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Research paper

Enhancing mechanism of Labrasol on intestinal membrane permeability of the hydrophilic drug gentamicin sulfate

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Abstract

The aim of this study was to clarify the mechanism by which caprylocaproyl macrogol-8 glyceride (Labrasol) enhances the intestinal absorption of gentamicin sulfate (GM), a drug that has poor permeability but relatively high solubility. We studied the following characteristics: (i) the phase behavior of Labrasol in aqueous solution, (ii) the affinity of GM to Labrasol micelles, and (iii) the interaction between Labrasol and membrane lipids. We measured the critical micelle concentration of Labrasol in aqueous solution to be approximately 0.01%. The average diameters of Labrasol micelles in 2% and 25% solutions were approximately 10 nm and 20 nm, respectively, indicating that micelles increase in size with increasing Labrasol concentration. Although GM dissolved in 5% Labrasol solution was dialyzable, GM dissolved in either 25% or 50% Labrasol solutions was not, suggesting that GM exists in the hydrophilic region of the Labrasol micelle or in the high affinity region of the micelle surface where GM is retained. In membrane permeability experiments and electrophysiological studies conducted with rat ileum, only 25% Labrasol solution enhanced GM permeability, but did not remarkably affect membrane resistance. Furthermore, Labrasol increased membrane lipid fluidity as determined by fluorescence anisotropy in porcine intestinal brush border membrane liposomes. These results suggest that high concentrations of Labrasol solution enhance membrane permeability of GM via a transcellular rather than a paracellular route. We propose the following mechanism: Labrasol micelles grow when the concentration of Labrasol exceeds 20%, at which point GM shows high affinity for the hydrophilic region of the micelles. Since Labrasol micelles increase membrane lipid fluidity, the membrane permeability of GM is concomitantly enhanced.

Keywords: Caprylocaproyl macrogol glyceride; Micelle shape; Fluorescence anisotropy; Intestinal absorption; Membrane lipid fluidity

1. Introduction

Caprylocaproyl macrogol-8 glyceride (Labrasol) is a safe, nonionic surfactant developed by Gattefosse Corp. (Saint-Priest, France). Labrasol is synthesized by an alcoholysis/esterification reaction using medium chain triglycerides from coconut oil and PEG400 as starting materials. Thus, Labrasol is comprised of a well-defined mixture of mono-, di- and triglycerides and mono- and di-fatty acid esters of polyethyleneglycol, with the predominant fatty acids being caprylic and capric acids. While Labrasol has been reported to increase the solubility of water-insoluble drugs by emulsification [1], it has also been demonstrated to facilitate the oral bioavailability of water-soluble drugs, such as gentamicin sulfate (GM) [2], and amphiphilic drugs, such as diclofenac diethylamine [3].

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The absorption-enhancing mechanism of Labrasol responsible for increasing membrane permeability for water-soluble drugs remains unclear and requires clarification. Recently, it has been reported that Labrasol of low concentrations (0.1% and 1%) enhanced remarkably the membrane permeability of D-mannitol in the chamber studies using Caco-2 cells [4], suggesting that Labrasol has a tight junction opening action. Further, as one of the biological actions of Labrasol on the absorption of GM from intestinal tract, our group indicated that Labrasol inhibited the efflux of GM from the enterocytes to the gastrointestinal lumen [5]. This result suggests that Labrasol inhibits the function of efflux pump in intestinal epithelial cells. From the viewpoint of Labrasol concentration in formulation, the membrane permeability of GM using a rat intestine was significantly enhanced by the addition of 33% Labrasol in formulation [5]. And the bioavailability of GM in vivo absorption studies was significantly enhanced in formulation containing 75% Labrasol [2]. Namely, it is clear that the enhanced absorption of GM from intestinal tract occurred only at the high concentration of Labrasol.

In the present study, we focused the reason why GM showed the high membrane permeability and intestinal absorption in the formulation of the highly concentrated Labrasol (>30%), although Labrasol has tight junction opening action at the low concentration of Labrasol (0.1–1%). To solve this problem, we sought to understand the relationship between biomembranes, GM, and Labrasol. Our goals were to examine the phase behavior of Labrasol in aqueous solution, the affinity of GM to Labrasol micelles, and the interaction between Labrasol and membrane lipids.

2. Materials and methods

2.1. Materials

Labrasol (Gattefosse Corp., Saint-Priest, France) was a gift from CBC Co., Ltd (Tokyo, Japan). Gentamicin sulfate was purchased from Nacalai Tesque (Kyoto, Japan); 1,6-diphenyl-1,3,5-hexatriene (DPH), auramine, polyethyleneglycol 400 (PEG400), hyaluronidase (500 IU/mg activity), and antipyrine were purchased from Wako Pure Chemical Industries (Osaka, Japan); and 1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene iodide (TMA–DPH) was purchased from Molecular Probes Inc. (Eugene, OR, USA). Other chemicals were of reagent grade.

2.2. Animals

Male Wistar rats weighing 220–300 g and a small pig weighing approximately 20 kg were fed standard laboratory chow and had free access to water. Food was withheld from the rats for 16 h before they were used in the membrane permeability and electrophysiological studies. Food was withheld from the pig overnight prior to being used in the membrane lipid fluidity study. All animals received human care and were treated in accordance with the Guidelines for Animal Experimentation from Kyoto Pharmaceutical University and Hokuriku University.

2.3. Determination of critical micelle concentration of Labrasol

Labrasol solutions were prepared at concentrations of 0.001, 0.01, 0.1, 0.5, 1, 2, 3, 4, 5, 10, 20, 25, 30, 40, 50, and 60% (v/v). Two microliters of the fluorescent probe DPH (0.25 mM) was added to each Labrasol solution (5 ml), then incubated at 37 °C for 30 min. The fluorescence intensity of all samples and a blank sample was measured with a Hitachi Spectrofluorometer F-4500 (Hitachi Seisakusho Corp., Tokyo, Japan) at $\lambda_{ex} = 363$ nm and $\lambda_{em} = 428$ nm.

2.4. Shape of Labrasol micelle in aqueous solution

To determine the point at which Labrasol micelles change in shape from spheres to rods, we measured the viscosity of various concentrations of Labrasol using different methods. First, the kinematic viscosity of Labrasol (0.001–60%, v/v) was measured with a Ubelode Viscometer at 25 °C, and the density of each solution was determined by measuring the weight of 1 ml of each Labrasol solution at 25 °C. We calculated the viscosity of these solutions by multiplying the kinematic viscosity by the density.

Second, we used the fluorescent dye, auramine, which reflects minute changes in the viscosity [6,7], to measure the viscosity of various concentrations of Labrasol (20–60%, v/v). Auramine (1×10^{-7} mol/l) was added to the Labrasol solutions, and then fluorescence intensity was measured at $\lambda_{ex} = 440$ nm and $\lambda_{em} = 500$ nm.

To determine whether Labrasol micelles adopt a liquid crystalline structure in highly concentrated aqueous solutions, we measured the fluorescence intensity of DPH embedded in Labrasol micelles. DPH-labeled Labrasol micelles were prepared by incubating Labrasol (20–60%, v/v) with DPH (1×10^{-7} mol/l) at 37 °C for 30 min. The fluorescence intensity of all samples and a blank sample was measured with a polarized plate reader set at $\lambda_{ex} = 363$ nm and $\lambda_{em} = 428$ nm. The anisotropy (r) of DPH embedded in the Labrasol micelles was calculated using the following equation,

$$r = \frac{I_V - I_H}{I_V + 2I_H}$$

where I_V and I_H represent the fluorescence intensities perpendicular and parallel, respectively, to the polarized excitation plane.

2.5. Micelle size and size distribution

The average particle size and size distribution of Labrasol micelles in 2%, 5%, 25%, and 50% (v/v) Labrasol aqueous solution were determined using a Dynamic Light

Scattering (DLS) Analyzer (DLS-7000, Otsuka Electronics Co., Osaka, Japan). The average diameter of the micelles was determined using the average weight-weighed diameter (d_w) and average number-weighed diameter (d_n). The particle size distribution was calculated as a ratio of d_w to d_n and the polydispersity index using a multimodal method and an exponential sampling algorithm [8,9].

2.6. Dialysis

We prepared 1 mg/ml GM solutions (2 ml) with or without Labrasol (5%, 25%, and 50%) and pipetted these solutions into individual dialysis membrane tubes (Spectra Por CE, MWCO 1000, Spectrum Lab. Inc., Los Angeles, CA, USA). The tubes were placed into beakers containing 48 ml of either distilled water or Labrasol (5%, 25%, and 50%), and the concentration of Labrasol inside and outside of the dialysis membrane tubes was allowed to equalize in order to maintain the osmotic pressure gradient. Dialysis was initiated by stirring the fluid in the beaker with a magnetic stir bar for 48 h, and then the amount of dialyzed GM was determined as described previously [5,10].

2.7. Membrane permeability and electrophysiological studies using rat ileum

Rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal administration) and killed by cutting the main abdominal artery. Immediately thereafter, the ileum was removed, cut into 4-cm lengths with scissors, and opened up into a flat sheet. The longitudinal muscle was removed from the flattened sheet of ileum with forceps, and then the sheet was set between Ussing-type diffusion chambers (No. 3440-s, Navicyte Inc., Sparks, NV, USA). The ileum sheet retains mucous layer. On the other hand, the ileum sheet which was removed the mucous layer was prepared by the pretreatment with hyaluronidase [11]. Namely, 320 U/ml hyaluronidase dissolved in phosphate buffered saline (pH 7.4) was singly circulated via perfusion through the canula at the speed of 1.0 ml/min for 30 min using a peristaltic pump before killing the rats. Glass barrel reference microelectrodes were placed into the chambers. The serosal and mucosal sides of the chambers were immediately filled with 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)/2-amino-2-hydroxymethyl-1,3-propanediol (Tris) buffer (pH 7.4) containing 100 mM D-mannitol and 100 mM KCl (3 ml). During the experiments, both chambers were incubated at 37 °C and bubbled with O_2/CO_2 (95:5, v/v). The membrane potential difference (PD) and short-circuit current (I_{sc}) were measured at 5 min intervals for 20 min during a pre-incubation period. After pre-incubation, we replaced the solution in the mucosal side with 1.5 mg/ml GM (3 ml) with or without Labrasol (5% and 25%, v/v), and PD and I_{sc} were measured successively at 5 min intervals for 60 min.

Antipyrine was selected as a compound transported via a passive transcellular route [12]. As well as the membrane permeability experiment of GM, the membrane permeability of antipyrine was examined using Ussing-type diffusion chambers that rat ileum pretreated with hyaluronidase is mounted. The concentration of antipyrine in mucosal side chamber was set to 0.5 mM.

To assess the membrane permeability of GM and antipyrine, we removed a 100 μl aliquot from the serosal chamber at 30, 60, and 90 min for GM and at 20, 30, 40, 50, and 60 min for antipyrine after incubation, filtered the sample with an Advantec filter (Dismic-13HP, PTFE 0.20 μm pore size, Toyo Roshi Kaisha, Tokyo, Japan), and injected 30 μl of this sample into an HPLC system. HPLC parameters for GM and antipyrine analyses were described previously in reference numbers [10] and [13], respectively. We calculated the membrane resistance (R_m) according to the following equation:

$$R_m = \frac{PD}{I_{sc}}$$

2.8. Preparation of liposomes from porcine intestinal mucosa

2.8.1. Preparation of brush border membrane vesicles (BBMVs)

A small pig was anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal administration), and its small intestine was excised. The intestinal mucosa was carefully scraped with a glass slide, and BBMVs were prepared from the scrapings according to the method of Kessler et al. [14]. The BBMVs were ultimately suspended in 20 mM HEPES–Tris buffer (pH 7.4) containing 100 mM D-mannitol and 100 mM KCl, and were adjusted to a final protein concentration of 10 mg protein/ml. Protein was quantified with a BCA assay kit (Pierce Chemical Co., Chicago, IL, USA). The quality of the preparation was determined by assay of the marker enzyme activities, i.e., alkaline phosphatase and sucrase. The activities of alkaline phosphatase and sucrase were enriched about 12- and 10-fold compared to those of the original mucosa, respectively.

2.8.2. Extraction of lipid from BBMVs

A glass centrifuge tube containing a suspension of BBMVs (1 ml), 0.2 M acetic acid (1 ml), methanol (5 ml) and chloroform (2.5 ml) was mixed for 30 s with a vortex mixer, and then allowed to remain undisturbed for 30 s. Upon completing 5 repetitions of this mix-and-rest procedure, the mixture was left at room temperature for 10 min. Chloroform (2.5 ml) was then added to the tube, which was vortexed for 2 min; and 0.1 M acetic acid (2.5 ml) was added to the tube, which was shaken for 2 min. After remaining undisturbed for 10 min, the tube was centrifuged at 1500*g* for 10 min. The upper phase solution was removed with a glass pipette and discarded. The remaining lower phase solution containing the lipids was evaporated in a rotary evaporator under vacuum at 45 °C until dry. After cooling at room temperature with N_2 gas, the tube containing the lipid was stored at 4 °C for later use.

2.8.3. Preparation of brush border membrane liposomes (BBMLs)

Brush border membrane liposomes (BBMLs) were prepared by dispersing the lipids lining the glass tube (see above). This was accomplished by washing the inside of the tube with 20 mM HEPES–Tris buffer (pH 7.4) containing 100 mM D-mannitol and 100 mM KCl. This was carried out using a 1 ml syringe and 26-gauge 1/2″ needle. We called the dispersed lipids BBMLs. We adjusted the final lipid concentration of the BBML suspension to 12 mg lipid/ml.

2.9. Membrane lipid fluidity assessment

2.9.1. Labeling BBMLs with fluorescent probes

BBMLs were labeled with the fluorescent probes, diphenyl hexatriene (DPH) and trimethyl ammonium diphenyl hexatriene (TMA–DPH) by incubating a suspension of BBMLs (1 ml) with either 1 μmol/l DPH or 1 μmol/l TMA–DPH (2 ml) for 30 min at 37 °C in the presence of gaseous N_2. The mixture was centrifuged at 70,000*g* for 30 min at 4 °C. After discarding the supernatant, we carefully re-suspended the precipitated BBML pellet in 1 ml of 20 mM HEPES–Tris buffer (pH 7.4) containing 100 mM D-mannitol and 100 mM KCl, using a 1 ml syringe and 26-gauge 1/2″ needle.

2.9.2. Fluorescence measurement

The intensity of fluorescence was measured at 25 °C with a Hitachi Spectrofluorometer F-4500 equipped with a rhodamine B quantum counter (Hitachi Seisakusho Corp., Tokyo). The excitation and emission wavelengths used during the measurements were 363 and 428 nm, respectively, for DPH and 365 and 428 nm, respectively, for TMA–DPH. The fluorescence anisotropy (r) of DPH and TMA–DPH embedded in the BBMLs was calculated using the following equation:

$$r = \frac{I_V - I_H}{I_V + 2I_H}$$

where I_V and I_H represent the fluorescence intensities perpendicular and parallel, respectively, to the polarized excitation plane [15]. We also measured the fluorescence intensity of solutions containing either DPH- or TMA–DPH-labeled BBMLs with or without Labrasol or solutions containing labeled BBMLs with PEG400 (0.01–40%, v/v) at 25 °C.

3. Results and discussion

3.1. Phase behavior of Labrasol in aqueous solution

3.1.1. Critical micelle concentration of Labrasol

Although the fluorescent probe DPH is water insoluble, it becomes soluble when mixed with surfactants by linking to the hydrophobic region of surfactant micelles; during this linking process, DPH emits fluorescence [16].

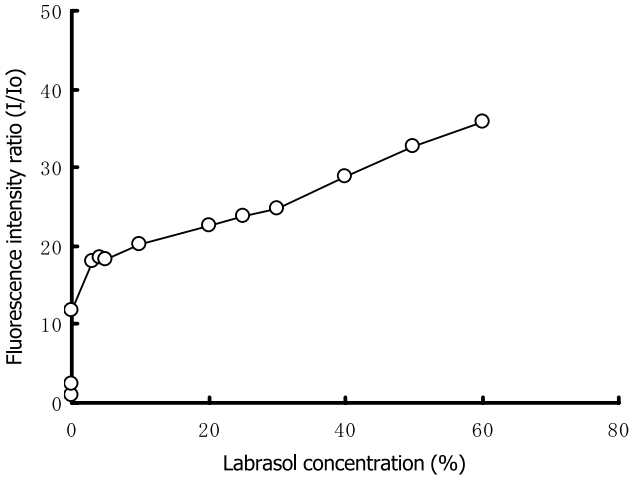

Fig. 1. Effect of Labrasol on DPH fluorescence intensity. I and I_o represent the fluorescence intensities of DPH in the presence or absence of Labrasol, respectively. The I_o value was 23. All samples contained DPH at a final concentration of 1×10^{-7} M. Data represent means of three experiments. Standard deviations (SDs) were very small and thus not indicated in the graph.

We determined the critical micelle concentration of Labrasol using this fluorescence depolarization technique. The fluorescence intensity ratio (I/I_0) of DPH in Labrasol solution containing 1×10^{-7} M DPH is shown in Fig. 1. The I/I_0 of a 0.001% Labrasol solution was 2.31, but the I/I_0 of a 0.01% Labrasol solution increased remarkably to 11.7. The I/I_0 of Labrasol solutions ranging from 0.1% to 60% increased gradually. These results suggest that the critical micelle concentration of Labrasol was approximately 0.01% and that the gradual increase in the fluorescence intensity ratio was proportional to the quantity of micelles present.

3.1.2. Shape of Labrasol micelle in aqueous solution

In general, micelles of nonionic surfactants in aqueous solution change from spherical to rod-like shapes to liquid crystal structures (e.g., lamellar) with increasing concentrations [17–19]. We examined changes in the micellar shape of Labrasol in aqueous solution by measuring the viscosity of different concentrations of Labrasol. This was based on data indicating that changes in viscosity of solutions containing surfactants reflect changes in micellar shape [20,21]. The viscosity of Labrasol solution increased with increasing concentrations of Labrasol (Fig. 2A). The viscosity of 20% Labrasol increased abruptly, indicating that this is the concentration at which micellar shape changes from spherical to rod-like (i.e., growth point). The fact that we detected only one abrupt shift in viscosity among the different concentrations of Labrasol solutions we tested indicates that the Labrasol micelles did not form hexagonal or lamellar structures.

To confirm that Labrasol micelles remain rod-like in 20–60% solutions, we measured the fluorescence intensity of auramine when added to various concentrations of

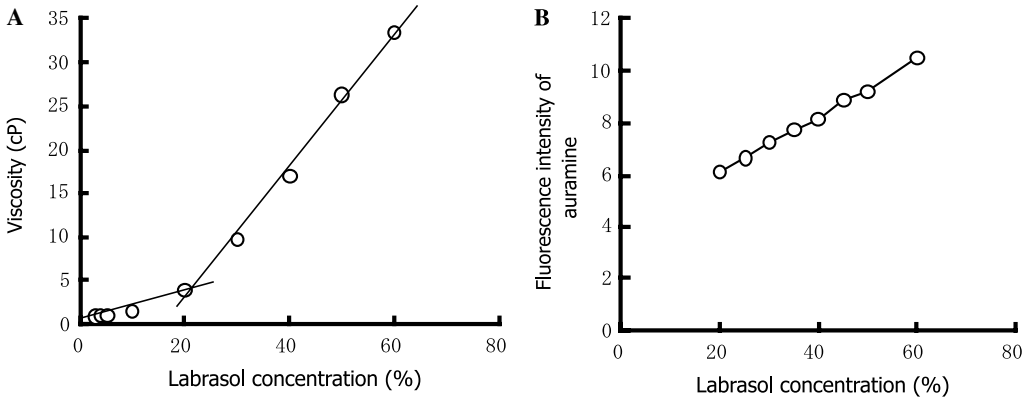

Fig. 2. (A) Viscosity of various concentrations of Labrasol solutions at 25 °C and (B) the effect of Labrasol on the fluorescence intensity of auramine. We measured viscosity using a Ubelode viscometer. The final concentration of auramine in all samples was 1×10^{-7} M. The fluorescence intensity of auramine in the absence of Labrasol (I_o) was 0.670. Data represent means of six experiments. SDs were very small and thus not indicated in the graphs.

Labrasol solutions. Fig. 2B shows the relationship between Labrasol concentration and the fluorescence intensity of auramine. The curve was linear, confirming the results of the viscosity experiments that the micellar shape of Labrasol did not change at concentrations ranging from 20% to 60%. This was further confirmed by examining the DPH fluorescence anisotropy as a function of Labrasol concentration (Fig. 3). If micelles in concentrated Labrasol solutions exist as liquid crystal structures, we would expect the fluorescence anisotropy of DPH linked to the micelles to be high, since other liquid crystal structures such as phospholipid bilayers of DPH-labeled BBMVs have fluorescence anisotropy values of 0.18–0.22 [22,23]. The fluorescence anisotropy values were almost the same, regardless of the concentration of Labrasol. The fluorescence anisotropy of DPH in 0.001% Labrasol was 0.057, whereas that in 60% Labrasol was 0.085. Because these anisotropy values were remarkably lower than those of DPH-labeled BBMVs, we concluded that concentrated Labrasol solutions did not form liquid crystal structures. In summary, from the auramine and DPH experiments, we conclude that, at concentrations ranging from 20% to 60%, Labrasol micelles did not change in shape.

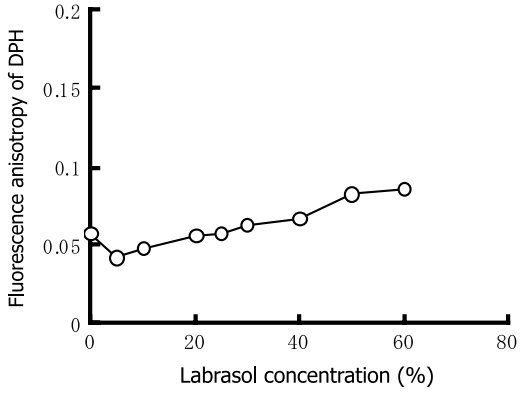

Fig. 3. Effect of Labrasol on the fluorescence anisotropy of DPH. The final concentration of DPH in all samples was 1×10^{-7} M. Data represent means of two experiments.

3.1.3. Micelle size and size distribution

Average particle size and size distribution of micelles are important factors that influence the membrane permeability of a drug [24]. To determine the concentration of Labrasol that would best facilitate the permeability of a drug, we used DLS analysis, which is frequently used for measuring particles of nano-order size [9,25–27]. Fig. 4 shows the weight-weighed and number-weighed size distribution of 2%, 5%, 25%, and 50% Labrasol solutions, and Table 1 shows the average micelle sizes and polydispersity indices of these solutions. The d_w values of 2%, 5%, 25%, and 50% Labrasol were 10.4, 12.9, 22.3, and 23.1 nm, respectively, and the d_n values were 6.5, 10.2, 18.0, and 14.0 nm, respectively, indicating that average particle size increased with increasing concentrations of Labrasol at the range of 2–25%. The average sizes of particles in 25% Labrasol were approximately 2-fold greater than those in 2% and 5% Labrasol solutions. The polydispersity index values of these four Labrasol solutions were low, ranging from 0.23 to 0.32. Taken together, these results indicate that micelle size increased in solutions having concentrations greater than 20% Labrasol (growth point concentration).

In general, particle shape can be estimated by calculating the ratio of hydrodynamic diameter determined by DLS assay and inertia diameter determined by static light scattering (SLS) assay. However, we could not calculate the inertia diameter of Labrasol, because particle size was too small to measure using a SLS device. Although it was not possible to definitively identify the shape of micelles in 25% Labrasol, when considering our findings thus far, we believe that the micellar shape is likely to be rod-like.

3.2. Dissolution of GM in Labrasol micelles

The aim of these experiments was to determine the affinity of GM to Labrasol micelles. Because GM is a hydrophilic drug, we hypothesized that, in aqueous solutions of Labrasol, GM may occupy the aqueous region surrounding Labrasol micelles and the hydrophilic surface region

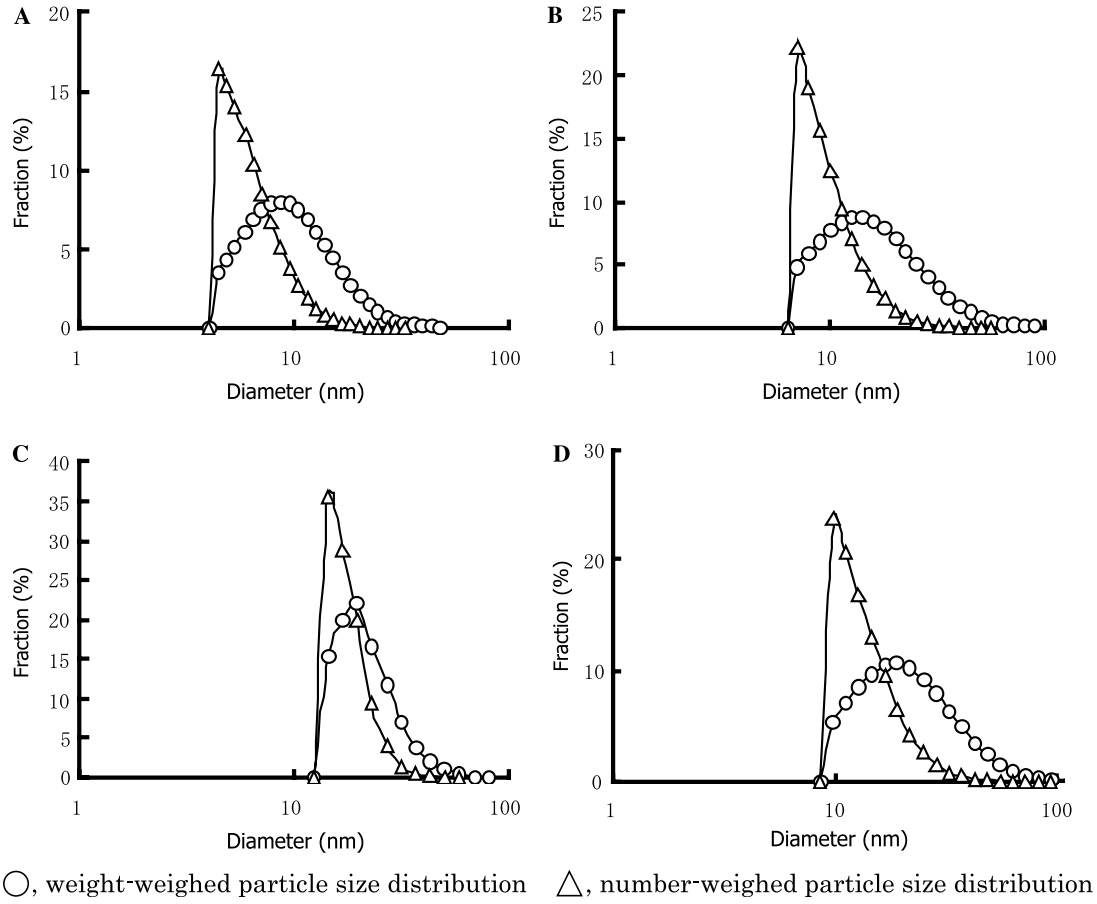

Fig. 4. Weight- and number-weighed particle size distribution of different concentrations of Labrasol in aqueous solution at 25 °C. (A) 2% Labrasol, (B) 5% Labrasol, (C) 25% Labrasol, and (D) 50% Labrasol. Accumulation times for scattering analysis were all 100.

Table 1
Average diameter and polydispersity index of Labrasol micelles

Labrasol concentration (%, v/v)	d_w (nm)	d_n (nm)	d_w/d_n	PI
2	10.4 ± 5.0	6.5 ± 2.0	1.60	0.305
5	12.9 ± 11	10.2 ± 4.0	1.26	0.279
25	22.3 ± 7.5	18.0 ± 4.3	1.24	0.231
50	23.1 ± 12	14.0 ± 5.0	1.65	0.316

Abbreviations: d_w, weight-weighed; d_n, number-weighed average diameter; PI, polydispersity index.

of the micelles. This hypothesis seems reasonable given that GM has a slender molecular structure and a small molecular diameter [calculated in the longest dimension to be 1.62 nm with Chem3D software (MathStat, Victoria, Australia)]. Additionally, since the diameter of micelles in 25% Labrasol was approximately 20 nm, it seems plausible that the GM molecule would easily fit within the hydrophilic region of Labrasol micelles. If the affinity of GM to Labrasol micelles is low, we predicted that GM would be easily dialyzed. Conversely, if the affinity of GM to Labrasol micelles is high, we expected that GM would not be dialyzed.

We found that the amount of dialyzed GM in the absence of Labrasol was 181 μg, whereas the amount of dialyzed GM in 5% Labrasol was 8.1 μg. This quantity was correspondent to 4.5% of that in the GM solution lacking Labrasol. Very little if any GM was dialyzed from the 25% and 50% Labrasol solutions containing GM, suggesting that almost all GM molecules occupy the aqueous region surrounding Labrasol micelles and/or the hydrophilic surface region of the micelles. A similar relationship between bile salt micelles and cholesterol has been reported [28]. Namely, in aqueous solutions containing surfactant, cholesterol molecules not only exist within the hydrophilic surface of the micelles, but also occupy the hydrophilic region between micelles in the aqueous solution. Based on our observations, we conclude that GM has a high affinity for Labrasol micelles.

3.3. Electrophysiological assessment of membrane permeability using Ussing-type diffusion chambers

The membrane permeability of drugs dissolved in micelles is believed to occur according to the aqueous transfer model [29–31], which posits that micelles cause the membrane resistance to diminish along the hydrophilic region of the membrane surface, thereby increasing permeability to the drug. Hence, according to this model, the membrane permeability of a drug specifically depends on

the drug-micelle binding constant and the drug's dissociation constant, and is enhanced by increased affinity of the drug to the micelles. However, this model does not take into account how micellar shape affects drug permeability. Because it is unclear how Labrasol micelles affect the membrane permeability of GM, we sought to determine whether the membrane permeability of GM is influenced by the different micellar shapes that present in 5% or 25% Labrasol system.

Fig. 5 shows the membrane permeability of GM in the absence and presence of 5% or 25% Labrasol. In order to know exactly the enhancing effect of Labrasol on the membrane permeability of GM, the permeability of GM was similarly examined using the ileum pretreated with hyaluronidase. The permeated amount of GM crossing from the mucosal to the serosal side of the diffusion chambers increased with time. After 90 min of incubation in the absence of Labrasol, the permeated amounts of GM in the condition without and with pretreatment of hyaluronidase were 3.90 ± 0.44 μg/cm^2 and 3.96 ± 0.28 μg/cm^2. After 90 min of incubation in 25% Labrasol, the permeated amounts of GM in both conditions were 5.52 ± 0.61 μg/cm^2 and 7.42 ± 0.67 μg/cm^2, respectively. These results suggested that mucous layer inhibited the increase of GM permeability by Labrasol, but not significant. Although the difference in membrane permeability rates (slope in graph) of GM among three kind test solutions in the condition without pretreatment of hyaluronidase was slight, the difference in those in the condition with pretreatment of hyaluronidase was observed clearly. Namely, the permeability rate of GM in the presence of 25% Labrasol was 1.6-fold compared with those in the absence and presence of 5% Labrasol. These results indicate that the micellar shape of Labrasol affected the membrane permeability of GM, since 25% Labrasol enhanced the GM permeability through rat ileum and 5% Labrasol did not. The latter finding suggests that the affinity of GM to Labrasol micelles may be closely involved in the permeability mechanism. These findings emphasize that interactions between Labrasol micelles and membrane components, such as lipids, as well as the affinity of GM to Labrasol micelles, are required to enhance the membrane permeability of GM.

With the exception of specialized routes such as peptide transporters, hydrophilic drugs are generally absorbed through the intestinal mucosa via a paracellular route. The integrity of the paracellular space is maintained by tight junctions between cells. The permeability of hydrophilic drugs is affected by the degree that tight junctions open [32–34]. Although it has been reported that Labrasol induced the opening action for tight junction of Caco-2 cells at the Labrasol concentration of 1% [4], we found that 25% Labrasol, not 5% Labrasol, enhanced the membrane permeability of GM across rat ileum. Therefore, it was assumed that concentrated Labrasol (25%) in formulation has an enhancing effect for transcellular route except for the tight junction opening action. Labrasol has capric acid as a predominant fatty acid. The report that the capric acid enhances the permeability via a transcellular route [35] supports the result which Labrsol enhanced the GM permeability. In order to clarify the enhancing effect of Labrasol on a transcellular route, we investigated the membrane permeability of antipyrine, a drug transported via a transcellular route [12], using Ussing-type diffusion chambers (Fig. 6). The permeable amount of antipyrine with 25% Labrasol was higher than those in the absence and presence of 5% Labrasol, suggesting that the concentrated Labrasol (25%) enhanced the permeability of drug via a transcellular route in rat ileum. Since the change in the membrane permeability is closely related to the membrane resistance, we investigated changes in membrane resistance in the absence and presence of 5% or 25% Labrasol using Ussing-type diffusion chambers fitted with rat ileum.

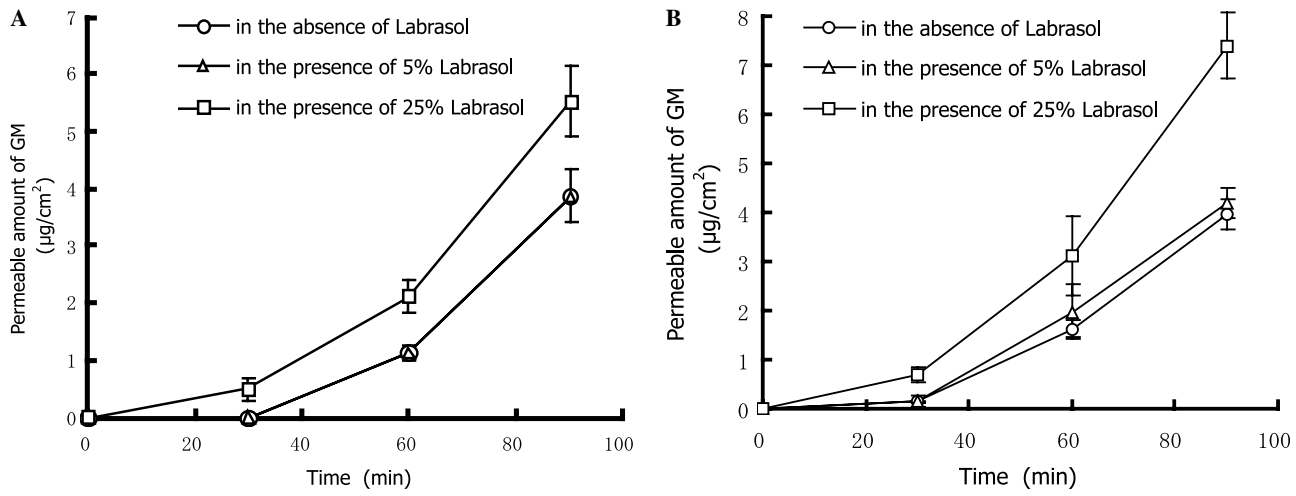

Fig. 5. Changes in the membrane permeability of GM at 37 °C by rat ileum without (A) and with (B) pretreatment of hyaluronidase. Permeability was assessed with Ussing-type diffusion chambers. The chamber on the mucosal side was filled with a GM solution containing 0%, 5%, or 25% Labrasol. Data represent means ± SD of three experiments.

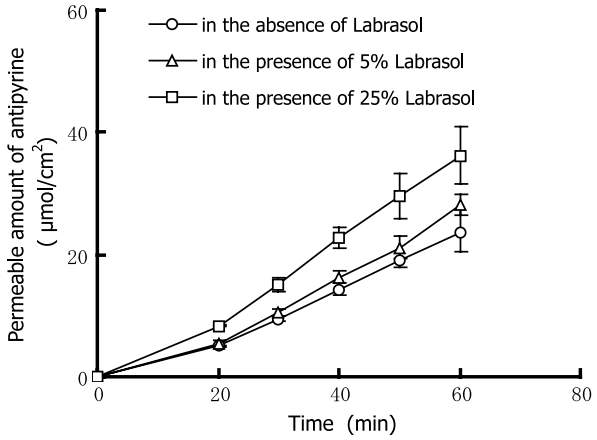

Fig. 6. Changes in the membrane permeability of antipyrine at 37 °C by rat ileum with pretreatment of hyaluronidase. Permeability was assessed with Ussing-type diffusion chambers. The chamber on the mucosal side was filled with 0.5 mM antipyrine solution containing 0%, 5%, or 25% Labrasol. Data represent means ± SD of three experiments.

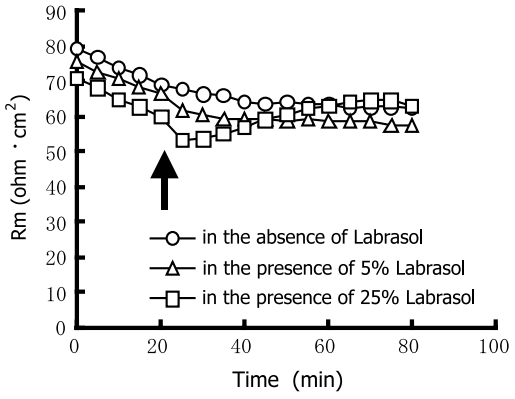

Fig. 7. Effect of Labrasol on membrane resistance at 37 °C. Membrane resistance was assessed with Ussing-type diffusion chambers separated by a sheet of rat ileum. The chamber on the mucosal side was filled with 20 mM HEPES–Tris buffer (pH 7.4) containing 100 mM D-mannitol and 100 mM KCl. After 20 min, the solution was replaced with 0%, 5%, or 25% Labrasol. Data represent means of three experiments. All SD values were within 10% of each mean.

As shown in Fig. 7, the membrane resistance in the absence of Labrasol was approximately 80 ohm cm^2 initially, gradually decreased, and then was maintained at 65 ohm cm^2. The membrane resistance–time curve in the presence of 5% Labrasol was the same as that when Labrasol was absent. On the other hand, with 25% Labrasol, membrane resistance temporarily decreased initially, and then gradually recovered to the control value (65 ohm cm^2). These results indicate that 5% Labrasol had no measurable effect on membrane resistance in rat ileum, and that 25% Labrasol only transiently affected membrane resistance.

3.4. Membrane lipid fluidity

Because Labrasol did not remarkably decrease membrane resistance, we hypothesized that Labrasol crosses the intestinal mucosa via a transcellular rather than a paracellular route. We tested this hypothesis by assessing membrane lipid fluidity in the presence of different concentrations of Labrasol. Generally, intestinal BBMVs are used to study membrane lipid fluidity; however, the solubility of concentrated Labrasol solutions in membrane proteins is poor. Therefore, we chose BBMLs prepared from porcine small intestine in order to reduce the lowering of turbidity of lipid dispersion based on solubilization by the addition of Labrasol during fluorometry. The effect of Labrasol on membrane lipid fluidity was assessed by labeling the membrane lipid layer with fluorescent probes (DPH and TMA–DPH), measuring fluorescence intensity, and calculating fluorescence anisotropy [36–38]. For comparison, we also performed these experiments with PEG400 in place of Labrasol.

Fig. 8 shows the fluorescence anisotropy of DPH and TMA–DPH embedded within BBMLs. The fluorescence anisotropy of DPH decreased sharply with Labrasol concentrations of 0–5%, leveling off with Labrasol concentrations of 5–40% (Fig. 8A). The fluorescence anisotropy of DPH remained constant in the presence of PEG400,

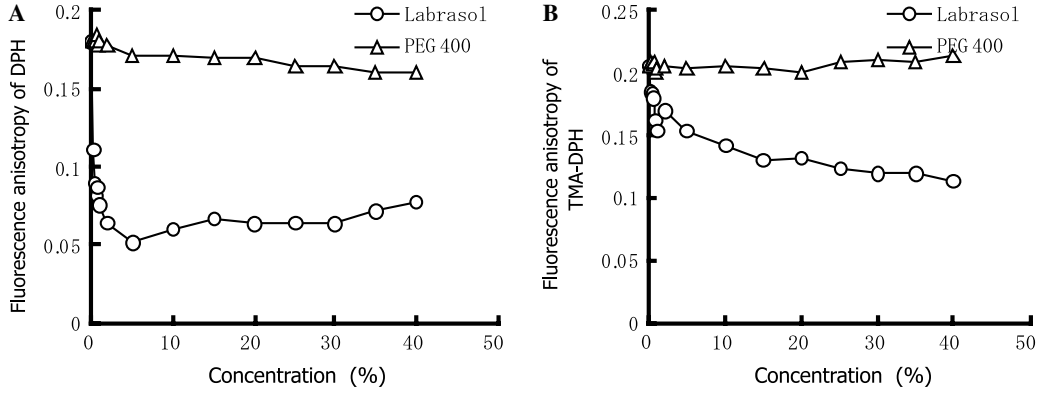

Fig. 8. Effects of Labrasol and PEG400 on the fluorescence anisotropy of (A) DPH and (B) TMA–DPH embedded in brush border membrane liposomes (BBMLs) at 25 °C. The BBMLs were prepared from porcine small intestines. Data represent means of three experiments. All SD values were within 10% of each mean.

regardless of PEG400 concentration. These results indicate that the membrane fluidity within the inner side of the lipid bilayer reached the highest degree of fluidity when exposed to 5% Labrasol.

We repeated the experiment with the DPH derivative TMA–DPH, which is more sensitive to changes in the surface side of the lipid bilayer than is DPH [38]. The fluorescence anisotropy of TMA–DPH embedded within BBMLs remained unchanged with Labrasol concentrations of 0–0.2% (Fig. 8B). The fluorescence anisotropy in 0.2% Labrasol was 0.177. The fluorescence anisotropy of TMA–DPH decreased gradually with increasing concentrations of Labrasol. As we observed with DPH, the fluorescence anisotropy of TMA–DPH remained more or less constant in the presence of PEG400, regardless of PEG400 concentration. These results suggest that Labrasol strongly interacted with the inner region of lipid bilayer than the surface region of membrane lipid.

The results of the membrane fluidity analysis appear to be at odds with those of the phase behavior of Labrasol, since the latter indicated that Labrasol solutions with concentrations greater than 20% are required to enhance membrane permeability to GM. One explanation for this apparent discrepancy is that the membranes of BBMLs are structurally weaker than those of rat ileum, because cholesterol and protein makes it more stable in the epithelial membrane does not exist in BBMLs [39–41]. If this were the case, one would expect that Labrasol would affect BBMLs much more rapidly and at lower concentrations than what would be needed to achieve the same affect in rat ileum. Namely, the effect of 5% Labrasol on BBMLs may be similar to that of 25% Labrasol on rat ileum. Nonetheless, it is clear that Labrasol strongly affected the fluidity of membrane lipids.

3.5. Conclusion

The critical micelle concentration of Labrasol was approximately 0.01% in aqueous solution, and the growth point for micellar size and shape occurred at a concentration of 20%. Although the micellar shape of Labrasol was not accurately caught, when Labrasol concentration exceeds 20%, the particle size was approximately 20 nm in diameter (DLS measurements). GM displayed a high affinity for Labrasol micelles when dissolved in solutions with concentrations greater than 20% Labrasol. This is consistent with our diffusion chamber experiments showing that membrane permeability to GM increased when dissolved in 20% or more Labrasol.

From the results of the membrane resistance and membrane lipid fluidity analyses we propose that Labrasol enhances membrane permeability to GM according to the following mechanisms: (i) GM may exist in the hydrophilic region of Labrasol micelles when dissolved in solutions of 20% or more of Labrasol; (ii) since Labrasol increases membrane lipid fluidity, GM associated with Labrasol micelles can enter epithelial cells; (iii) GM is likely to be transported through the intestines via a transcellular route based on an aqueous transfer model.

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