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Combinative promotion effect of Azone and fusogenic fatty acid on the large intestinal absorption in rat

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Summary

The combinative effect of Azone (AZ) and oleic acid (OA) on the large intestinal absorption of poorly absorbable drugs was investigated using the in situ closed loop method in rat. 6-Carboxylfluorescein (CF) was used as a poorly absorbable model drug. We used two formulae as follows: (1) HCO-60 micellar solution of AZ and/or OA; and (2) ufasomes, oleic acid vesicles, incorporating AZ (OA-AZ/ufasomes). The micellar solution of OA or AZ extremely enhanced the absorption of CF. Either the absorption promoting effect of OA or AZ in micellar solutions was dependent on the concentration of the adjuvant and reached the maximum level over about 20 mM. The combination of OA and AZ in micellar state showed approximately an additive effect. Also OA-AZ/ufasomes promoted the absorption of CF, as well as the micellar solutions, but these effects were smaller than that obtained with micellar solutions. Moreover, from the results of CF-free ufasomes compared with ufasomes encapsulating CF, it was suggested that CF encapsulated in ufasomes could hardly be absorbed, and that the absorption of CF leaking out from ufasomes could be extensively enhanced.

Introduction

There are numerous drugs which should be administered only via injection route because of poor absorbability from the gastrointestinal tract. Many investigators, therefore, have been searching to find out an absorption promoting adjuvant so that the bioavailability of these poorly absorbable drugs from the gastrointestinal tract can be improved.

We have already reported that the lipid–surfactant mixed micelles and ufasomes (i.e. the unsaturated fatty acid closed vesicles) improved the bioavailability of poorly absorbable drugs from the gastrointestinal tract (Muranishi et al., 1977, 1979; Yoshikawa et al., 1984; Murakami et al., 1986). On the other hand, Murakami et al. found out that AZ, whose strong promoting effect on the percutaneous absorption was recently studied by us, considerably improved the bioavailability of poorly absorbable drugs from the intestine (Murakami et al., submitted). In this study, we examined the combinative effect of OA and AZ in micellar solutions as a fundamental investigation of the action mechanism of AZ in the intestine. Further we incorporated AZ into ufasomes in

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order to examine whether it could enhance their absorption promoting effect.

Materials and Methods

Materials

CF was purchased from Eastman Kodak Co. (Rochester, NY). OA and AZ were supplied from Nippon Oil and Fats Co. and Teikoku Seiyaku Co., respectively. HCO-60, polyoxyethylene hydrogenated castor oil, supplied from Nikko Chemical Co. was used. All other chemicals used were of reagent grade quality.

Preparation of test solutions

In all the test solutions, CF was dissolved in 0.1 M tris(hydroxymethyl)aminomethane (Tris)-HCl buffer and adjusted to pH 8.5. The used dispersion systems were: (1) the micellar solutions using HCO-60; (2) OA-AZ/ufasomes encapsulating CF (OA-AZ/CF-ufasome); and (3) empty OA-AZ/ufasomes + free CF (OA-AZ/empty-ufasomes).

In micellar solutions of CF (100 μg/ml) using HCO-60, a clear solution was obtained upon sonicating the buffer solution mixture of OA, AZ and HCO-60 with Ohtake model 5202 sonicator (Ohtake Seisakusho Co., Tokyo, Japan) at 30 W for 5 min. The concentration ratio of HCO-60 to the adjuvant concentrations was 1 : 10.

OA-AZ/CF-ufasomes were prepared according to the method described in previous paper (Murakami et al., 1986a). OA and AZ were dissolved in chloroform and stored at $-20°$C. The mixture of the appropriate percentage of OA and AZ in chloroform was completely evaporated to dryness with a stream of nitrogen gas by warming it to around 40°C. After the adequate amount of 1 N NaOH was added to neutralize the fatty acid, the residual film was sonicated under nitrogen in ice-water at 15 W for 2 min with CF solution (20–70 mg/ml buffer). On the other hand, OA-AZ/empty-ufasomes were prepared using phenol red (PR) as a marker instead of CF. Immediately after sonication, the suspension of ufasomes was applied to a coarse Sephadex G-25 column (18 × 2 cm) to separate from non-encapsulated CF or PR. Eluate used was 0.1 M Tris-HCl buffer, pH 8.5, beforehand bubbled with nitrogen gas.

In the case of OA-AZ/empty-ufasomes, the OA-AZ/ufasomes were mixed with the adequate concentration of free CF solution, and the mixture was used for animal experiment after measuring the OA concentration of the void volume fraction. In the case of OA-AZ/CF-ufasomes, on the other hand, the CF concentration in the void volume fraction was measured first and then the ufasomes diluted with eluate were used for animal experiment. When ufasomes were administered to animals, after the whole procedure, the concentration of CF was adjusted to 100 μg/ml and the total concentration of OA and AZ was adjusted to about 16 mM.

Procedure of in situ absorption experiment

Absorption experiments were performed by the in situ closed loop technique according to our previous report (Hashida et al., 1984). The large intestine of male Wistar albino rats weighing 230–280 g was used. Animals were fasted for about 16 h prior to experiments (but given water ad lib.) and anesthetized with intraperitoneal sodium pentobarbital, 32 mg/kg of body weight, during the experiment. Bile was excluded from the body during the experiments by fistula. The dose of CF was 1 mg/kg of rat body weight. The warmed test solution at 37°C was introduced into the intestinal loop. Blood samples (ca. 200 μl) were collected periodically through polyethylene tubing cannulated into the carotid artery until 4 h after administration and then centrifuged at 1500 $\times g$ for 5 min. The area under the concentration–time curve (AUC) until 4 h was primarily used as an index of the absorption efficiency.

In vitro stability of OA-AZ / CF-ufasomes

The in vitro stability of OA-AZ/CF-ufasomes was studied according to our previous method (Hashida et al., 1984). OA-AZ/CF-ufasomes or OA/CF-ufasomes was incubated in a water bath (37°C). Then, 1 ml aliquots of the ufasome suspension were periodically taken and immediately ultrafiltrated through YMB membrane (Amicon Co.) at 700 $\times g$ for 8 min. CF that had leaked out from ufasomes was determined by measuring the CF concentration of the filtrates.

Analytical methods

Determination of the plasma CF concentration in the in situ absorption experiments was performed according to our previous report (Hashida et al., 1984). Briefly, after 50 μl of plasma sample was mixed with 100 μl of 12.5% Triton X-100 and was acidified with 1 N HCl, CF was extracted with 6 ml iso-amyl alcohol. After shaking, centrifugation followed and the CF in the organic phase was re-extracted with pH 10 Na_2CO_3-$NaHCO_3$ buffered solution. Then the aqueous phase was measured spectrofluorimetrically at 520 nm at an excitation wavelength of 490 nm. The measured sensitivity of plasma CF concentration by above methods was high and the detected limit was around 5 ng/ml. Fluorescence of CF in the in vitro study was measured after diluting the sample with pH 7.4 KH_2PO_4-Na_2HPO_4 buffered solution following by treatment with 12.5% Triton X-100. Determination of OA concentration was carried out using a commercial kit for the measurement of non-esterified fatty acids by the ACS(Acyl-CoA synthetase)-ACO(Acyl-CoA oxidase) method (NEFA kit-U, Nippon Shoji Co., Osaka, Japan).

Reversibility of the absorption promoting effect of AZ

The recovery of the intestinal barrier function after administration of the micellar solution of AZ was investigated according to the method described below. The micellar solution of 10 mM AZ + 1 mM HCO-60, whose concentrations were sufficient to exhibit the absorption promoting effect, was incubated for 30 min within the large intestinal loop of rat. Then after the whole loop was thoroughly washed with 0.1 M Tris-HCl buffer (pH 8.5), at an appropriate time free CF solution was administered and the plasma CF concentration measured.

Results

Promoting effect of OA or AZ in micellar solution

Fig. 1 shows the relationship between the concentration of adjuvants and the AUC after dosing of OA or AZ micellar solutions using HCO-60 to rat large intestine. Absorption promoting effect of

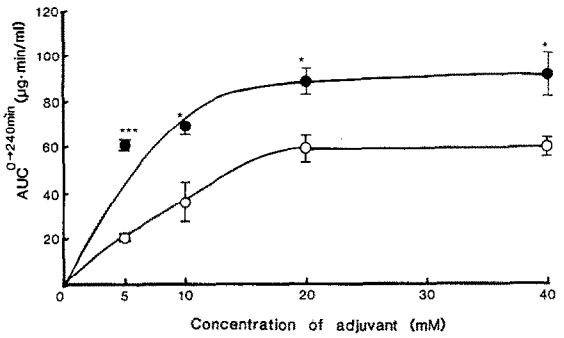

Fig. 1. Effect of OA or AZ concentration on the absorbability of CF from the large bowel in the micellar solutions. The concentration ratio of HCO-60 to the adjuvants (OA or AZ) was 1 : 10 at molar ratio. ○, OA + HCO-60; ●, AZ + HCO-60. Each value is the mean ± S.E. of 3–5 animals. Statistical comparison of AUC at each concentration was done by Student's *t*-test: $*P < 0.05$; $***P < 0.001$.

OA or AZ micellar solutions was dependent on the concentration of these adjuvants. By increasing the concentration of the both adjuvants to 20 mM, the promoting effect was increased, and reached a maximum level over the concentration of about 20 mM. In the comparison of OA and AZ, the promoting effect of AZ was significantly larger than that of OA in the series of this experimental system. Fig. 2 shows a typical plasma CF concentration–time curve after administration of CF in OA or AZ micellar solutions. In all cases, the peak plasma CF concentration of the curves appeared at a very early stage immediately after administration (15–30 min).

Combinative effect of OA and AZ in micellar solutions

The combined effect of OA and AZ in micellar solutions using HCO-60 was investigated by the following two methods: one was to change the compositional ratio of OA and AZ under the constant total molar concentration of these adjuvants, and the other was that the concentration of AZ was increased at 5 mM OA concentration.

Fig. 3 shows the promoting effect in the former case. AUC showed an increase by combination of AZ, but there was little difference in AUC by changing the compositional ratio of OA and AZ. In the latter way, as shown in Fig. 4, the absorp-

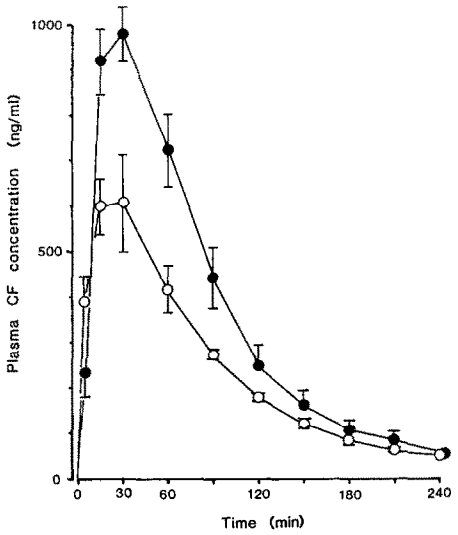

Fig. 2. A typical plasma CF concentration–time curve for a period of 240 min after administration of OA or AZ micellar solutions into the large intestine. ○, 20 mM OA+2 mM HCO-60; ●, 20 mM AZ+2 mM HCO-60. Each value is the mean ± S.E. of 3–5 animals.

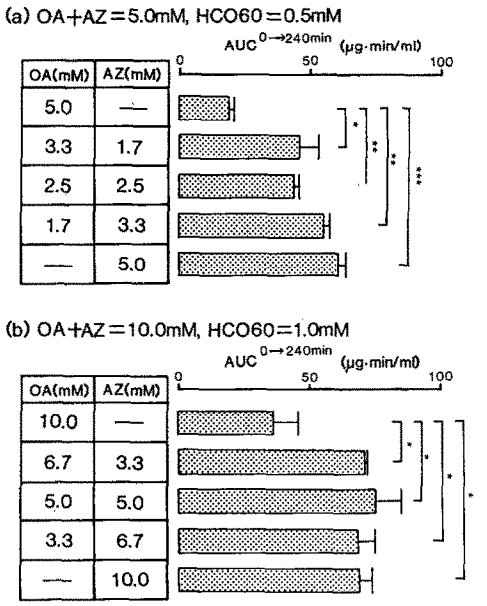

Fig. 3. Effect of co-administration of OA and AZ on the CF absorption at a constant sum concentration. Dotted bars represent AUC until 240 min after administration of the micellar solutions. Each value is the mean ± S.E. of 3–5 animals. Statistical comparison of AUC at each adjuvant's concentration was done by Student's *t*-test: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

tion promoting effect showed a gradual increase by increasing the added amount of AZ. This increase was approximately consistent with the increase of control which was administered the micellar solution of AZ. From the above experiments, it was likely that the combination of OA and AZ revealed the additive effect of both agents in the case of micellar solutions.

Preparation and stability of ufasomes incorporating AZ

The gel filtration pattern of OA-AZ/CF-ufasomes using a coarse Sephadex G-25 column was shown in Fig. 5. In the molar ratio of OA : AZ = 9 : 1 and 8 : 2, the ufasome fraction of CF fraction was fairly separated from free CF. The recovery percentage of OA within the ufasome fraction was sufficiently high (more than 90% in both OA-AZ/CF-ufasomes). When the molar ratio of OA : AZ was 7 : 3, however, there was a little tailing of CF in the ufasome fraction, and the recovery percentage of OA was lower than the other ratios (about 80%).

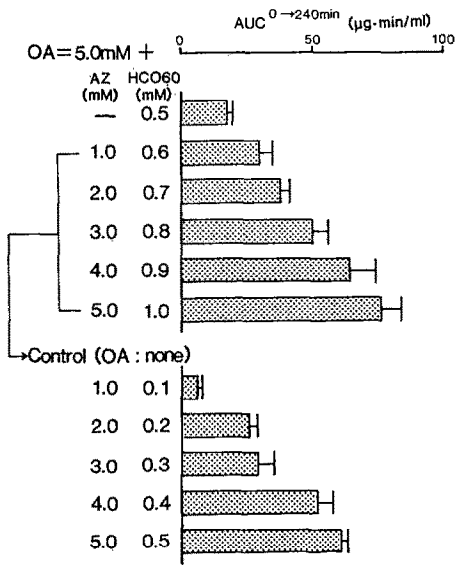

Fig. 4. Effect of AZ concentration on CF absorption at a constant 5 mM concentration of OA. Controls represent AUC of each AZ+HCO-60 micellar solution. Each value is the mean ± S.E. of 3–5 animals.

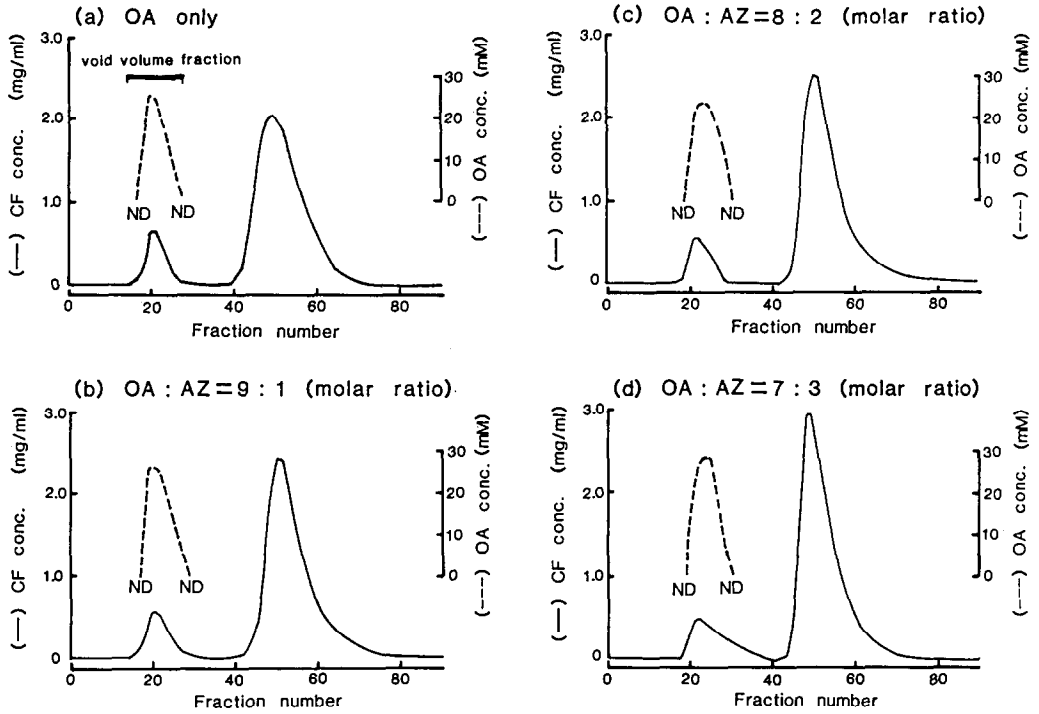

Fig. 5. Gel filtration pattern of OA-AZ/CF-ufasomes. CF concentration, suspension volume and OA concentration of ufasomes suspension at preparation were 25 mg/ml, 1.3 ml and 136 mM, respectively. Chromatography was performed on a coarse Sephadex G-25 column (length = 18 cm) with the flow rate ca. 20 ml/h (1 ml/tube). ND < 0.01 mM.

Then, we investigated the stability of OA-AZ/CF-ufasomes under in vitro conditions, as shown in Fig. 6, which represented the time course curves of CF remaining within ufasomes. OA/CF-ufasomes became leaky by increasing the incorporated AZ into them. When the molar ratio of OA : AZ was 7 : 3, about 80% of CF leaked out for 4 h.

Promoting effect of ufasomes incorporating AZ

The promoting effect of OA/CF-ufasomes caused by incorporating AZ was investigated. AUC until 4 h after administration was used as an index of the absorption promoting efficiency. The total concentration of OA and AZ in the formulation was adjusted to 16 mM and the molar ratio of OA : AZ were 10 : 0 (OA only), 9 : 1, 8 : 2 and 7 : 3. In addition, two formulae were used as follows: ufasomes containing CF (OA-AZ/CF-ufasomes)

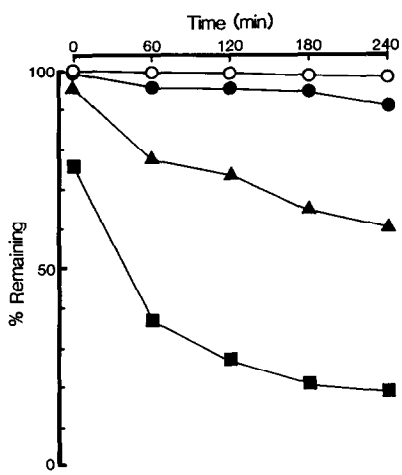

Fig. 6. In vitro stability of OA-AZ/CF-ufasomes. The symbols represent the mean for two experiments. ○, 16 mM OA; ●, 14.4 mM OA + 1.6 mM AZ; ▲, 12.8 mM OA + 3.2 mM AZ; ■, 11.2 mM OA + 4.8 mM AZ.

and empty ufasomes + free CF (OA-AZ/empty-ufasomes).

The result of OA-AZ/CF-ufasomes was shown in Fig.7a. As shown in Fig. 7a AUC of OA/CF-ufasomes (not containing AZ) showed the high value as 38.0 ± 3.3 (mean $\pm$S.E., $n = 4$) $\mu g \cdot min/ml$, but AUC of 9 : 1 and 8 : 2 OA-AZ/CF-ufasomes were 26.5 ± 2.6 ($n = 3$) and 22.7 ± 4.5 ($n = 4$) $\mu g \cdot min/ml$, respectively. These results indicated that both the AUC of OA-AZ/CF-ufasomes were lower than that of OA-CF-ufasomes. The peak plasma CF concentration during the early stages after administration, which was observed in OA/CF-ufasomes, was depressed in the case of both of the OA-AZ/CF-ufasomes. On the other hand, 7 : 3 OA-AZ/CF-ufasomes significantly enhanced the absorption promoting effect of OA/CF-ufasomes.

The effect of OA-AZ/empty-ufasomes on the absorption of CF from the large bowel was shown in Fig. 7b. Four different empty ufasomes (OA : AZ = 10 : 0, 9 : 1, 8 : 2 and 7 : 3) did not show significant a difference on the absorption of free CF. Besides, these AUC values of OA-AZ/empty-ufasomes were larger than that of OA/CF-ufasomes and were about the same values as 7 : 3 OA-AZ/CF-ufasomes. In 9 : 1 and 8 : 2 OA-AZ/empty-ufasomes, the steep plasma CF concentration during the early stage was clearly recognized, though it was apparently depressed in the case of 9 : 1 and 8 : 2 OA-AZ/CF-ufasomes. Hence, it was suggested that CF leaked from ufasomes were more absorbable than that encapsulated in ufasomes.

Reversibility of promoting effect of AZ

In our previous experiment, we observed that the promoting effect of AZ on the gastrointestinal tract was harmless in the light microscope observation (Murakami et al., 1986b). In this study, the recoverable efficiency of the intestinal barrier function after the administration of AZ was studied. Fig. 8 shows the recovery experiment after the pretreatment of AZ + HCO-60 for 30

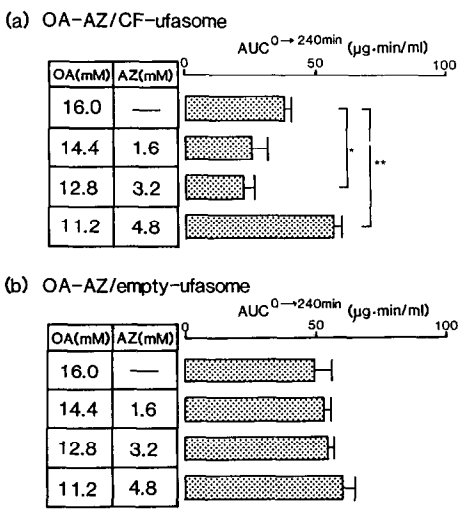

Fig. 7. Effect of OA-AZ/ufasomes on the absorption of CF from the large intestine. OA-AZ/CF-ufasome represents the vesicles encapsulated CF and OA-AZ/empty-ufasome represents ufasomes plus free CF. Dotted bars represent AUC until 240 min after administration. Each value is the mean $\pm$ S.E. of 3–4 animals. Statistical comparison of AUC at each adjuvant's concentration was done by Student's t-test: $*P < 0.05$; $**P < 0.01$.

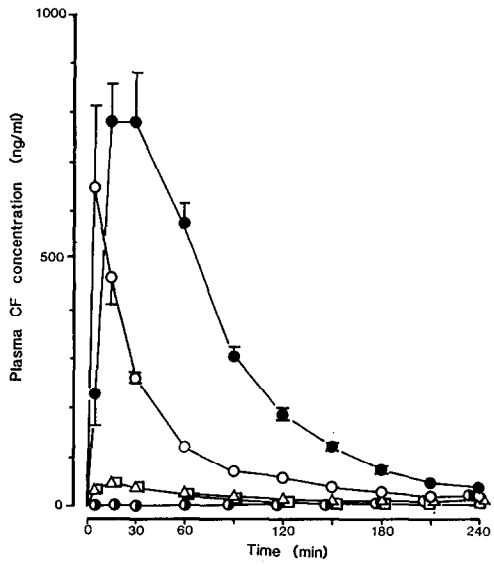

Fig. 8. Recovery study of the intestinal barrier function after pretreatment of AZ + HCO-60. ●, 10 mM AZ + 1.0 mM HCO-60; ○, free CF administered immediately after pretreatment; △, free CF administered at 30 min passed after pretreatment; □, free CF administered at 60 min passed after pretreatment; ◑, free CF (control). Each value is the mean $\pm$ S.E. of 3 animals.

min. As was evident from Fig. 8, slight differences were detected in the plasma CF levels between the control intestine (free CF administration without pretreatment) and the intestine passed 30 min as recovery time. We also observed the high plasma CF levels in the intestine which was administered with free CF immediately after pretreatment with AZ + HCO-60. This fact indicates that the intestinal barrier function after the administration of AZ + HCO-60 recovers rapidly.

Discussion

AZ has been known as the percutaneous absorption promoter and many investigators have been paying attention to AZ (Stoughton, 1982; Stoughton et al., 1983; Oshima et al., 1986). Recently we reported that AZ also considerably improved the bioavailability of poorly absorbable drugs in the intestine as well as fusogenic fatty acid. Although the pH 7.4 of the drug solutions was used in the previous paper, we chose the pH 8.5 because of the preparation of ufasomes in this study. When AUCs of CF were compared, the promoting effect of AZ + HCO-60 was significantly larger than that of OA + HCO-60 (Fig. 1). For this reason, it may be considered that AZ + HCO-60 exerts the absorption promoting effect irrelevant to the pH changes, in contrast to OA + HCO-60 which exerts the maximum effect at pH 6.5–7.5 (Murakami et al., 1985).

To study the mechanism of the promoter in the intestine is very important in these studies. The absorption promoting effect by combination of OA and AZ in micellar state was investigated as one of the fundamental studies on the absorption promoting mechanism of AZ. It was suggested that OA and AZ showed the additive effect in this state (Figs. 3 and 4). Hence, it is suggested that there is little difference in the mode of action between OA and AZ on the intestine. In addition, the mechanism for OA + HCO-60 in the intestine is currently under investigation in this laboratory. In the near future, therefore, it will be possible to suggest something on the mechanism for AZ in the large intestine. However, the promoting effect of OA and AZ does not always come to be the simple sum when OA and AZ were used in the combinative form (Fig. 3). It may be due to the interaction between each of the adjuvants, or between the adjuvants and the surfactant.

Ufasomes are closed vesicles consisting of unsaturated long-chain fatty acids reported by Gebicki and Hicks (Gebicki et al., 1973). In the previous paper, we reported that "OA/CF-ufasomes" were very stable in vitro at 37°C (the remaining percentage was about 97% even at 4 h), and the release of CF from them in the intestinal lumen might not be a function of its neutralization (Murakami et al., 1986a). "OA-AZ/CF-ufasomes", however, were rather unstable as seen in Fig. 6. Ufasomes became leaky with increasing the incorporated AZ into them. Further, it was not possible to disperse it by sonication and/or separate on column when AZ was incorporated more than 30%. In this stability experiment, CF adsorbed onto the YMB membrane was almost negligible.

The promoting effect of "OA-AZ/CF-ufasomes" did not show a definite tendency. Namely, there was a decrease in AUC with an increase in concentration of incorporated AZ from 0 to 20% into ufasomes, and conversely AUC significantly increased when AZ concentration increased further to 30%. In the case of 10% and 20% AZ concentration, these results might be caused by reinforcing the interaction between OA and AZ due to the absence of surfactant, and consequently by decreasing the relative amount of each adjuvant which could act on the intestine. On the other hand, as the reason why the bioavailability of CF increased significantly when AZ concentration was 30%, we considered a large amount of CF leaked from vesicles in comparison with the other "OA-AZ/CF-ufasomes". This consideration is supported by the fact that the absorption promoting effect of "OA-AZ/CF-ufasomes" is about the same with that of "OA-AZ/CF-ufasomes" when the molar ratio of OA : AZ is 7 : 3. That is, the absorption promoting effect of "OA-AZ/CF-ufasomes" would be controlled by the relation between the adjuvant's interaction and outside CF concentration of ufasomes.

As mentioned above, "OA-AZ/CF-ufasomes" became leaky with increasing the incorporated AZ

though the bioavailability was largest when it was 30%, which was the highest incorporatable percentage for AZ into "OA/CF-ufasomes". Hence, if ufasomes are utilized as drug carrier with the absorption promoting effect, we think that "OA/CF-ufasomes", none of AZ, are the optimal preparation.

As shown in the results, the absorption promoting effect of micellar solutions was larger than that of ufasomes. Therefore, we recommend the micellar solutions for merely enhancing the bioavailability of poorly absorbable drugs. But we have to keep in mind that ufasomes may be potential drug carriers with the absorption promoting effect for oral drug delivery if their stability are further improved.

Our previous experiment showed that the promoting absorption by AZ + HCO-60 occurred without injuring the intestinal mucosa. Also we observed in this study that the effect of AZ + HCO-60 on the intestine revealed the prompt recovery of the intestinal barrier function (Fig. 8). Accordingly, it is supposed that AZ may be a non-injurious and reversible absorption promoting agent on the intestine with resemblance to unsaturated fatty acid (Muranishi, 1985). In addition, since AZ micellar solutions are dispersed by surfactant, we anticipate that they are more stable than ufasome dispersion system.

In conclusion, the results obtained in this work were summarized as follows. In micellar solutions, the absorption promoting effect of AZ was significantly larger than that of OA in rat large intestine, and the combinative effect of both adjuvants was approximately additive. In the case of ufasome incorporating AZ, the absorption promoting effect was smaller than that of micellar solution. From the result of empty ufasomes, it was suggested that free CF leaked from ufasomes was predominantly absorbed, whereas the CF entrapped within the ufasomes was hardly absorbed.

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