Gastric Emptying-Limited Oral Absorption of \( \alpha \)-Linolenic Acid Administered as a Milk Fat-Globule Membrane (MFGM) Emulsion in Rats

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As a study to assess the potential application of milk fat-globule membrane (MFGM), which is of natural origin and expected to be a safer alternative to synthetic emulsifiers, to pharmaceutical dosage forms, the oral absorption of \( \alpha \)-linolenic acid was evaluated by the analysis of gastrointestinal disposition after oral administration as an MFGM emulsion to rats. A linear model incorporated with first-order gastric emptying followed by first-order intestinal absorption was fitted to the data of the remaining fraction of \( \alpha \)-linolenic acid versus time profiles for the stomach and small intestine to estimate the rate constants of gastric emptying (\( k_e \)) and intestinal absorption (\( k_i \)). The \( k_e \) (0.045 min\(^{-1}\)) was about 4 times larger than the \( k_i \) (0.011 min\(^{-1}\)). The \( k_i \) was comparable to the apparent oral absorption rate constant estimated by the pharmacokinetic analysis of plasma concentration data. These results suggest that the oral absorption of \( \alpha \)-linolenic acid administered as MFGM emulsion is gastric emptying-limited and, hence, any change in the intestinal absorption process would only modestly affect its oral absorption.

Keywords milk fat-globule membrane (MFGM); emulsion; \( \alpha \)-linolenic acid; oral absorption; gastrointestinal disposition; rat

The milk fat-globule membrane (MFGM), which encloses the fat droplets in milk, is known to contribute to stabilizing milk as an emulsion.\(^1\) Its pharmaceutical application as an emulsiﬁer has been of increasing interest, since it is of natural origin and could be safer than synthetic emulsifiers. The MFGM was suggested to have emulsifying capability comparable to synthetic emulsifiers such as HCO 60 and Tween 80.\(^2,3\) Recent studies have shown that the oral or intestinal absorption of vitamin A,\(^4\) vitamin D,\(^5\) and insulin\(^5\) was improved by administering them as MFGM emulsions, compared with the administration of simple solution or emulsions using synthetic emulsifiers, suggesting that MFGM could be a good alternative to synthetic emulsifiers. We recently reported that the oral absorption of \( \alpha \)-linolenic acid tended to be increased when it was administered as MFGM emulsion, compared with Tween 80 emulsion.\(^6\) We also reported that, comparing the emulsion preparation methods of homogenization only (method A) and homogenization with subsequent sonication (method B), the former provided an emulsion with larger oil droplet size and better oral absorption of \( \alpha \)-linolenic acid than the latter.\(^6\)

In the present study, we intended to focus on MFGM emulsion prepared by method A which provided the best absorption of \( \alpha \)-linolenic acid in our preceding study,\(^6\) and to further characterize the oral absorption of \( \alpha \)-linolenic acid, using our recently proposed method of gastrointestinal disposition analysis.\(^7,8\) The gastrointestinal disposition analysis allows us to differentiate between gastric emptying and intestinal absorption, two distinct rate processes involved in the oral absorption in vivo. The oral absorption has generally been characterized as an apparent single process because of technical difficulties in differentiating those processes. The differentiated information about the gastric emptying and intestinal absorption should facilitate a more reasonable interpretation of intestinal absorption data in situ and the development of strategies to be applied in vivo.

MATERIALS AND METHODS

Chemicals Milk fat-globule membrane (MFGM) was provided by Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). \([1-\text{\textsuperscript{14}C}]\alpha\)-Linolenic acid (2.0 GBq/mmol), \([\text{\textsuperscript{3}H}]\text{Glucagon} \) (15.8 GBq/g) and Bioﬂuor, scintillation cocktail, were purchased from DuPont-NEN Co. (Boston, MA, U.S.A.), and \( \alpha \)-linolenic acid was from Aldrich Chemical Company, Inc. (Milwaukee, WI, U.S.A.). All other chemicals were of analytical grade and commercially available.

Preparation of Emulsions MFGM was dispersed in 10 mm sodium phosphate buffer (pH 7.0) to make 27 mg/ml of suspension. In a test tube (internal diameter, 1.5 cm; length, 17 cm), 4.35 ml of the medium containing MFGM was mixed with 1.55 ml of cotton seed oil containing 59 mg of \( \alpha \)-linolenic acid and a trace amount (0.28 MBq) of \([\text{\textsuperscript{14}C}]\alpha\)-linolenic acid. The mixture was prewarmed in a water bath at 45°C for 5 min and homogenized for 1 min with a Polytron homogenizer with a PT 10 shaft (Kinematica GmbH, Switzerland) at the maximum speed (mark 10, 27000 rpm) to prepare an emulsion. This procedure was named method A in our preceding report,\(^6\) which provided an emulsion with larger droplet size and better oral absorption of \( \alpha \)-linolenic acid than method B, a method incorporated with sonication after the procedure for method A. Finally, a trace amount of \([\text{\textsuperscript{3}H}]\text{Glucagon} \) was added as a nonabsorbable water phase marker (25 kBq/ml).

Oral Absorption Experiments Male Wistar rats, weighing about 300 g and fasted overnight, were given an oral dose (50 mg/5 ml/kg) of \( \alpha \)-linolenic acid as MFGM emulsion through a gastric tube, left free in a metabolic cage at an ambient temperature of 25°C, and sacrificed 10, 20, 40 or 60 min after administration by having the
abdominal aorta cut for blood loss under light ether anesthesia. To prevent the loss and extensive mixing of gastrointestinal contents, ligations were made at the cardia, pylorus and duodenoejunal flexure, and clamps were placed to divide the small intestine below the duodenum into 3 roughly equal segments before the stomach and small intestine were isolated. The small intestine below the duodenum was then divided into 3 equal segments of jejunum, midgut and ileum. The stomach was cut open and washed in 10 ml of saline to collect the gastric contents. The lumen of each intestinal segment was washed with 2 ml of saline to collect its contents. Each sample of gastrointestinal content was collected in a tared counting vial to determine its weight, and homogenized with a Polytron homogenizer with a PT10 shaft (Kinematics GmbH, Switzerland). One hundred microliters of the homogenized sample was placed in a counting vial, to which was added 3 ml of Biofluor, a scintillation cocktail, to determine its radioactivity with a liquid scintillation counter (LSC-1000, Aloka Co., Tokyo). The tissue of each gastrointestinal segment was placed in a tared counting vial to determine its weight, then 3 ml of saline was added for the duodenum and 5 ml each for all the rest, and all were homogenized with the Polytron homogenizer. Roughly 200 µl of the homogenized sample was placed in a tared counting vial to determine its weight, to which was added 1 ml of Soluene-350, a tissue solubilizer, to solubilize it at 55 °C for 2 h. Then, 0.2 ml of 30% hydrogen peroxide was added to decolorize it at 55 °C for 30 min. After adding 3 ml of Biofluor, and then 0.5 ml of 0.5 M HCl, the radioactivity was determined.

The amount of α-linolenic acid in the contents of each gastrointestinal segment was estimated as the sum of its amount in the contents sample and the amount adhered to the tissue. The adherent contents volume (V_{ad}) was estimated by dividing the amount of inulin associated with the tissue sample by its concentration in the contents sample, because inulin can be assumed to be distributed only in the adherent contents without being absorbed. The expression for the remaining fraction of dose (FR) for α-linolenic acid is as follows:

$$FR = \frac{C_{t}}{C_{i}} \left( \frac{V_{t}}{V_{ad}} \right) \frac{1}{FR_{int}}$$

(1)

where

$$V_{ad} = \frac{C_{t}}{C_{i}} \times \frac{V_{t}}{D}$$

(2)

and where $C_{t}$ and $C_{i}$ are the concentrations of α-linolenic acid and inulin, respectively, in the contents sample; $C_{i}$ is the concentration of inulin in the tissue sample; $V_{t}$ and $V_{t}$ are the volumes of contents and tissue samples, respectively; $D$ is the dose of α-linolenic acid; $FR_{int}$ is the total fraction of inulin recovered from the gastrointestinal tract, which was used to correct the remaining fraction of α-linolenic acid. The specific gravity of all biological samples was assumed to be 1.

**Gastrointestinal Disposition Analysis** As discussed in our preceding report, assuming that the apparent intestinal membrane permeability clearance (CL_{a, app}) and the intestinal lumen volume for the unit length ($V$) are constant along the small intestine, the absorption rate from the small intestine can be described as a first-order rate process with a rate constant of $k_{a}$, regardless of the distribution pattern of a given solute in the intestinal tract. Therefore, further assuming that the gastric emptying is described by the first-order rate constant of $k_{g}$, and that the gastric absorption and the transfer from the small intestine to the large intestine are negligible, the same model equations as those for a linear compartment model consisting of the stomach and small intestine compartments can be used to describe the remaining fractions of dose in the stomach ($FR_{s}$) and small intestine ($FR_{int}$) as follows:

$$FR_{s} = e^{-k_{g}t}$$

$$FR_{int} = \frac{e^{-k_{a}t} - e^{-k_{g}t}}{1 - k_{a}k_{g}}$$

(3)

(4)

Equations 3 and 4 were simultaneously fitted to the data of $FR_{s}$ and $FR_{int}$ of α-linolenic acid to estimate $k_{a}$ and $k_{g}$ using a nonlinear regression program, PCNONLIN (Statistical Consultants, Inc., Lexington, KY), and the reciprocal of variance as the weight.

**Measurements of Intestinal Lumen Volume** The small intestine was isolated from the rats 20 min after the administration of 5 ml/kg of MFGM emulsion without inulin, in the same way as the gastrointestinal absorption experiments. Ligations were made to separate the duodenum, jejunum, midgut and ileum. After injecting the saline containing a trace amount of [3H]inulin into each segment, 0.5 ml for the duodenum and 2 ml for the other segments, the contents were immediately collected for the determination of radioactivity.

Assuming the mass preservation of inulin, the intestinal lumen volume of the unit length ($V$) was estimated from the dilution of inulin with the intestinal contents by using the following equation:

$$V = \frac{V_{in}}{L} \left( \frac{C_{in}}{C_{i}} - 1 \right)$$

(5)

where $V_{in}$ is the injected volume of the inulin solution, $C_{in}$ and $C_{i}$ are the concentrations of inulin in the injected solution and in the sample diluted with the intestinal contents, respectively, and $L$ is the length of the intestinal segment. The longitudinal-averaged intestinal lumen volume for the unit length of absorption site ($V_{ad}$) was estimated as the sum of the luminal volumes, $V_{i}$ for $i$th segment, weighted with the distribution of the nonabsorbable marker as follows:

$$V_{ad} = \sum_{i} \frac{FR_{ri}}{FR_{int}} \cdot V_{i}$$

(6)

where $FR_{ri}$ and $FR_{int}$ are the remaining fractions of inulin in $i$th segment and in the entire small intestine, respectively.

**RESULTS AND DISCUSSION**

**Gastrointestinal Disposition Analysis** Figure 1 shows the time-dependent gastrointestinal distribution profiles of α-linolenic acid and inulin after oral administration to rats.
Although a significant amount of inulin, a nonabsorbable marker, reached the most distal segment of the ileum 60 min after administration, the total recovery of inulin was about 100% up to 60 min, suggesting its restricted distribution within the stomach and small intestine without transit to the large intestine. The gastric emptying process appeared to be similar for both α-linolenic acid and inulin, as suggested by the α-linolenic acid to inulin ratio of the remaining fraction in the stomach of approximately 1 throughout the experimental period of 60 min (Fig. 2). Assuming that the intestinal transit was also similar for the both compounds, the lower recovery of α-linolenic acid than inulin from the small intestine can be attributable to the absorption of α-linolenic acid, but not to the transit to the large intestine. Inulin, which is hydrophilic and is distributed mainly in the water phase, also serves as a marker of the transit of the water phase. A slight increase, though statistically insignificant, in the α-linolenic acid to inulin ratio in the stomach at 40 and 60 min may suggest some delay in the gastric emptying of the oil phase, where α-linolenic acid is mainly distributed, compared with the gastric emptying of the water phase. However, such a delay appeared to be minimal. At least, it would be safe to assume that the gastric emptying and intestinal transit of α-linolenic acid would not be faster than that of inulin, and its gastrointestinal distribution was restricted to the stomach and small intestine in the same way as the distribution of inulin. In separate experiments, it was confirmed that the gastric absorption of α-linolenic acid was negligible 60 min after administration to the closed stomach. It was also confirmed that the biliary excretion was negligible 60 min after administration to the closed intestinal loop.

The data of remaining fractions of α-linolenic acid versus time profiles for the stomach and the small intestine were successfully illustrated in Fig. 3 by the proposed model incorporating first-order gastric emptying followed by the first-order intestinal absorption (gastrointestinal disposition analysis). The gastric emptying rate constant (kₑ) of 0.011 min⁻¹ was about 4 times smaller than the intestinal absorption rate constant (kₛ) of 0.045 min⁻¹, suggesting the gastric emptying-limited oral absorption of α-linolenic acid (Table 1). The kₛ was about 20% of the kₑ of 0.058 min⁻¹ for the similar dosing volume of saline (1ml/rat),¹ suggesting a slower gastric emptying for emulsion than for saline.

To further confirm the gastric emptying-limited absorption of α-linolenic acid, the apparent oral absorption rate constant (kₑ) was estimated by the pharmacokinetic analysis of plasma concentration data from our preceding report.² Figure 4 shows the plasma concentration versus time profile after the oral administration of α-linolenic acid in MFGM emulsion in rats. A one-body compartment model with first order absorption, expressed

![Fig. 1. Gastrointestinal Distribution of α-Linolenic Acid (A) and Inulin (B) after Oral Administration as MFGEM Emulsion in Rats](image)

The data are represented as the mean ± S.E. (n=3).

![Fig. 2. α-Linolenic Acid to Inulin Ratio of the Remaining Fraction in the Stomach after Oral Administration as MFGEM Emulsion in Rats](image)

The data are represented as the mean ± S.E. (n=3).

![Fig. 3. The Remaining Fraction of α-Linolenic Acid versus Time Profiles for the Stomach (○) and Small Intestine (●) after Oral Administration as MFGEM Emulsion in Rats](image)

The data are represented as the mean ± S.E. (n=3). The solid lines represent the computer-fitted profiles.

<table>
<thead>
<tr>
<th>Method</th>
<th>kₑ (min⁻¹)</th>
<th>kₛ (min⁻¹)</th>
<th>kₚ (min⁻¹)</th>
<th>Vₑ (µl/cm)</th>
<th>CLₑ,app (µl/min/cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vivo (Cp)⁶</td>
<td>0.011 ± 0.004</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>In vivo (GI)⁷</td>
<td>NA</td>
<td>0.011 ± 0.002</td>
<td>0.045 ± 0.019</td>
<td>56 ± 5</td>
<td>2.5</td>
</tr>
<tr>
<td>In situ⁸</td>
<td>NA</td>
<td>NA</td>
<td>0.020 ± 0.002</td>
<td>100</td>
<td>2.0</td>
</tr>
</tbody>
</table>

kₑ, apparent oral absorption rate constant; kₛ, gastric emptying rate constant; kₚ, intestinal absorption rate constant; Vₑ, average luminal volume; CLₑ,app, apparent membrane permeability clearance; NA, not applicable. a) Pharmacokinetic analysis of plasma concentration data in vivo from our preceding report⁶; b) analysis of gastrointestinal disposition in vivo; c) data from our preceding report in the intestinal loop.⁷ Data are represented as the computer-fitted parameters with S.E. for kₑ and kₛ in vivo (GI), and the mean with S.E. from 3 rats for kₑ in vivo (Cp), kₛ in situ and Vₑ in vivo (GI).
in the following equation 7, was fitted to the concentration (C) versus time (t) profile using a nonlinear regression program, PCNONLIN, to estimate $k_e'$ with the elimination rate constant of $k_{e1}$ and a lumped constant of $A$.

$$C = A \cdot (e^{-k_{e1}t} - e^{-k_e't})$$

The $k_{e1}'$, $k_{e1}$, and $A$ were estimated to be $0.011 \pm 0.004$ min$^{-1}$, $0.0024 \pm 0.0004$ min$^{-1}$ and $7.0 \pm 1.4$ μg/ml, respectively, as the mean ± S.E. from 3 rats. The $k_e'$ was in good agreement with the $k_{e1}$ estimated by the analysis of gastrointestinal disposition of α-linolenic acid, supporting the suggestion of gastric emptying-limited absorption. The $k_e'$ was arbitrarily assigned to the faster phase, and the possibility of flip-flop cannot be excluded. However, if the slower phase represents the absorption phase, $k_e'$ would then be 5 times smaller than the current estimate of 0.011 min$^{-1}$ and 20 times smaller than the $k_{e1}$ estimated by the analysis of gastrointestinal disposition. Therefore, the intestinal absorption would not be the rate limiting process, and any change in the intestinal absorption process would only modestly affect the oral absorption of α-linolenic acid.

**In Vivo-in Situ Relation in the Intestinal Absorption**

The $k_e$ was about 2 times larger than that in the intestinal loop in situ in our preceding report (Table I). However, the difference in $k_e$ was mostly accounted for by the difference in the intestinal lumen volume. The average intestinal lumen volume ($V_{sw}$) was estimated to be $56 \pm 5$ μl/cm in vivo, about a half of the experimentally set volume of $100$ μl/cm in situ. The apparent intestinal membrane permeability clearance ($CL_{a,app}$) as the product of $k_e$ and $V_{sw}$ was estimated to be $2.5$ μl/min/cm in vivo, which was close to the $CL_{a,app}$ of $2.0$ μl/min/cm in situ. The orally administered emulsion was presumed to be mixed with the gastrointestinal fluid, while the effect of gastrointestinal fluid should be negligible in the intestinal loop in situ because the intestinal contents were washed out in advance and the secretion of digestive fluid should be negligible in the closed loop of the midgut. Therefore, the similar values of $CL_{a,app}$ in vivo and in situ may suggest an insignificant contribution of gastrointestinal or digestive fluid in the absorption of α-linolenic acid from MFGM emulsion, and also that the rate of intestinal drug absorption in vivo from orally administered emulsion can be predicted from the apparent membrane permeability clearance in situ, considering the difference in the luminal volume.

The underlying mechanism for the agreement in $CL_{a,app}$ between in vivo and in situ could be complicated. The dilution of emulsion, without a gastrointestinal fluid component, would be expected to increase the ratio of the water phase concentration to the apparent (or total) concentration by decreasing the relative volume of the oil phase, which works as a kind of reservoir. On the other hand, assuming that the drug is mainly absorbed from the water phase rather than oil phase, the $CL_{a,app}$ theoretically represents a clearance obtained by dividing the absorption rate on the basis of water phase concentration by the apparent concentration. Thus, $CL_{a,app}$ would be expected to increase by simple dilution. Therefore, the agreement in $CL_{a,app}$ between in vivo and in situ may suggest an inhibitory effect of gastrointestinal fluid components on the absorption of α-linolenic acid from MFGM emulsion, and the overall effects of dilution and gastrointestinal fluid components might have been insignificant. The extent of involvement of gastrointestinal fluid in the absorption of α-linolenic acid might be different for different emulsions or emulsifiers, as suggested in our preceding report, in which the extent of oral absorption of α-linolenic acid was slightly larger for MFGM emulsion than Tween 80 emulsion, while the absorption in the intestinal loop was comparable. However, the suggested inhibitory effect of gastrointestinal fluid on the intestinal absorption is so far not consistent for different solutes. The intestinal absorption of vitamin D$_3$ administered as MFGM emulsion has recently been reported to be enhanced by taurocholate, a component of digestive fluid. These issues of in vivo-in situ relation and the involvement of gastrointestinal fluid in the intestinal drug absorption after administration as emulsions, including MFGM emulsion, may need to be examined in more detail in the future. The $V_{sw}$ may also need to be re-evaluated, which was tentatively estimated as the longitudinally-averaged value for the absorption site 20min after administration, when approximately half (25% of dose) of the total emulsion emptied from the stomach in 60min (50% of dose) was emptied.

We previously reported that the extent of oral absorption of α-linolenic acid tended to be increased when it was administered as MFGM emulsion, compared with administration as Tween 80 emulsion. However, any further modification or manipulation to enhance intestinal absorption may not lead to any more drastic enhancement or improvement of the extent of oral α-linolenic acid absorption, because α-linolenic acid appeared to be almost completely absorbed before reaching the distal end of small intestine, as suggested by the negligible recovery of α-linolenic acid from ileum in comparison with the recovery of insulin, as typically shown 60min after administration. The rate of oral absorption may not be further improved either, because it was suggested to be gastric emptying-limited. This also means any perturbation in the intestinal absorption process would only modestly affect the oral absorption. Thus, it was suggested that the oral absorption of α-linolenic acid from MFGM emulsion in our present and preceding studies is practically maximized. This study also demonstrated the usefulness.
of the gastrointestinal disposition analysis in understanding oral drug absorption by differentiating between gastric emptying and intestinal absorption processes.

REFERENCES