters of fatty acids	Propylene glycol esters of fatty acids	Propylene glycol esters of fatty acids	Propylene glycol esters of fatty acids	Propylene glycol esters of fatty acids
	Ethanol	Propylene glycol			Triethyl citrate	
	Corn oil	Ethanol			Sorbitan monolaurate	Ethanol
Surfactant		Polyoxyethylene hydrogenated castor oil	Polyoxyl 35 castor oil	Polyoxyl 35 castor oil	Polyoxyl 35 castor oil	Polyoxyl 35 castor oil
Other		Tocopherol	Other two components	Other two components		Other five components

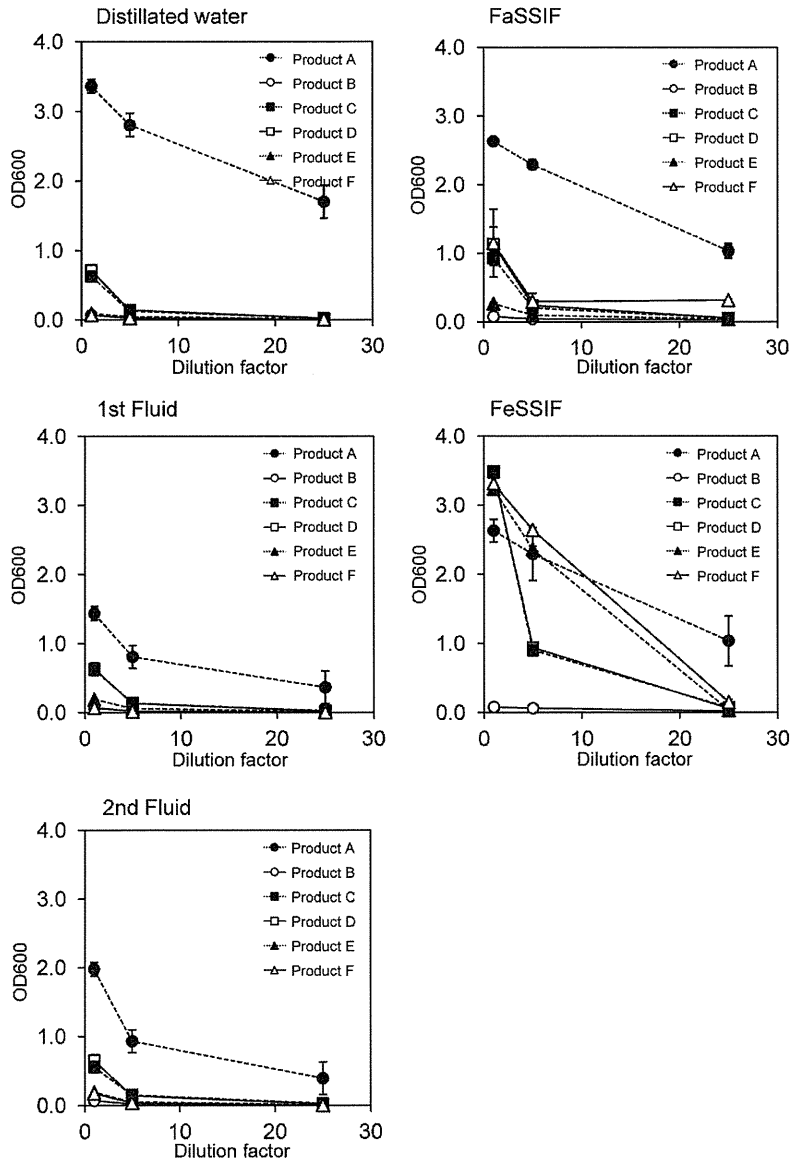

Fig. 2. Absorbance (600 nm) of the Suspension of the Contents of 1 CsA Capsule in 10 mL of Test Media and Diluted Solutions Thereof
Data are represented as the mean±S.D.

Pharmacokinetics of CsA and Its Metabolites in Rats
The results described above demonstrate that, although all generic products were dispersed in each test medium unlike Product A, the physical appearance and particle diameter of the generic dispersion solutions were different from those of the innovator Product B. Therefore, we next assessed the blood concentration profiles of CsA and its metabolites (AM1, AM9, AM1c, and AM4N) after oral administration in rats (Fig. 5). The blood concentration was measured by liquid chromatography-mass spectrometry (LC-MS) analysis, and the

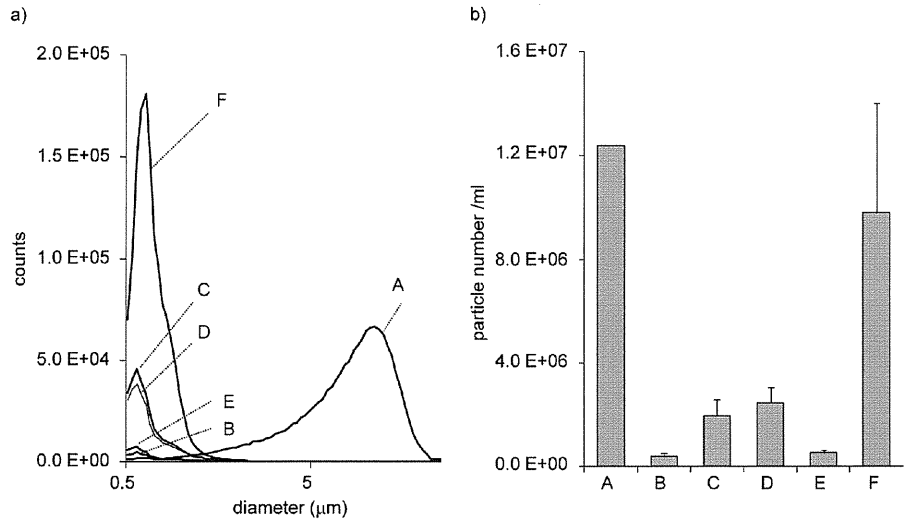

Fig. 3. Particle Distribution (a) and Particle Number (>0.5 μm) (b) of CsA Lipid Particles in Water
Each value represents the mean±S.D. (n=3).

pharmacokinetic parameters *AUC*, C_{max}, and T_{max} were analyzed as indicated in Table 3. The pharmacokinetic parameters of AM4N are not shown in Table 3 because its blood concentration in many samples was below the lower limit of quantification. The blood concentration of CsA increased rapidly, and that of its metabolites increased subsequently (Fig. 5). The C_{max} (671±95 ng/mL) and *AUC* (7194±507 h·ng/mL) of the innovator Product B was obviously higher than the C_{max} (474±60 ng/mL) and *AUC* (5839±371 h·ng/mL) of the oil-based formulation Product A (Table 3), a finding that was consistent in principle with previous reports.[21,22] The C_{max} and *AUC* of the generic products also tended to be higher than those of Product A. The C_{max} of the 4 generic products tended to be slightly lower than those of Product B. Again, however, there were no significant differences between Product B and the generic products in either C_{max} or *AUC*. Likewise, no significant differences were observed in the pharmacokinetics of CsA metabolites AM1, AM9, or AM1c. Koehler *et al.* reported that the bioavailability of a CsA generic product (Eon Labs) in rats was lower than that of Neoral, whereas the plasma AM4N level was significantly elevated in groups receiving Eon compared to that in another group receiving Neoral.[17] In our data, a significantly elevated AM4N blood level was not observed in groups treated with generic products compared with that of the group treated with Neoral, Product B. In rats, CsA undergoes first-pass metabolism by CYP3A, which is located in the gastrointestinal mucosa and in the liver. Therefore, these results suggest that the CsA contained in the generic products tested in this study was absorbed *via* the same pathway used for the CsA in the innovator. We performed the same examination again and confirmed that the bioavailability of the generic products was similar to that of the innovator. Only the T_{max} differed significantly between Product B and the generic products. This same significant delay in T_{max} of the generic products was also observed in the second experiment.

Discussion

Regions corresponding to different phases of the formulation, such as microemulsion, emulsion, micelles, or reverse micelles, are described in a ternary phase diagram according to different concentrations of each component (such as water, surfactant, and oil).[6] The variations in components, such as the presence or absence of co-surfactants/co-solvents and different types of oil, also result in the formation of different phase regions. Additive compositions of generic products of CsA are different from that of the innovator (Table 1). Actually, in this study, the physical appearance of the generic products in water was different from that of the innovator. Therefore, the phase of dispersion solution of the generic products might be near the emulsion phase, or might be a mixture of emulsion and microemulsion.

In a study of microemulsion formulation, when the optimized microemulsion pre-concentrate was dispersed in FeSSIF, the particle size remained small in the dispersion solution (20–50 nm).[23] In our study, the dispersion solution of the generics in FeSSIF was cloudy white like milk, whereas that of the innovator was clear as in water. Although the details were unclear, the formulations of the generic product could be susceptible to taurocholate and lecithin in FeSSIF, and the phase regions of their solution dispersed in FeSSIF could be shifted to another phase region. From these points, we also hypothesize that the 4 generic products are self-emulsifying formulations, but their phase states are different from those of the innovator Product B and the oil-based Product A.

The relationship between particle size and bioavailability in CsA microemulsion or emulsion formulations has been investigated in humans.[24] In this previous report, the *AUC* increased as the particle size decreased, and only the formulation whose particle size was under 100 nm exhibited a desirable bioavailability. However, the type of surfactant used for the formulation with large particles (>150 nm) was different from the formulation with small particles (<60 nm); thus, as reported in another study,[25] bioavailability can be affected not only by particle size but also by the characteristics of surfactants on the particle surface. The improved bioavailability provided by self-emulsifying formulations is believed to be due to a larger particle surface area, improved aqueous solubility of drugs, and the enhancement of intestinal membrane permeability produced by local disturbance of the cell membrane.[26] These mechanisms will be enhanced by the properties of the

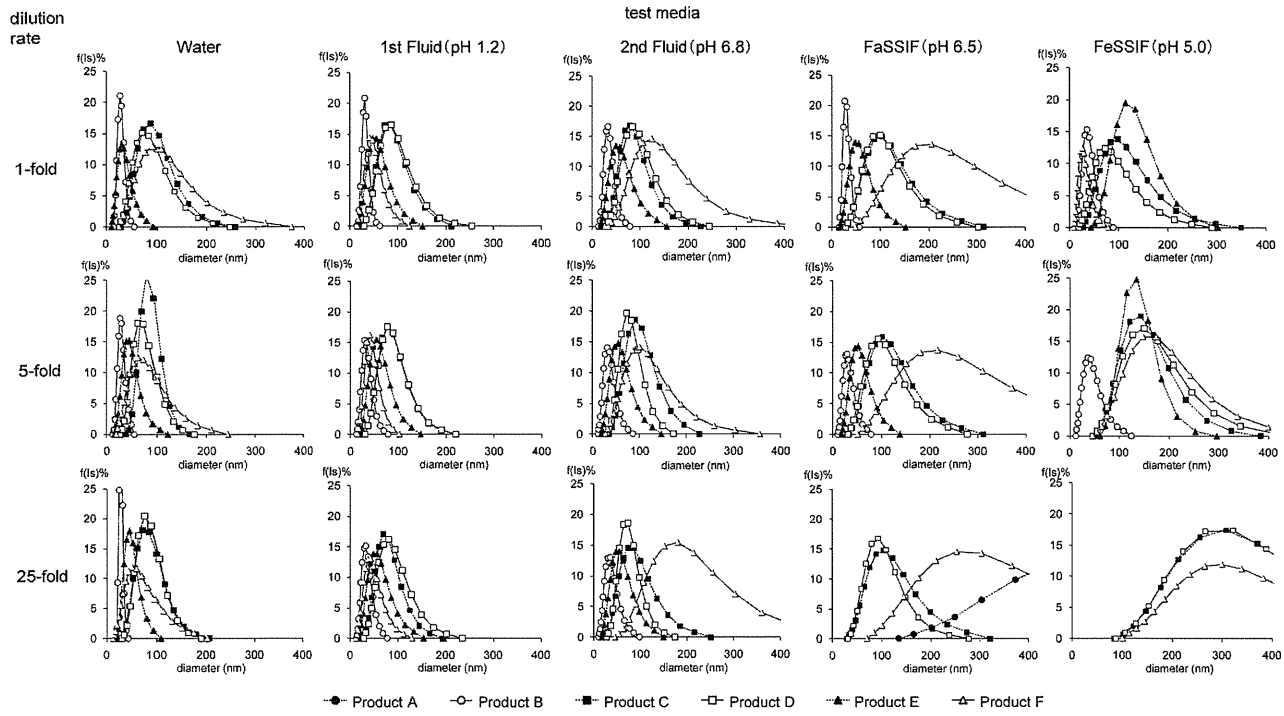

Fig. 4. Effects of Dilution and Test Media on the Size Distribution of CsA Lipid Particles

The contents of each capsule were dispersed in 10 mL of test medium, and further diluted 5-fold and 25-fold with the test medium. The size distribution of CsA lipid particles in the suspension was measured by a dynamic light scattering method.

Table 2. Particle Size of CsA Lipid Particles in Each Solution

	Dilution factor	Product A	Product B	Product C	Product D	Product E	Product F
Water	1	ND[a]	27.1±0.5	74.3±4.1[b]	68.7±3.7[b]	27.0±1.2	92.8±17.0[b]
	5	ND[a]	26.4±1.3	74.8±6.6[b]	64.5±8.7[b]	39.7±2.1	79.2±17.2[b]
	25	1423.6±1369.8	27.2±2.7	67.9±10.0[b]	65.1±6.7[b]	43.6±2.7[c]	67.7±12.3[c]
1st fluid (pH 1.2)	1	ND[a]	28.6±1.1	71.1±3.0[b]	69.8±2.0[b]	44.3±0.3[b]	80.8±61.7
	5	ND[a]	28.5±0.3	70.3±4.9[b]	72.5±1.9[b]	47.5±1.4[b]	102.3±106.1
	25	3075.2±1617.3	28.3±0.7	65.9±4.5[b]	66.7±3.8[b]	46.2±1.9[b]	43.2±0.8
2nd fluid (pH 6.8)	1	ND[a]	28.3±1.3	72.3±8.0[c]	67.6±3.0[c]	45.5±3.0	137.8±45.8[b]
	5	ND[a]	28.2±1.6	74.5±6.6[c]	68.8±2.6	45.7±2.0	121.7±62.1[b]
	25	4900.3±4128.6	29.3±2.2	68.4±4.6	66.5±2.9	46.8±2.6	197.6±88.8[b]
FaSSIF (pH 6.5)	1	ND[a]	26.5±0.9	83.4±10.9[b]	74.0±5.7[b]	42.0±1.0	136.3±23.4[b]
	5	ND[a]	25.3±1.6	84.4±7.3[b]	80.2±5.3[b]	41.3±1.3	151.4±29.5[b]
	25	388.6±65.0	ND[a]	79.5±11.5	78.7±4.5	ND[a]	204.4±23.1
FeSSIF (pH 5.0)	1	ND[a]	30.0±1.1	72.6±3.5[b]	58.7±1.1[b]	103.6±3.0[b]	22.2±3.7[c]
	5	ND[a]	29.8±0.9	122.1±4.5[b]	123.5±4.7[b]	122.4±1.7[b]	137.9±8.56[b]
	25	1995.4±2169.9	ND[a]	254.9±6.4[b]	256.9±9.0	ND[a]	264.1±18.1

Each value represents the mean±S.D. (n=3). *a*) Not determined. *b*) $p<0.01$ compared to Product B. *c*) $p<0.05$ compared to Product B.

surfactant, as will particle size. Although the oil or other components of generics are different from those of the innovator, the same type of surfactant, a polyoxyethylene castor oil derivative, is used for all of these products. Therefore, it is possible that there were no significant differences in the bioavailability of CsA between the innovator and the generics because the same type of surfactant is used.

On the other hand, the T_{max} of the generic products was longer than that of the innovator. The particle size of the innovator Product B can remain small in gastrointestinal tract, because, unlike the generic products, the particle size of the innovator Product B did not vary in any test solutions. Thus, we think that the difference in particle size between the innovator and the generics can affect T_{max}, the pre-concentrate of the generics can be dispersed homogeneously in gastrointestinal fluid with the same degree of small particle size, with sufficiently low variability to prevent differences in AUC or C_{max} at the site of drug absorption. In the bioequivalence guidelines (BE guidelines) published in Japan ("Guideline for Bioequivalence Studies of Generic Products" http://www.nihs.go.jp/drug/DrugDiv-E.html), T_{max} is generally not required to be equivalent because T_{max} is a variable parameter. The interval of administration of CsA capsules is long (about 12 h) and the maximal change in the rate of approximate $T_{1/2}$ was less than

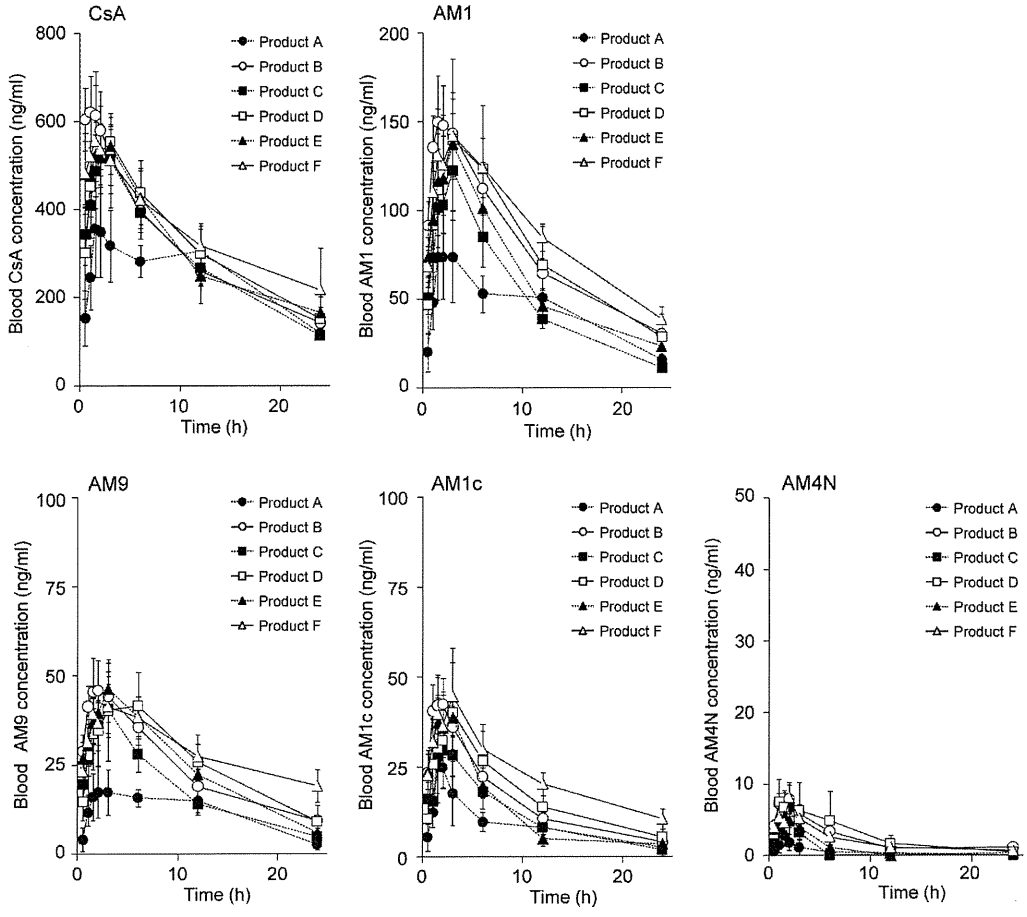

Fig. 5. Pharmacokinetics Profiles of CsA and Its Metabolites (AM1, AM9, AM1c, and AM4N) after Oral Administration (3.5 mg/kg) of CsA Lipid Particles in Water to Rats

Each point is the mean of values obtained from 5 rats, and the vertical bar represents the standard error.

Table 3. Pharmacokinetics Parameters of CsA after Oral Administration of CsA Lipid Particles to Rats

	Product A	Product B	Product C	Product D	Product E	Product F
CsA						
T_{max} (h)	7.00±2.14	1.10±0.21[a)]	2.30±0.44[a,b)]	2.90±0.87[a,b)]	2.70±0.89[a,b)]	2.85±1.01[a,b)]
C_{max} (ng/mL)	474±60	671±95	559±74	611±197	565±69	615±107
AUC (h·ng/mL)	5839±371	7194±507	6625±541	7454±2185	7105±721	7653±1502
AM1						
T_{max} (h)	7.00±2.14	3.15±1.55	2.70±0.30	3.80±0.97	2.70±0.89	3.17±0.79
C_{max} (ng/mL)	106±45	164±21	127±18	161±42	135±35	164±19
AUC (h·ng/mL)	1033±112	1675±99	1188±101	1798±472	1509±170	1743±212
AM9						
T_{max} (h)	7.00±2.14	3.10±1.55	3.00±0.84	4.80±0.73[b)]	3.00±0.84	4.30±1.49[b)]
C_{max} (ng/mL)	30±4	52±8[a)]	44±6[a)]	50±13[a)]	47±5[a)]	48±6[a)]
AUC (h·ng/mL)	278±27	519±56[a)]	414±26[a)]	604±142[a)]	566±30[a)]	607±107[a)]
AM1c						
T_{max} (h)	4.10±1.99	1.35±0.18	2.90±0.81[b)]	3.40±0.68[b)]	1.80±0.34[b)]	2.85±0.81[b)]
C_{max} (ng/mL)	29±7	46±8	33±4	45±17	39±6	50±9
AUC (h·ng/mL)	197±38	336±36	261±42	401±100[a)]	290±34	478±100[a)]

Each value represents the mean±S.E. (n=5). *a*) $p<0.05$ compared to Product A. *b*) $p<0.05$ compared to Product B.

10% between the innovator and the generics based on our results. Therefore, the difference in T_{max} would not be predicted to have a significant effect on the dosage schedule.

Our data show that the physical appearance of the 4 tested generics in FeSSIF was clearly different from that of the innovator, suggesting the possibility of variation in CsA absorption in the intestine under fed conditions, while in our study the administration was performed under the fasted condition only.