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The impact of quantity of lipid based formulations with different compositions on the oral absorption of ritonavir: A trade-off between apparent solubility and permeability

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ABSTRACT

In this study, the effect of the quantity of lipid-based formulations (LBFs) on the oral absorption of ritonavir (RTV), a model for poorly water-soluble drugs, was investigated. Two types of LBFs, comprising short- and medium-chain lipids (LBF-SMC) and long-chain lipids (LBF-LC) loaded with different masses of RTV, were prepared. Then, the respective LBFs were dispersed in distilled water at concentrations of 1.0, 2.0, and 3.0% w/w, which provided the same drug concentration for all formulations. When 1.0% LBF-SMC and LBF-LC were orally administered to rats, the oral absorption was significantly improved compared with that of the suspension (a reference formulation) because of enhanced solubilization of RTV in the gastrointestinal tract; however, this improvement was lower for LBF-LC than for LBF-SMC. The oral absorption decreased with increasing LBF concentration for both LBF-SMC and LBF-LC. The *in vitro* permeation in sequence with *in vitro* digestion revealed that this phenomenon was caused by a reduction in the free drug concentration in the gastrointestinal tract. Moreover, the effect of decreasing the free concentration was more remarkable for LBF-LC than for LBF-SMC because of the greater solubilization capacity of LC digestion products. These findings may be useful for designing improved drug delivery systems.

1. Introduction

Lipid-based formulations (LBFs) provide a means to enhance the oral absorption of drugs with low-solubility via pre-dissolution of the drug in the formulation (Jo et al., 2020; Liu et al., 2020; Sun et al., 2021). After oral administration, the LBF undergoes endogenous digestion by gastric and pancreatic lipases, resulting in the generation of a series of colloidal species composed of amphiphilic digestion products (typically fatty acids, monoacylglycerides, and diacylglycerides) and endogenous components (bile salts and phospholipids) in the gastrointestinal (GI) tract (Koziolek et al., 2018; Yeap et al., 2013a, 2013b). This lipid digestion pathway results in a reduced solubilization capacity of LBFs and the generation of a transient supersaturation of drugs that promotes oral absorption. However, the supersaturated state also carries the risk of driving drug precipitation, which may restrict oral absorption (Williams et al., 2014; Kuentz, 2019).

Tri-, di-, and mono-glycerides with different chain lengths of fatty acids are typically used as components of LBFs. Aqueous colloidal species generated during LBF digestion also have the ability to solubilize lipophilic drugs (but typically lower than lipids) (Tanaka et al., 2020). The nature of colloidal structures differs depending on the LBF composition, which leads to differences in solubilization capacity. Conversely, the incorporation of drugs into the colloidal structures also stimulates a decrease in thermodynamic activity in the GI tract, which is likely to reduce free concentration and therefore apparent permeability. Indeed, when ritonavir (RTV), a biopharmaceutics classification system (BCS) class II drug clog P base (Takagi et al., 2006) or a class IV drug considering its lower Caco-2 permeability (Aungst et al., 2000; Patel et al., 2004) relative to that of metoprolol (Incecayir et al., 2013), was orally administered as LBF with long-chain (LC) lipids, the *in vivo* AUC was not improved compared to that of the control suspension (Tanaka et al., 2021a). This was due to a reduction in the free drug concentration

Abbreviations: AP, aqueous colloidal phase; 4-BPB, 4-bromophenylboronic acid; GI, gastrointestinal; IS, internal standard; LBF, lipid based formulation; LBF-LC, long-chain lipid-based formulation; LBF-SMC, short-chain and medium-chain lipid-based formulation; LC, long-chain; MC, medium-chain; NaTC, sodium taurocholate; OP, oil phase; PP, pellet phase; RTV, ritonavir; SC, short-chain; SR, super saturation ratio; SRM, maximum super saturation ratio.

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caused by the incorporation of RTV into the LC colloidal structures. On the other hand, the AUC drastically increased after administration of the LBF with medium-chain (MC) lipids, which did not reduce the free drug concentration to the same degree as the LC-LBF (Tanaka et al., 2021a). In addition, the extent of the free concentration of saquinavir depended on the solubilization capacity of digestion products generated from LBFs with different compositions, leading to different oral absorptions *in vivo* (Tanaka et al., 2021b). Thus, both the solubilization effect of LBFs and the effect on intestinal permeation should be evaluated to accurately predict oral absorption.

In addition to LBF composition, determining the quantity of formulation excipients (lipids and surfactants) is also important for the development of optimal LBFs. Generally, an increase in the quantity of formulation is effective for increasing the drug load and drug solubilization in the GI tract. However, it is considered that formulation quantity may also change the nature of colloidal species, and thus, it may have a great impact not only on solubilization but also on the apparent permeability of drugs. Lee et al. (2013) have reported the effect of administered doses of MC and LC lipids on the oral absorption of cinnarizine, a poorly water-soluble drug, at a constant cinnarizine dose in rats. The *in vivo* exposure to cinnarizine increased when the mass of co-administered MC and LC lipids increased from 125 to 250 mg. However, further increase in the lipid dose to 500 mg had no impact on the oral bioavailability of cinnarizine for MC lipids and reduced oral bioavailability of the LC lipid (although it was not significantly different). Although they discussed that the possible reasons may be delay in gastric emptying (because the T_{max} increased at 500 mg co-administration of both lipids), insufficient lipid digestion, and/or a decrease in thermodynamic activity at higher dose of lipids, experiments to prove these assumptions have not been performed. From our reports described above (Tanaka et al., 2021a, 2021b), it is expected that as lipid dose increases, thermodynamic activity in the GI tract may decrease, leading to reduction in apparent intestinal permeability. However, mechanisms of drug absorption from LBFs with different quantity and compositions has not yet been fully elucidated.

In vitro digestion experiments have been extensively utilized to assess LBF processing and drug solubilization behavior in the GI tract (Karavasili et al., 2020; Klitgaard et al., 2020; Sahbaz et al., 2017). However, as the system lacks an absorption sink, the data obtained for drug solubilization sometimes fail to predict oral absorption (Larsen et al., 2013; Feeney et al., 2016). To improve the predictability of oral absorption, we performed simple *in vitro* permeation experiments with a dialysis membrane in sequence with *in vitro* digestion experiments and successfully predicted the *in vivo* performance of LBFs (Tanaka et al., 2021b).

In this study, we attempted to evaluate the effect of the quantity of LBF with different compositions on the oral absorption of RTV. First, different masses of LBFs were dispersed in a simulated rat intestinal fluid (where drug concentrations were identical), and solubilization behavior *in vitro* was examined using a conventional *in vitro* digestion model. Subsequently, *in vitro* permeation experiments were performed using a dialysis membrane and pre-digested LBF to evaluate the impact of the colloidal species generated during LBF digestion on free drug concentration. Finally, *in vitro* data were correlated to *in vivo* oral exposure to determine the possibility of predicting bioperformance in this *in vitro* system.

2. Materials and methods

2.1. Materials

RTV, tributyrin, a short-chain (SC) triglyceride, Tween 85, egg-lecithin, and 4-bromophenylboronic acid (4-BPB) were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Saquinavir was purchased from LGM pharma (Erlanger, KY, USA). Capmul MCM C8 EP/NF, a medium-chain (MC) mono- and di-glyceride, was obtained from Abitec Corporation (Columbus, OH, USA). Maisine CC, a long-chain (LC) mono-, di-, and triglyceride, was purchased from Gattefossé (Lyon, France). Corn oil, a LC triglyceride, and sodium taurocholate (NaTC) were bought from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Porcine pancreatin extract (P7545, 8 × USP specifications) was obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade.

2.2. Preparation of LBF-SMC and LBF-LC loaded with various masses of RTV

A lipid mixture of tributyrin and capmul MCM C8 EP/NF [1:1 (w/w)] was mixed with Tween 85 at a ratio of 4:1 (w/w) to produce the SC and MC lipid-based formulation (LBF-SMC). LC lipids composed of corn oil and Maisine CC [1:1 (w/w)] were mixed with Tween 85 at a ratio of 4:1 (w/w) to produce the LC lipid-based formulation (LBF-LC). Then, different masses of RTV were suspended in the respective LBFs at 5, 7.5, and 15 mg/g, and the drug was completely solubilized by sonication to produce drug loaded LBFs. The digestion rate of SC- and MC-lipids is typically much faster than that of LC-lipids (Han et al., 2009; Devraj et al., 2013). Comparing SC/MC LBFs to LC LBFs enables to investigate the effect of quantity of LBFs with different digestion rates on oral drug absorption.

2.3. Measurement of equilibrium solubility in LBFs

Blank LBF (0.3 g) was added to plastic tubes containing excess crystalline drug. The tubes were vortexed and incubated at 37 °C. Tubes were centrifuged at 15,100 × g for 10 min (FLD2012, AS ONE Corporation, Osaka, Japan) at 3, 4, 5, 6, and 7 days, and the supernatant (20 mg) was diluted at 50-fold with chloroform and methanol (2:1 v/v), and further diluted 100-fold with water and methanol (1:1 v/v). The final samples were mixed with an internal standard (IS) solution (1 μg/mL saquinavir in 50% methanol) at 1:1 v/v, and then the RTV in the samples was analyzed using LC-MS/MS. Drug solubilities in LBFs were defined as the values when drug concentrations in LBFs across two consecutive days were less than 5%.

2.4. Evaluation of in vivo oral absorption after administration of RTV

2.4.1. Animals

All animal experiments were performed in accordance with the Guideline for Animal Experimentation from the Animal Experimentation Ethics Committee of Hiroshima International University (approval number: AE20–032). Sprague-Dawley male rats were purchased from Japan SLC (Hamamatsu, Japan). Rats weighing 205–232 g were made to fast overnight but were allowed free access to water. During the experiment, rats were deprived of water 1 h prior to and 3 h after dosing.

2.4.2. Preparation of formulations

LBF-SMC and LBF-LC loaded with 15, 7.5, and 5 mg/g RTV were pre-dispersed in distilled water at different concentrations of 1.0, 2.0, and 3.0% w/w, respectively, to a final RTV concentration of 0.15 mg/mL for all pre-dispersed formulations. In addition, methylcellulose solution (0.5% w/v) was added to a glass vial containing crystalline RTV, and the vial was stirred overnight to prepare the control suspension (0.15 mg/mL).

2.4.3. Oral administration and blood sampling

A polyethylene tube was inserted into the left femoral artery of rats anesthetized with isoflurane to enable collection of the blood samples. After the rats were recovered from anaesthetized state, rats were orally administered 1 mL of the suspension and six pre-dispersed formulations. The dose of RTV was 0.15 mg/body for all dosing groups, but dosing concentrations of LBFs were different (1.0%, 2.0%, or 3.0% w/w), which enabled the evaluation of the impact of the quantity of LBFs on oral

absorption. Thereafter, the rats were kept in Bollman cages (KN-326–2, Natsume Seisakusho Co., Ltd., Tokyo, Japan), and blood samples were obtained using the cannula at 0.25, 0.5, 1, 1.5, 2, 2.5, 3, and 4 h after drug administration. The obtained blood samples were then centrifuged at 5400 × g for 5 min. The resultant plasma samples (50 μL) were mixed with 50 μL of saquinavir in methanol (200 ng/mL, IS solution) and 50 μL of methanol. The samples were centrifuged again at 15,100 × g for 10 min to remove the precipitated proteins generated during the mixing process. The drug concentration in the supernatant was analyzed using LC-MS/MS.

2.5. Experiments to evaluate the fate of RTV after in vitro digestion

In vitro digestion studies were carried out using a pH-stat apparatus (AT-710 equipped with a main control unit MCU-710, Kyoto Electronics Manufacturing Co., Ltd., Kyoto, Japan) using a previously described method (Tanaka et al., 2021a; 2021b) with a slight modification. In this model, LBFs were dispersed in a simulated intestinal fluid, and digestion was stimulated by adding porcine pancreatin extract. After centrifugation of the digested samples, samples were typically separated into pellet phase (PP), colloidal aqueous phase (AP), and undigested oil phase (OP). The drug solubilization behavior in the GI tract was evaluated by analyzing the drug distribution in each phase.

In brief, 0.075, 0.15, and 0.225 g of LBF-SMC and LBF-LC loaded with 15, 7.5, and 5 mg/g of RTV were dispersed in 13.5 mL of a lipolysis buffer, a model rat intestinal fluid (2 mM Tris maleate, 1.4 mM $CaCl_2{\cdot}H_2O$, 150 mM NaCl, 50 mM NaTC, and 3.7 mM egg-lecithin, adjusted to pH 6.5 with NaOH), in a thermostated glass vessel (MTA-118–5, Kyoto Electronics Manufacturing Co., Ltd., Kyoto, Japan). The concentrations of NaTC and egg-lecithin in the simulated rat intestinal fluid were based on the actual concentrations of bile acid and phospholipid in the rat upper small intestine that previously measured (Tanaka et al., 2012). Following 10 min equilibration using a magnetic stirrer (length: 3 cm) at 450 rpm at 37 °C, 1.5 mL pancreatin extract was added to the mixture to initiate digestion (final lipase activity was 1000 TBU/mL in the digest). Similar to the experimental design of the oral absorption study, the final RTV concentration in the medium was the same (0.075 mg/mL) for all formulations, and the concentrations of formulations differed in the medium (0.5, 1.0, and 1.5% w/w for LBF-SMC and LBF-LC loaded with 15, 7.5, and 5 mg/g RTV, respectively). The concentration of the drug and LBFs in the buffer was set at 50% of the dosing concentrations for the *in vivo* pharmacokinetic study, reflecting the 2-fold dilution by biliary secretion in the duodenum (Tanaka et al., 2020). Thereafter, the fatty acids liberated by lipolysis were automatically titrated with 1.2 M NaOH for LBF-SMC and 0.6 M NaOH for LBF-LC to maintain the physiological pH in the intestine (6.5). During digestion of LBF-SMC, 1 and 3 mL digests were sampled at 15 and 30 min from the glass vessel, and digestion was inhibited by adding 0.5 M 4-BPB, which is a lipase inhibitor, in methanol at 5 μL/mL. Next, 1 mL of each sample was centrifuged at 15,100 × g for 5 min to obtain the supernatant. Because LBF-SMC was quickly digested, the supernatant was regarded as AP. Collected AP was then diluted 20-fold with 50:50 v/v methanol-water. The PP remaining in the plastic tube was dissolved with 1 mL of methanol to measure the mass of RTV in the PP.

For LBF-LC, 4.8 and 3 mL digests were sampled at 15 min from the glass vessel, and digestion was inhibited by adding 0.5 M 4-BPB. Because of the limited volume of digest (15 mL) for sampling, the study was performed again to obtain 4.8- and 3 mL samples after 30 min of digestion. As an undigested OP was present in the LBF-LC digests, samples (4.8 mL) were transferred to polyamide tubes, and ultracentrifuged (350,000 × g, 37 °C, CP 70MX, P65ST rotor, Koki Holdings Co., Ltd., Tokyo, Japan) for 30 min to isolate the PP, AP, and OP. The OP was carefully taken using a 1-ml syringe with a 23 G needle and added into a volumetric flask (10 mL) followed by a chloroform–methanol mixture (2:1 v/v) up to the volume. The OP samples were further diluted 5–10-fold with methanol. The AP remaining in the tube was obtained and diluted 20-fold with 50:50 v/v methanol-water. Finally, the PP remaining in the tube was completely dissolved in methanol (10 mL). Saquinavir in 50:50 v/v methanol-water (1 μg/mL, IS solution) was mixed with the final samples (1:1 v/v) to determine drug amount using LC-MS/MS.

Separately collected 3 mL of the LBF-SMC and LBF-LC digests at 15 and 30 min were used as donor solutions for the *in vitro* permeation experiment (see Section 2.8.1).

2.6. Experiments to evaluate fate of RTV after in vitro dispersion

LBF-SMC and LBF-LC containing different masses of RTV were dispersed in 15 mL of lipolysis buffer to the same concentration of drug (0.075 mg/mL) and formulation (0.5, 1.0, and 1.5% w/w) as in the *in vitro* digestion study (Section 2.5). In this case, lipolysis was not performed by adding pancreatin. After 15 min dispersion at 37 °C, 4.8 and 3 mL dispersions were sampled from the reaction vessel, and 4.8 mL of the samples were ultracentrifuged (350,000 × g, 37 °C) for 30 min to separate into PP, AP, and OP. Each phase was processed in the same way as the digestion samples for LBF-LC (Section 2.5.). The mass of the RTV in each phase was measured by LC-MS/MS. The samples (3 mL) were utilized as a donor solution for the *in vitro* permeation experiment (see Section 2.8.1).

2.7. Estimation of supersaturation ratio (SR) and maximum supersaturation ratio (SR^M)

Drug-free LBFs were dispersed or digested, and drug-free AP was obtained under the same experimental conditions as described in Sections 2.5 and 2.6. Then, blank AP (0.5 mL) was added to a plastic tube with crystalline RTV (1 mg). After 24 h incubation at 37 °C, the samples were centrifuged at 15,100 × g for 15 min, and the resultant supernatant was diluted 20-fold with 50:50 v/v methanol-water.

In addition, to measure the solubility of RTV in the lipolysis buffer, crystalline RTV was added to the lipolysis buffer at 0.075 mg/mL and equilibrated at 37 °C for 24 h. Then, the suspension was centrifuged at 15,100 × g for 15 min, and the resultant supernatant was diluted 20-fold with 50:50 v/v methanol-water.

All final samples were mixed with a solution of saquinavir in 50:50 v/v methanol-water (1 μg/mL, IS solution) at 1:1 v/v, and the drug amount in the samples was determined using LC-MS/MS.

The solubility data in blank AP were used to estimate the SR and SR^M using Eqs. (1) and (2), as previously described (Tanaka et al., 2021a, 2021b).

$$SR = \text{solubilized concentration of RTV in the AP during dispersion or digestion/RTV solubility in AP} \quad (1)$$

$$SR^M = AP^{MAX} / \text{RTV solubility in the AP}, \quad (2)$$

where AP^{MAX} is the total mass of RTV contained in LBFs/the volume of lipolysis buffer in the digestion vessel. The ratio between AP^{MAX} and RTV solubility in the corresponding AP provides the theoretical maximum SR in the AP.

2.8. In vitro permeation across dialysis membrane

2.8.1. Measurement of RTV concentration on the receiver side of diffusion chamber during in vitro permeation experiments

An *in vitro* permeation experiment was performed using a diffusion chamber (5G-00–00–09–3.4, PermeGear, Inc., Pennsylvania, USA) mounted with a dialysis membrane (molecular weight cut-off of > 1000 Da, Spectrum Laboratories, Inc., California, USA) as an absorptive membrane. NaTC solution (50 mM, pH 6.5) was added (2.7 mL) to the receiver chamber. The permeation study was initiated by adding 2.7 mL of donor solutions containing PP, AP, and OP (if any) collected from the *in vitro* dispersion and digestion experiments described above. In

addition, an equilibrated suspension of RTV in lipolysis buffer (0.075 mg/mL) was investigated as a control. Both sides of the diffusion chamber were agitated at 450 rpm using a magnetic stirrer. During the experiment, 50 μL of the samples were collected from the receiver side at 10, 20, 30, 40, 50, 60, and 70 min. After dilution with 50:50 v/v methanol-water, the samples were mixed with saquinavir in 50:50 v/v methanol-water (200 ng/mL, IS solution) at 1:1 v/v for quantification.

2.8.2. Calculation of the apparent first-order permeation rate constant (k_{app})

The k_{app} of the RTV was estimated using Eq. (3):

k_{app}=the flux of RTV on the receiver side (μg·mL^{-1}·min^{-1}) /total concentration of RTV [PP + AP + OP (if any)] in the donor solution (μg/mL) (3)

Since only free drug is permeable across the dialysis membrane, the k_{app} is an indicator of the free fraction of drugs in donor solutions (Yano et al., 2010; Tanaka et al., 2021b). Therefore, the fluctuation in k_{app} values directly reflects the fluctuation of the free fraction in donor solutions.

2.9. LC-MS/MS analysis of RTV in the samples

LC-MS/MS analysis was performed using a Shimadzu 8040 UPLC triple quadrupole mass spectrometer (UPLC-MS/MS, Shimadzu, Kyoto, Japan). A Zorbax Eclipse XDB-C18 column (2.1 × 50 mm, I.D., 5 mm, Agilent Technologies, CA, USA) was used at 40 °C for chromatographic separation. An isocratic mobile phase comprising 10 mM ammonium formate containing 0.1% formic acid in purified water and methanol at a ratio 33:67 (v/v) was made to flow at 0.3 mL/min. The electrospray ionization was operated in the positive ion mode. The precursor-product ion pairs monitored were *m/z* 721.1 > 139.9 for RTV and 671.3 > 570.2 for saquinavir.

For analysis of samples obtained from *in vitro* dispersion/digestion and solubility study, RTV standards in 50:50 v/v methanol-water (0.1–5 μg/mL) were mixed with IS solution (1 μg/mL saquinavir in 50:50 v/v methanol-water) at 1:1 v/v. It provided 0.05–2.5 μg/mL of standard solutions. Inter-assay variability was accurate to 111.3, 102.9 and 101.7% and precise to 9.4, 6.3, and 4.7% at low (0.05 μg/mL), medium (1 μg/mL), and high (2.5 μg/mL) concentrations of quality control standards ($n = 6$), respectively.

For assay of samples for *in vitro* permeation study, RTV standards in 50:50 v/v methanol-water (5–100 ng/mL) were mixed with IS solution (200 ng/mL saquinavir in 50:50 v/v methanol-water) at 1:1 v/v. It provided 2.5–50 ng/mL of standard solutions. Inter-assay variability was accurate to 109.5, 103.0 and 98.9% and precise to 9.1, 1.9, and 3.0% at low (2.5 ng/mL), medium (30 ng/mL), and high (50 ng/mL) concentrations of quality control standards ($n = 6$), respectively.

For assay of plasma samples, two sets of RTV standards in methanol were prepared to cover the different ranges of plasma concentrations after oral administration of different formulations (2–50 ng/mL for 1.0% LBF-SMC and 2–25 ng/mL for the other formulations). Each of RTV standards (50 μL) and 200 ng/mL saquinavir in methanol (50 μL) were added to blank plasma (50 μL). Then, precipitated protein was removed by centrifugation, and the obtained supernatants were used as standards. It provided 0.67–16.7 ng/mL and 0.67–8.33 ng/mL of RTV concentration ranges. The assays were accurate and precise to within ±10% (± 15% at the LLOQ) for both 0.67–16.7 ng/mL ($n = 4$) and 0.67–8.33 ng/mL ($n = 6$) standards.

2.10. Statistical analysis

All statistical analyses were conducted using EZR (version 1.54, Saitama Medical Center, Jichi Medical University, Saitama, Japan) (Kanda, 2013), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). Statistically significant differences were determined using one-way ANOVA followed by Tukey's post-hoc test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Pharmacokinetic studies

The plasma concentration-time courses following oral administration of RTV (0.15 mg/rat) as a suspension and pre-dispersed formulations containing different concentrations of LBF-SMC and LBF-LC are shown in Fig. 1, and the pharmacokinetic parameters are presented in Table 1.

For suspension administration, the maximum concentration (C_{max}) and the area under the plasma concentration-time curve from 0 to 4 h ($AUC_{0–4\ h}$) were 8.2 ± 2.7 ng/mL and 7.9 ± 1.7 ng*h/mL, respectively. When RTV was administered as 1.0% LBF-SMC, the C_{max} and $AUC_{0–4\ h}$ values drastically increased to 36.9 ± 7.4 ng/mL ($p < 0.01$) and 22.4 ± 5.3 ng*h/mL ($p < 0.01$), respectively, relative to those in the group for suspension (8.2 ± 2.7 ng/mL for C_{max} and 7.9 ± 1.7 ng*h/mL for $AUC_{0–4\ h}$). However, improvement effect in oral absorption significantly decreased as administered concentration of LBF-SMC increased to 2.0 and 3.0% compared to that with 1.0% LBF-SMC (although $AUC_{0–4\ h}$ in 2.0% LBF-SMC was not significantly different).

For 1.0% LBF-LC, the absorption significantly increased compared with the suspension formulation ($p < 0.01$ for $AUC_{0–4\ h}$ and $p < 0.05$ for C_{max}), whereas no improvement was observed for 2.0 and 3.0% LBF-LC. The oral absorption of RTV tended to be higher for LBF-SMC than for LBF-LC when compared at the same dosing concentration of formulations, although only the C_{max} values for 1.0% of LBF-SMC were significantly different from that for the corresponding concentration of LBF-LC ($p < 0.01$).

3.2. Fate of RTV after in vitro dispersion

The results of *in vitro* dispersion for LBFs are presented in Fig. 2, and a summary of the concentration and solubility of RTV in AP, SR, and SR^M are presented in Table 2. For 0.5% LBF-SMC and LBF-LC, RTV was present at 65.3 ± 7.4 and 37.7 ± 4.7%, respectively, in OP (Fig. 2). Drug distributions in both OPs increased as the formulation concentration increased to 1.5%. Consistent with this finding, RTV concentration in AP and SR significantly decreased for both LBFs with increasing formulation concentration due to partition of drug into OP (Table 2) ($p < 0.01$; 1.0% and 1.5% LBF-SMC *vs*. 0.5% LBF-LC, and 1.0% and 1.5% LBF-LC *vs*. 0.5% LBF-LC). The extent of drug distribution in OP was lower for LBF-LC than for LBF-SMC when compared at the same formulation concentration. This is attributed to lower affinity of RTV to LBF-LC because the solubility of RTV in LBF-LC (17.0 ± 0.27 mg/g) was lower than that in LBF-SMC (77.7 ± 3.1 mg/g). Although the SR^M value, an indicator of drug precipitation from LBFs during digestion (Williams et al., 2012), exceeded 1 for all formulations (Table 2), no drug precipitation was observed regardless of LBF concentrations due to the presence of a large amount of OP that reduced drug concentrations in AP and SR.

3.3. Fate of RTV after in vitro digestion

The results of *in vitro* digestion are shown in Fig. 3, and the concentration and solubility of RTV in AP, SR, and SR^M are summarized in Table 3. In addition, the results of the statistical analysis are summarized in Tables S1–3. Precipitation was slight regardless of formulations and concentrations in the model rat intestinal fluid during LBF digestion (Fig. 3). For 0.5% LBF-SMC, OP completely disappeared within 15 min after onset of digestion, and 12.3 ± 1.6% and 9.1 ± 0.80% of RTV existed in the PP after 15 and 30 min, respectively (Fig. 3). Since the SR^M values at 15 and 30 min were 1.56 and 1.82 (Table 3), respectively, rapid lipid digestion and the higher SR^M drove precipitation, and the SR decreased to approximately 1 after precipitation. However, majority of

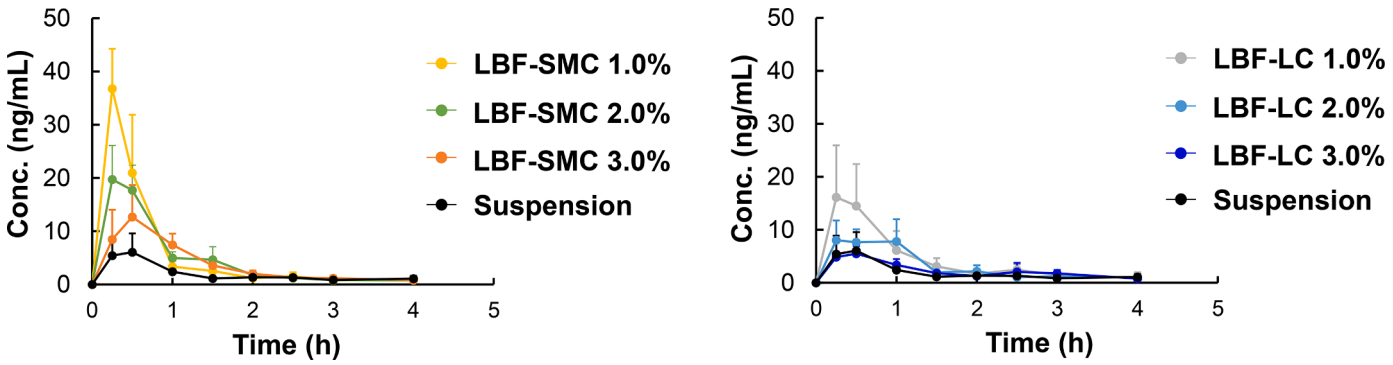

Fig. 1. Plasma concentration-time profiles after oral administration of RTV (0.15 mg/rat) as suspension and pre-dispersed formulations containing different concentration of LBF-SMC and LBF-LC ($n = 4$). Data are expressed as mean ± S.D. of four experiments.

Table 1
Pharmacokinetic parameters of RTV after oral administration as various formulations.

	Suspension	LBF-SMC 1.0%	LBF-SMC 2.0%	LBF-SMC 3.0%	LBF-LC 1.0%	LBF-LC 2.0%	LBF-LC 3.0%
$AUC_{0-4\ h}$ (ng*h/mL)	7.9 ± 1.7	22.4 ± 5.3[a]	18.9 ± 3.0[a]	15.3 ± 2.6[a,b]	17.7 ± 3.1[a]	12.7 ± 2.9[c]	9.2 ± 1.2[c,d,e]
C_{max} (ng/mL)	8.2 ± 2.7	36.9 ± 7.4[a]	21.4 ± 4.0[c,f]	13.2 ± 5.3[c]	20.7 ± 9.0[c,f]	10.1 ± 3.4[c]	5.8 ± 0.9[c,d,e]

Data are expressed as mean of ± S.D. of four experiments.
[a] Statistically different when compared with the data of suspension ($p < 0.01$).
[b] Statistically different when compared with the data of LBF-SMC 1.0% ($p < 0.05$).
[c] Statistically different when compared with the data of LBF-SMC 1.0% ($p < 0.01$).
[d] Statistically different when compared with the data of LBF-SMC 2.0% ($p < 0.01$).
[e] Statistically different when compared with the data of LBF-LC 1.0% ($p < 0.05$).
[f] Statistically different when compared with the data of suspension ($p < 0.05$).

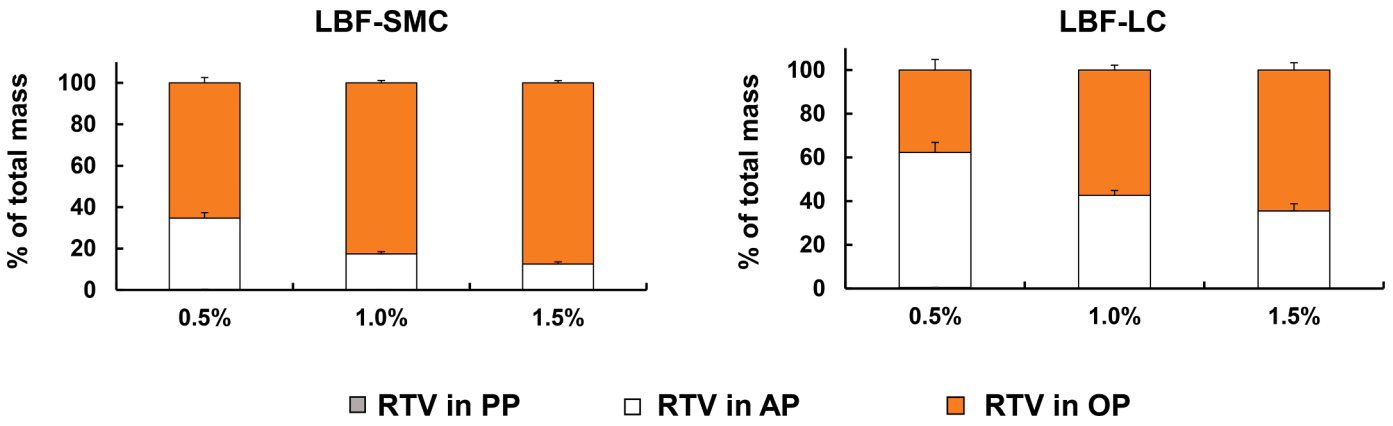

Fig. 2. Phase distribution of RTV after 15 min dispersion of LBFs at various concentrations. Data are expressed as mean ± S.D. of three experiments.

the RTV was still in a solubilized state in AP during digestion. Similar phase distributions were observed for 1.0 and 1.5% LBF-SMC.

For 0.5% LBF-LC, LBF was not completely digested, and 21.8 ± 6.7 and 20.8 ± 3.0% of RTV was distributed to undigested OP at 15 and 30 min, respectively (Fig. 3). The distribution into OP increased with an increase in the concentration of LBF-LC. As such, the RTV concentration in the AP decreased with increasing drug distribution to OP (Table 3).

The drug solubility values in AP (Table 3) were unchanged for 0.5% LBF-SMC (48.0 ± 1.5 μg/mL at 15 min and 41.1 ± 0.8 μg/mL at 30 min) and were significantly higher for LBF-LC [52.8 ± 6.8 μg/mL at 15 min ($p < 0.05$) and 61.8 ± 1.8 μg/mL at 30 min ($p < 0.01$)] than that in lipolysis buffer alone (36.5 ± 0.55 μg/mL). The solubility values in AP increased as the initial concentration of LBF-SMC and LBF-LC increased because of enhanced solubilization capacity caused by enhanced liberation of digestion products to AP. In addition, the solubility values in AP of LBF-LC were higher than those in AP of LBF-SMC, indicating that the solubilization capacity of LC digestion products was higher than that of SMC digestion products.

3.4. *In vitro* permeation across the dialysis membrane

In vitro permeation of RTV across the dialysis membrane was evaluated using suspensions and various dispersion and digestion fluids as donor solutions. The concentration profiles of the RTV as a function of time in the receiver compartment of the diffusion chamber are shown in Fig. 4. The drug concentration on the receiver side increased linearly for all the donor solutions. The k_{app} was then calculated by dividing the flux of RTV on the receiver side ($\mu g \cdot mL^{-1} \cdot min^{-1}$) by the total concentration of RTV in the donor solutions (μg/mL) (Fig. 5). This parameter reflects the free concentration of RTV in donor solutions. In addition, the results of statistical analysis for k_{app} are summarized in Table S4. For dispersion, the k_{app} values for 0.5% LBF-SMC and LBF-LC were 3.22 ± 0.65 and 5.44 ± 0.09 × 10^{-5} min^{-1}, respectively. Each value decreased with increasing concentration of each LBF on the donor side from 0.5 to 1.5%.

Table 2
Concentration and solubility of RTV in AP, SR and SR^M for *in vitro* dispersion experiment.

		RTV concentration in AP (μg/mL)	RTV solubility in AP (μg/mL)	SR	SR^M
LBF-SMC	0.5%	22.8 ± 1.97	38.5 ± 2.21	0.59 ± 0.051	1.95
	1.0%	12.0 ± 0.91	48.6 ± 0.81	0.25 ± 0.019	1.54
	1.5%	8.6 ± 0.49	61.3 ± 1.21	0.14 ± 0.008	1.22
LBF-LC	0.5%	41.4 ± 1.78	41.0 ± 1.01	1.001 ± 0.044	1.83
	1.0%	32.1 ± 0.69	45.4 ± 0.95	0.71 ± 0.015	1.65
	1.5%	26.5 ± 1.29	55.3 ± 1.34	0.48 ± 0.023	1.36

Data are expressed as mean ± S.D. of three experiments.
The following combinations of RTV concentration in AP are not statistically different.
LBF-SMC (0.5%) *vs.* LBF-LC (1.5%), LBF-SMC (1.0%) *vs.* LBF-SMC (1.5%).
The other combinations of RTV concentration in AP were statistically different ($p < 0.01$).
The following combinations of RTV solubility in AP are not statistically different:.
LBF-SMC (0.5%) *vs.* LBF-LC (0.5%), LBF-SMC (1.0%) *vs.* LBF-LC (1.0%).
The following combinations of RTV solubility in AP are statistically different ($p < 0.05$).
LBF-LC (0.5%) *vs.* LBF-LC (1.0%).
The other combinations of RTV solubility in AP were statistically different ($p < 0.01$).
The following combination of SR is statistically different ($p < 0.05$):.
LBF-SMC (1.0%) *vs.* LBF-SMC (1.5%).
The other combinations of SR are statistically different ($p < 0.01$).

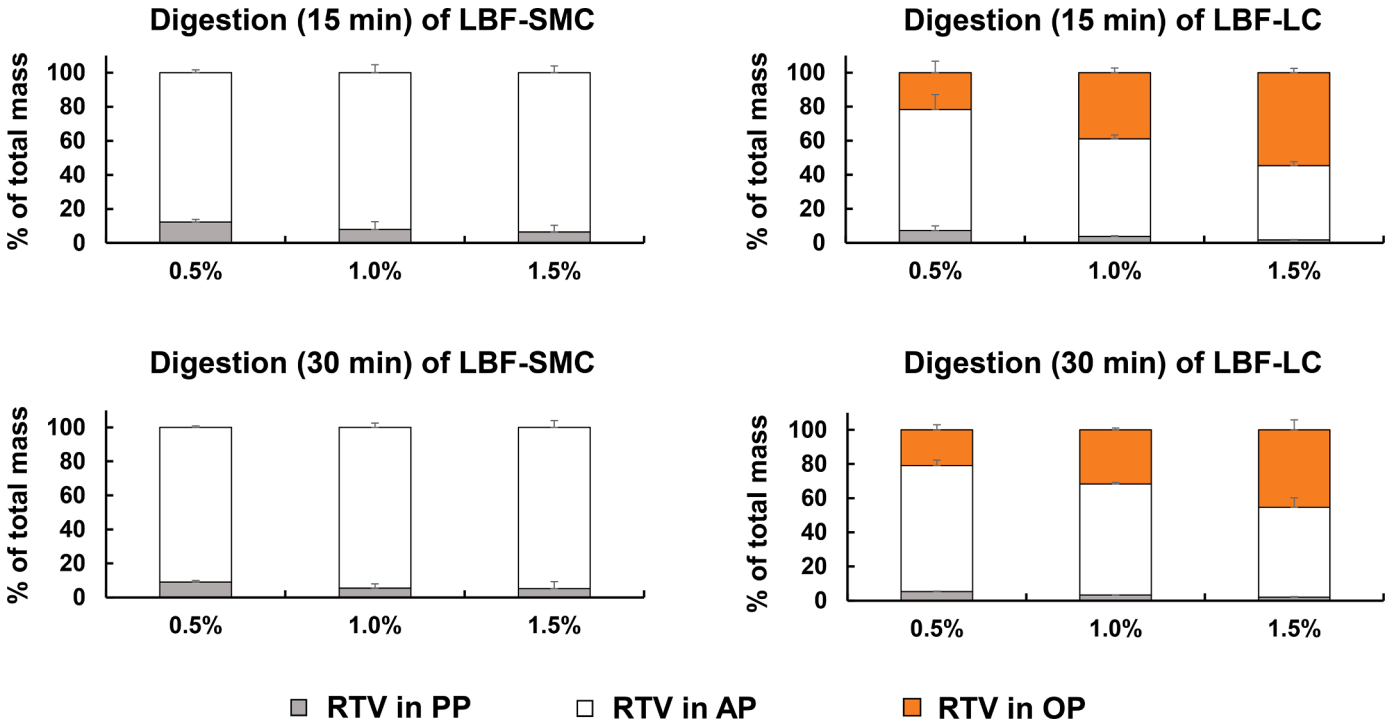

Fig. 3. Phase distribution of RTV after 15 and 30 min digestion of LBFs at various concentrations. Data are expressed as mean ± S.D. of three experiments.

This may be because the increased distribution of RTV into OP (Fig. 2), depending on each LBF concentration, led to a decrease in the free concentration of RTV in AP. In addition, the k_{app} values for the dispersion of LBF-SMC were lower than those for the dispersion of LBF-LC when compared at the same concentration on the donor side because of the higher affinity of RTV for SMC lipids.

Under digestion conditions, the k_{app} values for LBF-SMC significantly increased at the 15 and 30 min time points compared to those for the corresponding concentration of LBF-SMC under dispersion conditions ($p < 0.01$ for the all combinations). This was attributed to the increased free drug concentration in AP caused by the disappearance of OP by digestion of LBF-SMC (Fig. 3). In addition, the k_{app} for the 15 and 30 min digest of LBF-SMC decreased with an increase in the initial formulation concentration ($p < 0.01$; 1.5% LBF-SMC *vs.* 0.5% LBF-SMC for 15 min of digestion and 1.0 and 1.5% LBF-SMC *vs.* 0.5% LBF-SMC for 30 min of digestion). This is because the amount of liberated digestion products increased, leading to enhanced incorporation of the drug into the digestion product micelles.

On the other hand, the k_{app} values for LBF-LC were unchanged for both 15 and 30 min digestion compared to those for the corresponding LBF-LC concentrations under dispersion conditions, except for the combination of 0.5% dispersion and 0.5% digestion at 30 min ($p < 0.01$). In addition, k_{app} values for LBF-LC digestion were significantly lower than those for LBF-SMC digestion when compared at the same formulation concentrations and digestion time points ($p < 0.01$ for all combinations). This may be due to the substantial reduction in free drug concentration caused by the presence of undigested OP (Fig. 3) and the higher solubilization capacity of mixed micelles of liberated LC digestion products and bile components (see solubility data in Table 3). These data suggested that the composition and quantity of LBF greatly

Table 3
Concentration and solubility of RTV in AP, SR and SR^M for *in vitro* digestion experiment.

			RTV concentration in AP (μg/mL)	RTV solubility in AP (μg/mL)	SR	SR^M
LBF-SMC	0.5%	15 min	54.3 ± 1.5	48.0 ± 1.5	1.13 ± 0.031	1.56
		30 min	57.4 ± 3.3	41.1 ± 0.8	1.39 ± 0.079	1.82
	1.0%	15 min	61.5 ± 13.0	71.4 ± 5.9	0.86 ± 0.18	1.05
		30 min	76.6 ± 14.6	54.2 ± 1.9	1.41 ± 0.27	1.38
	1.5%	15 min	65.9 ± 7.2	103.0 ± 10.9	0.64 ± 0.070	0.73
		30 min	68.9 ± 9.6	76.6 ± 7.8	0.90 ± 0.13	0.98
LBF-LC	0.5%	15 min	42.4 ± 8.7	52.8 ± 6.8	0.80 ± 0.16	1.42
		30 min	53.3 ± 5.3	61.8 ± 1.8	0.86 ± 0.085	1.21
	1.0%	15 min	39.4 ± 2.4	99.7 ± 5.3	0.40 ± 0.024	0.75
		30 min	46.6 ± 0.65	96.5 ± 5.6	0.48 ± 0.0067	0.78
	1.5%	15 min	30.3 ± 1.3	140.8 ± 1.2	0.22 ± 0.010	0.53
		30 min	38.3 ± 5.7	157.2 ± 1.8	0.24±0.036	0.48

Data are expressed as mean ty .D. of three experiments.
The results of statistical analysis are summarized in Table S1–3.

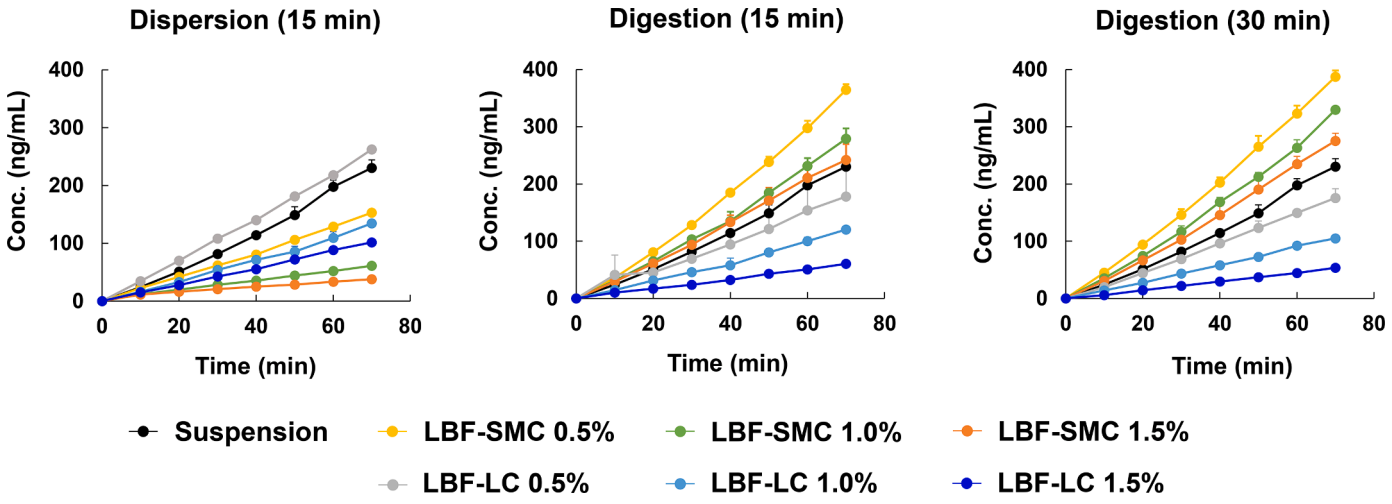

Fig. 4. Concentration-time courses of RTV in the receiver compartment of diffusion chamber during permeation experiments. Data are expressed as mean of ± S.D. of three experiments.

influenced the concentration of free drug in the intestinal fluid.

For suspension, although 45.3 ± 6.7% of RTV was present as solid form, the k_{app} ($5.98 \pm 6.0 \times 10^{-5}$ min^{-1}) was relatively high compared to the k_{app} values for dispersions and digests of LBFs where the drug was almost solubilized. This may be due to the higher free drug concentration in the suspension in which the lipid and digestion products were not contained.

4. Discussion

The oral absorption of drugs from LBFs is determined by several factors (Tanaka et al., 2021a; Yeap et al., 2013a), and thus, an accurate prediction of the *in vivo* performance is very difficult (Feeney et al., 2016). This makes it more difficult to design optimal LBFs. It is considered that the use of high amounts of excipients can enhance the solubilization of lipophilic drugs in the GI tract. However, this may also lead to a decrease in thermodynamic activity (Tanaka et al., 2021b), probably leading to a reduction in intestinal permeability. Therefore, the use of an appropriate quantity of excipients is considered an important factor for drug absorption from LBFs. In the present study, RTV was loaded into various masses of LBFs comprising SMC or LC lipids, and the effect of the administered quantity of two types of LBFs on drug absorption was investigated.

After oral administration of RTV (0.15 mg/rat) as pre-dispersed LBF-SMC and LBF-LC at low concentrations, the absorption was significantly improved compared to that of the suspension. However, the improvement effect decreased as the concentration of administered LBFs increased from 1.0% to 3.0% w/w for both LBFs, and the absorption for 2.0 and 3.0% LBF-LC was not improved compared to the suspension (Fig. 1 and Table 1).

To clarify the cause for the different pharmacokinetic profiles, *in vitro* dispersion and digestion studies were performed. As drastic precipitation was not observed during dispersion and digestion regardless of the formulation and concentration (Figs. 2 and 3), it is considered that the main cause of the different absorption profiles *in vivo* may be the fluctuation in the intestinal permeation of RTV rather than drug dissolution in the GI tract. In our previous study, the incorporation of drugs into the aqueous colloidal species generated from digestion products and undigested lipids decreased the free drug concentration in the GI tract, leading to a reduction in apparent drug permeability (Tanaka et al., 2021a, 2021b). As such, the impact of lipid digestion on free drug concentration was then evaluated using an *in vitro* permeation study with a dialysis membrane, and k_{app} was calculated as an indicator of free drug concentration in the GI fluid. The rank order of k_{app} for suspension and digest (both time points of 15 and 30 min of digestion) was 0.5% LBF-SMC > 1.0% LBF-SMC > suspension > 1.5% LBF-SMC > 0.5% LBF-LC > 1.0% LBF-LC > 1.5% LBF-LC. This was almost consistent with the rank of the $AUC_{0-4\ h}$ values *in vivo* (Table 1), although the rank of k_{app} for suspension was higher than that of *in vivo* AUC. These data clearly indicate that the variation in oral absorption profiles from different masses of LBFs was caused by a change in free concentration during lipid digestion in the GI tract.

The k_{app} values for the LBF-LC digest were significantly lower than those for the LBF-SMC digest when compared at the same LBF

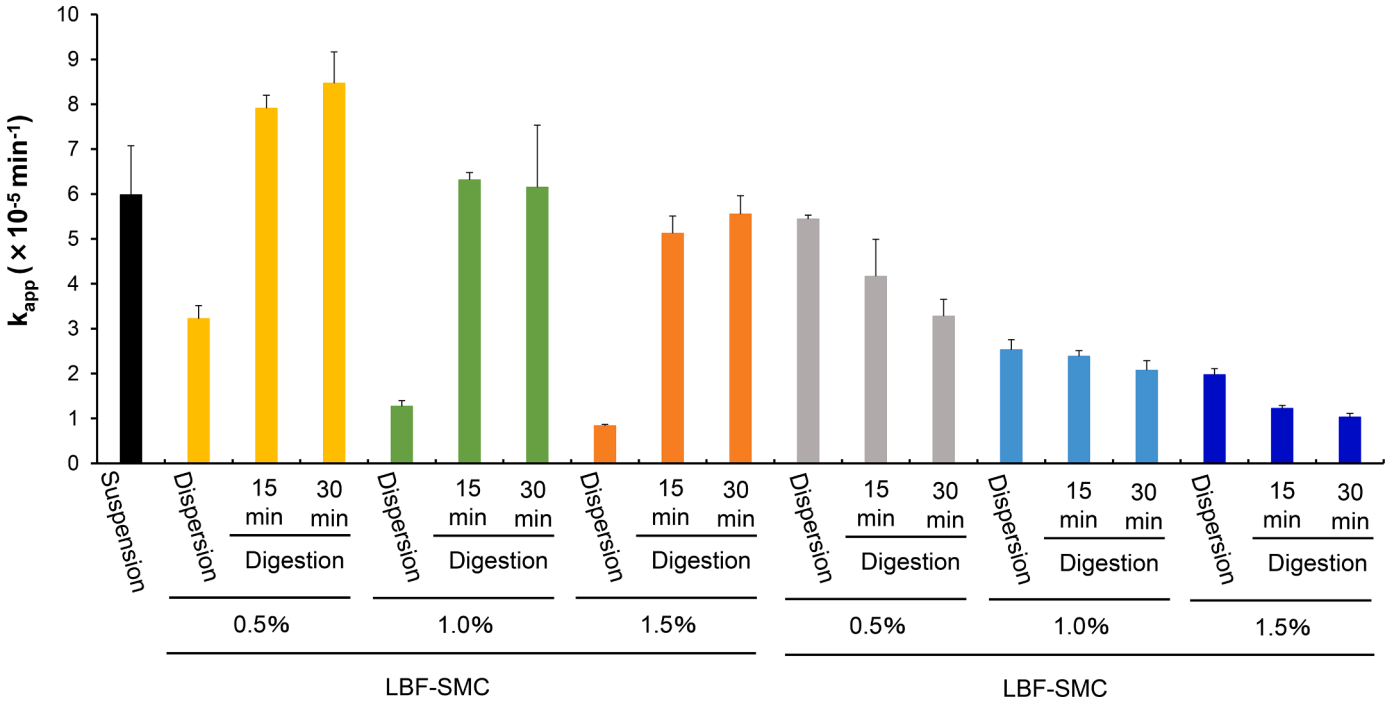

Fig. 5. Apparent permeation rate constants (k_{app}) calculated from permeation-time profiles of RTV. Data are expressed as mean ± S.D. of three experiments. The results of statistical analysis are summarized in Table S4.

concentrations on donor side and digestion time points ($p > 0.01$ for all combinations) (Fig. 6). This may be due to extensive incorporation of RTV into undigested LBF-LC [typically, the digestion rate of LC-lipid is much slower than that of SC- and MC-lipids (Han et al., 2009; Devraj et al., 2013)] and the aqueous colloidal species composed of LC digestion products with higher solubilization capacity than that of SMC digestion products. The extent of k_{app} is consistent with the extent of SR (Table 3), another indicator of free drug concentration in AP (McEvoy et al., 2017). Thus, the lower free drug concentration during digestion of LBF-LC may lead to lower absorption *in vivo* than that from LBF-SMC when compared to the corresponding administered concentrations (Table 1), although there was a significant difference in C_{max} between 1.0% of LBF-SMC and LBF-LC.

Moreover, the reduction in k_{app} led by an increase in LBF-LC concentration (k_{app} reduced 57.3 and 29.4% for 1.0 and 1.5% LBF-LC, respectively, relative to that for 0.5% LBF-LC for 15 min digestion, and 63.2 and 31.4% for 1.0 and 1.5% LBF-LC, respectively, relative to that for 0.5% LBF-LC for 30 min digestion) was more extensive than that for LBF-SMC (k_{app} reduced 79.8 and 64.8% for 1.0 and 1.5% LBF-SMC, respectively, relative to that for 0.5% LBF-SMC for 15 min digestion, and 72.6 and 65.6% for 1.0 and 1.5% LBF-SMC, respectively, relative to that for 0.5% LBF-SMC for 30 min digestion). The extent of reduction in k_{app} was consistent with that in SR (Table 3). The lower impact of LBF-SMC quantity on free drug concentration may reduce the effect of the formulation dose on the $AUC_{0-4\ h}$ values for LBF-SMC compared to those for LBF-LC ($AUC_{0-4\ h}$ reduced 84.3% and 68.3% for 2.0% and 3.0% LBF-SMC and 71.8% and 52.0% for 2.0% and 3.0% LBF-LC, respectively, compared to 1.0% of the corresponding LBF). However, dose-dependent

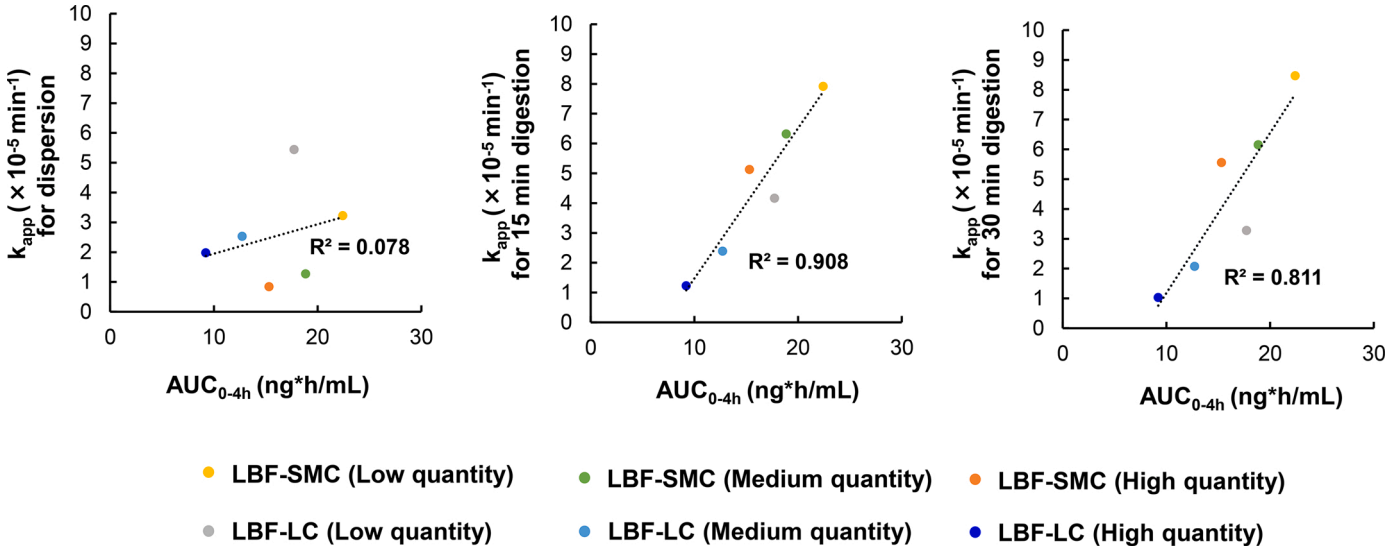

Fig. 6. *In vitro-in vivo* correlations of oral $AUC_{0-4\ h}$ and k_{app} after formulation dispersion and digestion.

reduction in $AUC_{0-4\ h}$ (71.8% and 52.0% reduction for 2.0% and 3.0% LBF-LC, respectively, compared to 1.0% LBF-LC) was weaker than that in k_{app}, especially for LBF-LC (e.g. 57.3 and 29.4% reduction for 1.0 and 1.5% LBF-LC, respectively, relative to that for 0.5% LBF-LC for 15 min of digestion). In the *in vitro* permeation study using the dialysis membrane employed here, the colloidal species are retained on the donor side, leading to entrapment/sequestration of drugs (although monomer components are permeable dialysis membranes). However, it is apparent that lipids and digestion products are absorbed along the length of the intestine, often via active transport (Yeap et al., 2013c). As such, the static *in vitro* model setup is likely to overestimate the entrapment effect, especially in LC digestion products with higher solubilization capacity.

The k_{app} values were then correlated with *in vivo* $AUC_{0-4\ h}$ values to determine the possibility of predicting oral absorption (Fig. 6). As mentioned above, the k_{app} for the suspension was an outlier for the correlation. Since suspension in model intestinal fluid was saturated before application to the donor side of the diffusion chamber for the *in vitro* permeation study, the overestimated k_{app} value for suspension may be caused by the absence of an initial dissolution process that is present *in vivo*. Under these circumstances, the suspension data were removed from the correlation plots. When the k_{app} from dispersions was correlated with *in vivo* AUC_{0-4}, a poor correlation was evident [coefficient of determination (R^2) = 0.078]. This may be because the lack of a digestion process for *in vitro* dispersion resulted in a different solubilization behavior from that in the actual GI tract. By contrast, although k_{app} seemed to be underestimated due to the overestimated entrapment effect *in vitro*, a good correlation was evident between the k_{app} for digestion at 15 min (R^2 = 0.908) and 30 min (R^2 = 0.811) and *in vivo* exposure. These data clearly indicate that the effect of lipid digestion on intraluminal free drug concentration is a key factor for the evaluation/prediction of drug absorption from LBFs.

When a drug is absorbed via active transporters, it is possible that this *in vitro* permeation study using a dialysis membrane with predigested LBFs may fail to predict oral absorption. RTV is known as a P-glycoprotein (P-gp) (Holmstock et al., 2010; Martinec et al., 2019). However, since the T_{max} was approximately 15–30 min regardless of the formulation administered (Fig. 1), RTV was absorbed mainly from the upper segment of the small intestine where P-gp expression is low (Takano et al., 2006) at the dose employed in this study. Thus, the LBFs used in this study did not significantly affect the transport of RTV via P-gp, resulting in good *in vitro–in vivo* correlations.

As previously described in introduction, it has been reported that oral absorption of cinnarizine increased with increasing the mass of co-administered MC and LC lipids from 125 to 250 mg (Lee et al., 2013). Since the dose of cinnarizine employed in their study was relatively high (14.81 mg/kg for MC lipids and 11.51 mg/kg for LC lipids), the absorption might be dominantly limited by solubilization; thus, an increased mass of lipids enhanced the solubilization capacity of the drug in the GI tract, leading to increased absorption. In the present study, a relatively low dose of RTV (0.15 mg/body) was employed, and most of RTV was solubilized during digestion even at the lowest LBF concentrations (Fig. 3). Thus, at the employed dose, the absorption of RTV may be dominated by the apparent permeability rather than by solubilization. As a result, increasing the dose of LBF did not improve the oral absorption of RTV. In the study by Lee et al., the oral absorption of cinnarizine was unchanged or reduced at the highest dose (500 mg) of MC and LC lipids, respectively. This phenomenon was consistent with our findings. Since the current study experimentally demonstrated that an increase in LBF dose reduced free drug concentration and ultimately oral drug absorption, the reduced bioavailability of cinnarizine at high lipid doses may be partly attributed to a decrease in free drug concentration in the GI tract.

Jacobsen et al. (2021) has investigated the effect of different quantities of phospholipids on the absorption of celecoxib, a BCS class II drug. Although phospholipids (monoacyl and diacyl phospholipids) were used as excipients in the formulations, similar to our study, oral absorption of the drug in rats decreased with increase in formulation content of both phospholipids. However, there was no formulation difference in the oral absorption. They also concluded that this phenomenon may be caused by drug incorporation into colloidal species at higher phospholipid contents, which could lead to a reduction in intestinal permeation. In addition, they performed *in vitro* permeation experiment using a 96-well Permeapad®, a biomimetic membrane, and the obtained *in vitro* data were correlated with *in vivo* oral AUC in rats. However, as with our result, *in vitro* study overpredicted the *in vivo* performance of the control suspension. In their *in vitro* study, dispersions of phospholipid formulations in model human intestinal fluid was directly applied to a donor side of the *in vitro* permeation set-up without digestion step. Therefore, they discussed that digestion and absorption of phospholipids may increase free concentration in the GI tract, leading to the result that the phospholipid formulation performed worse in the *in vitro* permeation compared to the *in vivo* pharmacokinetic study (i.e. performance of control suspension relatively increased in the *in vitro* permeation study.). In the present study, since fatty acids are actively absorbed *in vivo* but not in the *in vitro* permeation study, free drug concentration in digests may be underestimated in the *in vitro* system. This could be another reason why the suspension formulation performed better in the *in vitro* system compared to the *in vivo* absorption study.

5. Conclusion

Information on the impact of the quantity and composition of LBF on oral absorption is very limited, which makes it more difficult to predict oral bioavailability from LBFs. This study revealed that the improved oral absorption *in vivo* (relative to suspension) decreased as the administered concentration of LBF increased from 1.0% to 3.0% w/w for both LBF-SMC and LBF-LC, owing to the solubility-permeability trade-off. In addition, the reduction in thermodynamic activity was more pronounced for LBF-LC than for LBF-SMC because of the greater solubilization capacity of LC digestion products and incorporation of the drug into undigested lipids. This paradoxical effect on apparent solubility and permeability may be a key factor in determining the overall fraction absorbed. Therefore, the balance between solubility and permeability should be considered when developing the most effective LBFs. In addition, *in vitro* predictive tool mounted with a dialysis membrane successfully predicted the effect of the quantity of LBF with different compositions on oral absorption. In this system, unlike other *in vitro* model using biological membrane, preparation of isolated intestinal tissue and Caco-2 cell monolayer is not required. Thus, our *in vitro* model is time-saving. In addition, it is not necessary to validate the compatibility of these biological membrane with the pancreatic enzymes and LBF excipients. However, there are some limitations described above e.g. overestimation of entrapment effect. Also, this model is not suitable to compare bioperformance from different drugs with different lipophilicity.

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Appendix A

Supplementary material

CRediT authorship contribution statement

Yusuke Tanaka: Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Visualization, Supervision, Funding acquisition. **Hirotaka Doi:** Investigation, Writing –

original draft. **Takeru Katano:** Investigation, Writing – original draft. **Satoshi Kasaoka:** Resources, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejps.2021.106079.

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