Effects of Fatty Acid Sucrose Esters on Cefitibuten Transport by Rat Intestinal Brush-Border Membrane Vesicles

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The effects of fatty acid sucrose esters on membrane lipid dynamics and cefitibuten transport by rat intestinal brush-border membrane vesicles (BBMV) were examined to clarify the differences in the action of mono- and poly-acyl sucrose esters on the drug transport. Fatty acid sucrose mono-acyl ester (SS) inhibited cefitibuten transport by BBMV similar to the action of polyoxyethylene sorbitans (Tweens), while fatty acid sucrose poly-acyl ester mixtures (F-160 and F-140) did not affect the drug transport by BBMV. SS but not F-160 and F-140 caused an increase in the anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH)- and 1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene iodide (TMA-DPH)-labeled BBMV in a concentration-dependent manner. Thus, the uptake of cefitibuten by BBMV was strongly correlated with the lipid fluidity of BBMV, in the outer layer and in the inner hydrophobic regions; however, there was no strong correlation between the membrane lipid fluidity and the drug uptake by BBMV. The micelle size and the size distribution of F-160 and F-140 were larger and more widely dispersed, respectively, compared to those of SS and Tweens. These results suggest that the effects of fatty acid sucrose esters on cefitibuten transport by BBMV are related to the dispersion parameter of these pharmaceutical adjuvants.

Key words fatty acid sucrose ester; non-ionic surfactant; cefitibuten; membrane lipid dynamics; size distribution; dispersion parameter

The use of surface active agents has been a major strategy in improving drug absorption in the gastrointestinal (GI) tract because of their solubilizing and/or emulsifying effects on lipophilic substances. Ionic surfactants tend to have adverse effects on the mucosal integrity of the GI tract as well as on the stability of medicaments. Non-ionic surfactants, therefore, have been widely used as additives for food and medication. Besides Tween-, Span- and Cremophor- (or HCO-) type non-ionic surfactants, fatty acid sucrose or glycerol esters¹⁾ and ethers,²⁾ which are mild surfactants consisting of natural products, have been given much attention as pharmaceutical adjuvants for oral use because they are biodegradable and bioavailable. However, since few systematic and comparative investigations have been conducted,^{3,4)} little is known about the effects of non-ionic surfactants on the intestinal transport of drugs or nutrients which are normally absorbed *via* transporters in the GI tract. In the last decade attempts have been made to utilize some biosurfactants such as unsaturated fatty acids,⁵⁾ bile salt-fatty acid mixed micelles,⁶⁾ milk fat globule membranes⁷⁾ and fatty acid sucrose esters⁸⁾ as intestinal absorption enhancers for drugs with a low oral bioavailability.⁹⁾ While the detailed mechanisms of permeability enhancement due to these biosurfactants remain to be elucidated, it has been reported that the lipoidal adjuvants interact with the polar region as well as with the hydrophobic domains of the luminal membrane of the GI tract, depending on their hydrophile-lipophile balance (HLB) properties and/or steric properties such as components of acyl-chain.^{10,11)} In addition, there is evidence showing that active drug transports could be disturbed by chemically induced changes in membrane fluidity.¹²⁾ Recently, Borchardt and his co-workers demonstrated that polysorbate 80 and Cremophor EL inhibit P-glycoprotein-mediated active efflux of drugs towards the luminal side of the intestine.¹³⁾

From a biopharmaceutical point of view, we have investigated the effects of several series of non-ionic surfactants on membrane lipid dynamics in relation to transport of a drug.¹⁴⁾ We found that almost all Span-, Tween-type surfactants and fatty acid glycerol esters disturbed the transport of disopyramide, in rat intestinal brush-border membrane vesicles (BBMV). The modulation of disopyramide transport with the non-ionic surfactants was found to correlate with changes in the lipid fluidity of the membrane's outer lipid layer rather than that in the inner hydrophobic domains. Hence, it appears that an exploration of the appropriate surfactants for low water-soluble drugs which are predominantly absorbed *via* a carrier transport system in the GI tract is required.

Fatty acid sucrose esters, another series of non-ionic surfactants, are potential candidates as a pharmaceutical adjuvant for improving the low aqueous solubility of peroral medications. The objective of the present study was to examine the effects of mono- and poly-acyl sucrose esters on the uptake of cefitibuten by BBMV. The aqueous solubility of cefitibuten was practically insoluble, in fact, the volume of necessary water to melt one gram of cefitibuten was over 10000 ml at 20 °C. It has been reported that the transport mechanism of cefitibuten is proton-coupled and stereoselective (*cis*-isomer selective) by BBMV.^{15,16)} In addition, the effects of the sucrose esters on cefitibuten transport and on the membrane lipid dynamics were comparatively investigated from differences in their hydrophobic parameter such as HLB number and dispersion parameters of average size and size distribution of the esters.

MATERIALS AND METHODS

Materials Cefitibuten (Shionogi Co., Osaka, Japan) and fatty acid sucrose esters (SS, F-160 and F-140, Daiichi

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Table 1. Physicochemical Characteristics of Fatty Acid Sucrose Esters

Product name	Composition (%) ^{a)}		HLB	c.m.c. ^{b)} (10 ³ %)
	Monoester	Di- and tri-esters		
SS	100	0	19	1.3
F-160	74	26	15	3.1
F-140	62	38	13	4.8

a) The ratio of stearic acid and palmitic acid is 7:3. b) The c.m.c. was measured by the fluorescence method using pyrene.¹⁷⁾

Kogyo Seiyaku Co., Kyoto, Japan) were kindly donated by each manufacturer (see Table 1 for the characteristics of the sucrose esters). Polyoxyethylene (20) sorbitan monolaurate (Tween 20), polyoxyethylene (20) sorbitan monopalmitate (Tween 40), polyoxyethylene (20) sorbitan trioleate (Tween 85) and polyoxyethylene (6) sorbitane monolaurate (Tween 21) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 1,6-Diphenyl-1,3,5-hexatriene (DPH), which partitioned deep in the hydrophobic interior of the membrane lipid layer, and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene iodide (TMA-DPH), which partitioned in the hydrophilic region of the membrane outer lipid layer, were purchased from Wako Pure Chemicals Co. (Osaka). Other chemicals were of reagent grade.

Preparation of Rat Intestinal BBMV BBMV were isolated from rat intestine by the $MgCl_2$ precipitation method¹⁸⁾ as described previously.¹⁴⁾ The isolated BBMV (10 mg protein/ml as a final concentration) were finally suspended in 20 mM HEPES/Tris buffer (pH 7.5) containing 100 mM D-mannitol and 100 mM KCl for uptake studies. The quality of the preparation was determined by assay of the marker enzyme activities of BBMV, *i.e.*, alkaline phosphatase and sucrase were enriched about 15 and 10 fold compared to those of the original mucosa, respectively.

Stability of BBMV The solubilizing effects of the sucrose esters on the membrane components of BBMV were estimated according to the method of Ohyashiki *et al.*¹⁹⁾ It was decided that the sucrose esters would be used in 0.05% (w/v) or less, so that the turbidity of membrane suspensions was kept at more than 85% of the controls in order to give a reliable indication of the membrane integrity.

Measurement of Inside pH of BBMV 2',7'-Bis(carboxyethyl)-4(5)-carboxyfluorescein (BCECF) was used as a fluorescence pH indicator. After isolated rat intestinal mucous membranes were incubated with BCECF (5 μ M) for 40 min at 37 °C according to the method of Thwaites *et al.*,²⁰⁾ BBMV was prepared by the method described above. Furthermore, BBMV prepared was centrifuged at 70000 $\times g$ for 15 min to remove BCECF which was not trapped in BBMV, and then the precipitants were resuspended with 20 mM HEPES/Tris buffer, pH 7.5, containing 100 mM D-mannitol and 100 mM KCl (2 mg protein/ml as a final concentration). BCECF-loaded BBMV was added to the medium of the equivalence of 20 mM MES (2-(N-morpholino) ethanesulfonic acid)/Tris buffer, pH 5.5, containing 100 mM D-mannitol and 100 mM KCl or buffer solution of pH 7.5 mentioned above in the presence of the sucrose esters. After incubation of the mixture solution, the proton ion concentration of BBMV was quantified by fluorescence (excitation at 490 nm and emission at 526 nm) using a Hitachi spectrofluorometer

850 (Hitachi Seisakusho Co., Tokyo) for 5 min at 25 °C. The inside pH of BBMV was converted to pH_{in} by comparison with values from a calibration curve (pH 6.0–7.5).

Uptake Experiment with BBMV The uptake of ceftibuten by BBMV was measured by a rapid filtration technique as described by Iseki *et al.*²¹⁾ The uptake experiment was carried out by a modification of the method described by Sugawara *et al.*²²⁾; the reaction was initiated by the addition of 20 mM MES/Tris buffer (pH 5.5) containing 100 mM D-mannitol, 100 mM KCl, 1.2 mM ceftibuten and sucrose esters (0–0.05%, w/v) to the BBMV suspension (5 mg protein/ml as a final concentration) at 25 °C. The reaction was stopped by diluting the reaction mixture with a 5 ml ice-cold buffer (20 mM HEPES/Tris buffer (pH 7.5) containing 100 mM D-mannitol and 100 mM KCl). The resultant solution was immediately filtered through a Millipore filter (HAWP, 0.45 μ m, 2.5 cm diameter, Japan Millipore Co., Tokyo). Ceftibuten trapped on the Millipore filter was extracted with 0.3 ml of 0.5% (w/v) Triton X-100 and was measured by HPLC. Non-specific binding of ceftibuten to BBMV was determined using the test buffer in place of the membrane vesicle suspension in the presence and absence of the sucrose esters or Tweens used. The extent of each binding was subtracted from the uptake data.

Fluorescence Polarization Study DPH and TMA-DPH were used as fluorescence probes for monitoring the membrane lipid fluidity in the inner hydrophobic and the outer hydrophilic domains, respectively.²³⁾ Fluorescence polarization measurements were performed with a Hitachi spectrofluorometer 850 equipped with a rhodamine B quantum counter (Hitachi Seisakusho Co.) as described in a previous report.¹⁴⁾ Briefly, the probe-labeled BBMV was incubated in the presence or absence of the surfactants for 10 min at 25 °C. The results obtained in the steady-state depolarization experiments were expressed in terms of fluorescence anisotropy, γ , which was calculated using the following equation: $\gamma = (I_V - I_H)/(I_V + 2I_H)$, where I_V and I_H are the fluorescence intensities observed with the analyzing polarizer parallel and perpendicular to the polarized excitation beam, respectively.²⁴⁾

Dynamic Light Scattering (DLS) Study DLS measurements of the surfactants were made using a laser particle analyzer (LPA-3000/3100, Otsuka Electronics Co., Osaka) at 25 °C. It uses a 632.8 nm He–Ne laser at a power of 5 mW and the light scattering was measured at 90°. The average size and size distribution were evaluated as a Z-average using monomodal method (a cumulant analysis) and as a ratio of weight-weighted diameter for number-weighted diameter using a multimodal method (exponential sampling algorithm), respectively. Analysis range in the multimodal method was set at a diameter of from 3 nm to 5 μ m at the first run of each measurement. All surfactants used (0.05%, w/v, as a final concentration) were solubilized using 20 mM HEPES/Tris buffer (pH 7.5) containing 100 mM D-mannitol and 100 mM KCl filtered through 0.22 μ m filters.

Analytical Methods The concentration of ceftibuten was determined by a specific HPLC method using LC-9A (Shimadzu, Kyoto) equipped with a UV detector C-R6A at a wavelength of 262 nm. Separation of the drug was achieved on a reversed phase column (Cosmosil 5C18, 4.6 mm i.d. $\times$ 250 mm) with a mobile phase consisting of methanol: 50 mM citrate buffer, pH 2.5 (2:8, v/v) at a flow rate of 0.7

Fig. 1. Time Profiles of Ceftibuten Uptake in the Absence (●) or Presence of SS (○), F-160 (△) and F-140 (□) (A), and Effect of SS Concentration on Ceftibuten Uptake (B) by BBMVs under an Inward Proton-Gradient Condition ($pH_{in}=7.5$, $pH_{out}=5.5$)

Membrane vesicles were preincubated in 20 mM HEPES/Tris buffer, pH 7.5, containing 100 mM D-mannitol and 100 mM KCl. The vesicles were then incubated with 20 mM MES/Tris buffer, pH 5.5, containing 100 mM D-mannitol, 100 mM KCl and 1.2 mM ceftibuten at 25 °C. Data are the means $\pm$ S.E. of three to five experiments.

ml/min. Protein concentration was measured by a commercial assay kit (BCA assay kit[®], Pierce Chemical Co., IL, U.S.A.) using bovine serum albumin as a standard.

RESULTS

Ceftibuten Transport by BBMVs The uptake of ceftibuten by BBMVs in the presence of various concentrations of the sucrose esters was examined. An inward proton gradient for the uptake of ceftibuten was created by using different pH conditions inside (pH_{in}) and outside (pH_{out}) of BBMVs, i.e., 7.5 and 5.5, respectively. Figure 1A shows the time-profiles of ceftibuten uptake with or without 0.05% (w/v) sucrose esters. When ceftibuten was given without the sucrose esters, rapid uptake by BBMVs was observed with an initial overshoot phenomenon. SS, but neither F-140 nor F-160, proved to reduce the ceftibuten transport, especially at the earlier phase of the uptake. As shown in Fig. 1B, this suppressive effect of SS on ceftibuten transport was found to be concentration dependent. None of the sucrose esters used affected the drug transport without such a proton gradient (data not shown).

Effects of Sucrose Esters on Inside pH of BBMVs To elucidate the action of the sucrose esters on an inward H^+ -gradient condition of BBMVs, the pH_{in} of BBMVs was examined in comparison with that in the absence of the esters. Table 2 shows the pH_{in} of BBMVs in the absence and presence of the esters. In addition to the effects of the esters, all the pH_{in} of BBMVs were gradually decreased as time passed. Although the overshoot phenomenon of ceftibuten transport by BBMVs was not shown in the presence of SS, the pH_{in} of BBMVs with SS at 60 s was similar to that in the absence of the esters (control) and was greater than 0.44 compared to the pH_{in} of the control at 20 min. These results suggest that the disappearance of the H^+ -gradient condition was not particularly stimulated by SS.

Effects of Fatty Acid Sucrose Esters on Membrane Lipid Dynamics The steady-state anisotropy of DPH and TMA-DPH bound to BBMVs in the absence of adjuvants was 0.250 ± 0.002 and 0.285 ± 0.002 (mean $\pm$ S.E., $n=3$), respectively. The latter was significantly higher than the former, in good agreement with those in the literature.^{25,26} The anisotropy of DPH increased with the addition of increasing amounts of SS, but not with the addition of F-160 or F-140,

Table 2. Effects of Sucrose Esters on pH_{in} of BBMVs Loaded with pH Sensitive Fluorochrome, BCECF

Incubation time (s)	pH_{in}			
	Control	SS	F-160	F-140
30	7.10	7.09	7.10	7.10
60	7.01	6.88	6.90	6.89
120	6.88	6.84	6.88	6.87
300	6.84	6.84	6.85	6.84

The pH_{in} of BBMVs without the sucrose esters (control) at 20 min after incubation was pH 6.44. Fluorescence intensity measurements were carried out at 25 °C. Each concentration of sucrose esters was 0.05% (w/v).

Fig. 2. Changes in Fluorescence Anisotropy of DPH-(A) and TMA-DPH-(B) Labeled Membranes at Various Concentrations of SS (○), F-160 (△) and F-140 (□) at 25 °C

The S.E. of three experiments is not shown in each point since it is too small to indicate.

to BBMVs suspension (Fig. 2). Similar results were obtained in the experiments using TMA-DPH as a probe. These findings indicate that SS raises the order of the membrane lipid both in the inner hydrophobic and the outer superficial regions.

Contribution of Hydrophobicity of Fatty Acid Sucrose Esters to Ceftibuten Transport and Lipid Fluidity of BBMVs To clarify the contributions of hydrophobicity of adjuvants to ceftibuten transport and membrane lipid fluidity, another series of non-ionic surfactants with different HLB numbers, i.e., Tween 85 (HLB=11.0), Tween 21 (HLB=13.3), Tween 40 (HLB=15.6) and Tween 20 (HLB=16.7), was also employed in this study. Figure 3A shows the relationship between the HLB number of the surfactants and the

Fig. 3. Correlation between HLB Number of Surfactants and Cefitbuten Uptake by BBMV at 25 °C (A) or Fluorescence Anisotropy of DPH (O) or TMA-DPH (Δ)-Labeled Membranes at 25 °C (B)

The HLB numbers of SS, F-160, F-140, Tween 20, Tween 40, Tween 21 and Tween 85 are 19.0, 15.0, 13.0, 16.7, 15.6, 13.3 and 11.0, respectively. The extent of cefitbuten uptake in the absence of surfactants was 1.24 nmol/mg protein at 1 min. The values of the fluorescence anisotropy of DPH- and TMA-DPH-labeled membranes in the absence of surfactants were 0.250 and 0.285, respectively. Each concentration of sucrose esters was 0.05% (w/v). Data are the means $\pm$ S.E. of three to five experiments for figure A and the S.E. of figure B is not shown in each point since it is too small to indicate.

Table 3. Typical Size Parameters of Fatty Acid Sucrose Esters and Tweens

Product name	Size (nm)			dw/dn
	dw	dn	Average diameter	
SS	7	6	61	1.1
F-160 ^{a)}	490	115	396	4.3
F-140 ^{a)}	505	111	408	4.6
Tween 85	50	42	151	1.2
Tween 21	30	27	117	1.1

Each product concentration is 0.05% (w/v). There were 50 accumulation times.

a) Two peaks were observed in the size distribution.

uptake of cefitbuten by BBMV against the control (in the absence of the surfactants). Neither F-140 or F-160 affected the uptake of cefitbuten, whereas Tweens and SS-regardless of the HLB number-significantly and to a similar extent suppressed it. The values of the fluorescence anisotropy of DPH- and TMA-DPH-labeled BBMV, on the other hand, were reduced in the presence of the surfactants. The relationship between the anisotropy ($\Delta\gamma$) and the HLB number of the surfactants is shown in Fig. 3B. The ($\Delta\gamma$) was increased with a decrease in HLB number except for F-160 and F-140, and that of DPH-labeled BBMV was especially marked.

Micelle Size and Size Distribution of Fatty Acid Sucrose Esters Table 3 shows typical size distribution parameters, i.e., weight-weighted diameter (dw), number-weighted diameter (dn), average diameter and the dispersion parameter (dw/dn). SS, Tween 85 and Tween 21 gave a mono-dispersed distribution, while F-160 and F-140 showed two distribution peaks and a broad range of diameter. The average diameters of F-160 and F-140 were larger than those of the other surfactants. The average diameter ($d=2r$) can then be calculated from the Einstein-Stokes equation: $D=kT/3\pi\eta r$, where D is the mutual diffusion coefficient, k is Boltzmann constant, T is the absolute temperature, η is the viscosity of the medium and r is Stokes radius of substrate. The dw/dn values of SS, Tween 85 and Tween 21, but not F-160 and F-140, were approximately 1.

DISCUSSION

Some surface active adjuvants may affect the absorption efficiency of carrier-mediated transport of drugs and nutrients in the GI tract. It was reported that the uptake of D-glucose transport in rat BBMV²⁷⁾ and of L-leucine transport in the liposomes prepared from the extracted lipids of *Lactococcus lactis*²⁸⁾ were decreased with a decrease in the membrane lipid fluidity. Therefore, an appropriate choice of pharmaceutical adjuvants is required for the medicinal preparations of the drugs which are uptaken by the carrier-mediated transport system. In the present study, it was demonstrated that the maximum uptake of cefitbuten by BBMV was inhibited and the membrane lipid fluidity was decreased by the addition of the sucrose mono-acyl ester, SS (Fig. 1). F-160 and F-140 had no effect the drug uptake by BBMV or the lipid fluidity. From these results, it seems that the uptake of cefitbuten is related to changes in the membrane lipid fluidity. However, although Tween-type surfactants with HLB ranging from 11 to 17 caused a decrease in the drug uptake by BBMV, the membrane lipid fluidity was clearly increased by addition of Tweens, in contrast to the case of SS (Fig. 3). These results suggest that the cefitbuten transport by BBMV is affected by the membrane lipid fluidity, although the fluidity changes did not directly reflect the uptake of cefitbuten.

As a first hypothesis of the inhibitory effect of SS on the cefitbuten transport, the disappearance of an inward H^+ -gradient condition of BBMV with SS would be possible. However, no differences in changes in the pH_{in} of BBMV were seen in the absence and presence of the sucrose esters (Table 2). The initial decrease in the pH_{in} of BBMV at 30 s after incubation, i.e., pH 7.10, could be due to residual BCECF trapped on the membrane surface. These results suggest that SS did not enhance the disappearance of an inward H^+ -gradient, i.e., the driven force of cefitbuten transport by BBMV.

As a second possibility, physicochemical properties of non-ionic surfactants are mainly described in terms of the critical micelle concentration (c.m.c.) and HLB.^{2,29)} The c.m.c. values of SS, F-160 and F-140 were determined to be 1.3×10^{-3} , 3.1×10^{-3} and 4.8×10^{-3} % (w/v), respectively (Table 1). Hence, the sucrose esters should form micelles in the concentration range of 0.01 to 0.05% (w/v) under the ex-

perimental conditions. There was no correlation between the c.m.c. values of the esters and the uptake of ceftibuten by BBMV. Similarly, no correlation between the HLB number of the esters and the ceftibuten uptake was observed. These results appear to indicate that the effects of sucrose esters on the efficiency of ceftibuten transport by BBMV cannot be explained by the c.m.c. value or the HLB number as a hydrophobicity index of surfactants.

As a third and particularly strong possibility, we also investigated the effects of micelle size and size distribution of the surfactants on the uptake of ceftibuten by BBMV, because it was reported that hydrophobic interactions between plasma membrane lipids and non-ionic surfactants can be assessed by the dynamic packing shape²⁾ and micellar size distribution.³⁰⁾ The dw/dn value shown in Table 3 is known as the most suitable dispersion parameter in the case of a broad size distribution.³¹⁾ In fact, the dw/dn values of F-160 and F-140 were apparently above 1, while the dw/dn values of SS and Tweens were approximately 1. These results indicated that F-160 and F-140 were in a polydispersity as a widely dispersed distribution, and SS and Tweens were in a monodispersity under the experimental conditions. It has been reported that the theory of a multicomponent micelle system as a function of micelle distribution, *i.e.*, the state of polydispersity in size distribution of micelles which have sufficiently finite width, can be applied to a balance between the two factors: the entropy of mixing of the micelles and the formation terms as two- or three-component micelles.³²⁾ Furthermore, it seems that the state of polydispersity of surfactants strongly contributes to the interaction between the surfactants and ceftibuten transport system by BBMV.

In conclusion: i) sucrose mono-acyl ester, SS, suppressed the membrane lipid fluidity and inhibited ceftibuten transport by BBMV under an inward H^+ -gradient condition; ii) sucrose poly-acyl ester, F-160 and F-140, did not affect either the membrane lipid fluidity or the drug transport; iii) while the differences in ceftibuten transport by BBMV in the presence of the sucrose esters could not be explained from the hydrophobicity such as HLB number, the micelle size distribution of surfactants might be important for the interaction between the drug transport system and pharmaceutical adjuvants.

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