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β-Cyclodextrin as a suitable solubilizing agent for in situ absorption study of poorly water-soluble drugs

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Abstract

To evaluate the intestinal permeability of poorly water-soluble compounds, it is of importance to completely dissolve them in a medium and to avoid precipitation during experiments. This study was undertaken to find an agent possessing a high-solubilizing capacity and exhibiting minimal modulating impact on membrane integrity and absorption systems such as passive diffusion and carrier-mediated permeation. Phenytoin dissolution was compared in the presence of seven solubilizing agents at concentrations of 1, 2, or 5% using a centrifugation method. The capacity to dissolve phenytoin was great in β-cyclodextrin (β-CD) and hydroxypropyl β-cyclodextrin, followed by Tween 80. Those of methanol, dimethyl sulfoxide, dimethyl acetoamide, and polyethylene glycol 400 were much lower than expected. One percent β-CD did not alter the absorption of fluorescein isothiocyanate-dextran 4000 or the release of protein and lactate dehydrogenase into in situ loop contents, suggesting that 1% β-CD had no significant impact on the integrity of the intestinal membrane. One percent β-CD also did not alter the absorption of caffeine, ceftibuten, or rhodamine 123 from in situ jejunal loops, indicating no interference with passive diffusion and active transports mediated by a peptide transporter and P-glycoprotein. In conclusion, 1% β-CD is a suitable solubilizing agent for evaluating in situ intestinal absorption of poorly water-soluble compounds.

Keywords: Solubilizing excipient; β-Cyclodextrin; Phenytoin; Intestinal absorption; Poorly water-soluble drug; Solubilization

1. Introduction

Dissolution into the gastrointestinal fluids and intestinal membrane permeation are essential steps in the absorption of orally administered drugs and thus the lack of adequate water and lipid solubility in a drug often become crucial obstacles to the development of oral dosage forms. Many drug candidates currently in the developmental stage exhibit high lipophilicity and extremely limited water solubility (Lipinski et al., 2001). For evaluating the intestinal membrane permeability of a drug, it is important to completely dissolve it in a medium and to avoid its precipitation during experiments. Poor solubility seriously restricts in vitro, in situ, and in vivo absorption studies. Therefore, the usefulness of cosolvents, surfactants, and complexants as solubilizing agents has been widely studied to establish a system for examining the intestinal absorption of poorly water-soluble compounds. However, it is critical issues that they often cause

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alterations in the intestinal epithelial barrier functions to varying extents and, as a result, the absorption characteristics of test compounds could be modified in the presence of some solubilizing agents. Accordingly, the choice of an appropriate agent and the set up of the experimental concentrations are important in such approaches. Generally, the modulating effect of a solubilizing agent on the intestinal membrane appears to be concentration-dependent (Anderberg et al., 1992; Sakai et al., 1997; Quan et al., 1998; Watanabe et al., 2000) and thus their addition should be minimized. However, recommended concentrations of solubilizing agents varied widely among studies.

As reviewed previously (Tsuji and Tamai, 1996), various transporters such as a H^+-gradient-dependent peptide transporter (PEPT1), monocarboxylic acid transporters (MCTs), P-glycoprotein (P-gp) and multidrug resistance-associated proteins (MRPs) are closely and unexpectedly involved in the absorption and exsorption of structurally and pharmacologically unrelated drugs in the intestine. Therefore, in the case of poorly water-soluble compounds in the developmental stage, possible interactions with some transporters present in the intestinal wall are taken into consideration. In order to evaluate an interaction between test compounds and a membrane transporter precisely, coadministered solubilizing agent should exert no or only a minimal modulating impact on the function of transporters. However, it has been reported that various types of solubilizing excipients are capable of interfering with membrane transporters such as P-gp (Woodcock et al., 1990; Lo et al., 1998; Johnson et al., 2002). We have recently found that several pharmaceutical additives such as D-mannitol, microcrystalline cellulose, and carboxymethylcellulose calcium are capable of increasing methylprednisolone absorption by modulating P-gp (unpublished data). It is also likely that some solubilizing agents affect the intestinal metabolism mediated by cytochrome P450 (CYP) 3A (Johnson et al., 2002).

The effect of solubilizing agents on the integrity of intestinal membrane was widely evaluated in vitro experimental models such as Caco-2 cell monolayers (Sakai et al., 1997; Totterman et al., 1997; Takahashi et al., 2002) and excised rat intestinal segments (Sugiyama et al., 1997; Johnson et al., 2002). In contrast, such information is limited for in situ loop experiments, which are useful for examining transporter-mediated absorption and exsorption (Saitoh et al., 1998; Watanabe et al., 2003). The objective of this study was to look for a suitable solubilizing agent for the in situ absorption study of poorly water-soluble compounds.

2. Materials and methods

2.1. Materials

β-Cyclodextrin (β-CD) and hydroxypropyl β-cyclodextrin (HP-β-CD) were purchased from Seikagaku Corporation (Osaka, Japan) and TOCRIS (Ballwin, MO, USA), respectively. Polyoxyethylene (20) sorbitan monooleate (Tween 80), dimethyl sulfoxide (DMSO), *N*, *N*-dimethyl acetoamide (DMAA), 2,6-di-*O*-methyl β-cyclodextrin (DM-β-CD), phenytoin and verapamil hydrochloride were obtained from Wako Pure Chem. Ind. (Osaka, Japan). Polyethylene glycol 400 (PEG400) was obtained from Maruishi Pharmaceutical Co., Ltd. (Osaka, Japan). Rhodamine 123 and caffeine were purchased from ACROS ORGANICS (Morris Plains, NJ, USA) and Hoeiyakkou (Osaka, Japan), respectively. Fluorescein isothiocyanate-dextran 4000 (FD-4) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Ceftibuten was kindly supplied from Shionogi & Co., Ltd. (Osaka, Japan). All other chemicals and solvents were of analytical reagent grade. The average degree of substitution of hydroxypropyl group of HP-β-CD was 5.3.

2.2. Dissolution test of phenytoin in the media containing various solubilizing excipients

Phenytoin was used as a model compound with poor water solubility. The medium used here was Dulbecco's phosphate-buffered saline (D's PBS) (pH7.4). Solubilizing agents tested were methanol, PEG400, Tween 80, DMSO, DMAA, β-CD, and HP-β-CD. Phenytoin was dispersed into D's PBS containing these solubilizing excipients at concentrations of 1, 2, and 5% (v/v or w/v), except HP-β-CD. The final concentrations of phenytoin were targeted to be 1, 2, and 5 mM. Phenytoin dispersion was sonicated for 30 min and let stand for 30 min at room temperature, followed by centrifugation at 27,000 ×

g for 30 min at 25 °C. The supernatant fluid was taken to determine the concentration of phenytoin dissolved in a test medium. Since 100% methanol dissolved 1, 2, and 5 mM phenytoin completely, these methanol solutions were used as controls.

2.3. Absorption experiment using in situ rat intestinal loops

Absorption experiments were performed using an in situ loop technique. Tyrode's solution (137 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 12 mM $NaHCO_3$, and 0.4 mM NaH_2PO_4) including 6 mM D-glucose was used as an experimental medium. The pH of Tyrode's solution was adjusted to 6.5. After 15–18 h fasting, male Wistar rats weighing 350–450 g were anesthetized by injecting sodium pentobarbital (4 mg/100 g body weight) into the abdominal cavity. The procedure to make three loops in the duodenum, jejunum, and ileum was the same as that described previously (Saitoh et al., 1998). When two loops (each 10 cm) were prepared in the jejunal regions, the proximal ligature of the first loop was placed approximately 20 cm from the pylorus and that of the second loop approximately 35 cm from the pylorus. After the contents of the loop was gently flushed out with 10 ml of saline, which was warmed to approximately 37 °C in advance, 1 ml of drug solution with or without a solubilizing agent was injected into the loops with a syringe. The bile duct was tied in all experiments to prevent the influx of bile into the lumen. Then entire intestine was restored to the abdominal cavity and body temperature was maintained during anesthesia by heating with a lamp. At 10 or 30 min after drug administration, the loops were rapidly isolated from the body and the blood adhering to the surface of loop was washed away with ice-cold saline, and then the content of the each loop was emptied into a 10-ml volumetric flask. The inner side of the intestine was rinsed with saline to give a volume of 10 ml. For determining drug concentration, 2 ml were taken and mixed with an equal volume of methanol. The mixture was centrifuged at 1500 × *g* for 5 min at room temperature and the supernatant was applied to HPLC analysis.

In this study, principles of good laboratory animal care were followed and animal experimentation was performed in compliance with *the Guidelines for the Care and Use of Laboratory Animals* in Health Sciences University of Hokkaido.

2.4. Analyses

Phenytoin, caffeine, ceftibuten, and rhodamine 123 were assayed using a Shimadzu LC-10A HPLC system (Kyoto, Japan) equipped with a Shimadzu SPD-10A UV/VIS detector. Chromatographic conditions were as follows: column, Cosmosil $5C_{18}$-ARII (4.6 mm (i.d.) × 150 mm, Nakarai Tesque Inc., Kyoto, Japan); mobile phase: 0.05 M KH_2PO_4-CH_3CN (6:4 for phenytoin, 9:1 for caffeine and 1:1 for rhodamine 123) and 0.05M KH_2PO_4-CH_3OH (95:5 for ceftibuten); wave length, 210 nm for phenytoin, 270 nm for caffeine 500 nm for rhodamine 123 and 260 nm for ceftibuten; column temperature, 55 °C; flow rate, 0.8–1 ml/min; and injection volume, 10–20 μl. The concentration of FD-4 was determined with a fluorescence spectrophotometer (Hitachi F-4010, Hitachi, Tokyo, Japan) using excitation and emission wavelengths of 495 and 514 nm, respectively. The activity of lactate dehydrogenase (LDH) in the loop contents was determined with an LDH CII kit (Wako Pure Chem. Ind.). Protein concentration was assayed with a Bio-Rad Protein Assay kit. It was considered that β-CD included in the recovered loop contents minimally affected LDH and protein assay.

2.5. Statistical analysis

Data are expressed as the mean ± S.E. of at least three determinations. Statistical analysis was performed using Student's *t*-test, and $P < 0.05$ was considered to be significant.

3. Results and discussion

3.1. Dissolution of phenytoin in the media containing various solubilizing excipients

In the present study, we elucidated the solubilizing capacities of seven well-known agents, using phenytoin as a model compound with very limited water-solubility. In selecting the solubilizing agents to be tested, we omitted anionic surfactants and bile acids because they are reported to show significant

Table 1
Comparison of solubilizing capacities of seven agents for dissolving phenytoin

Test medium	Concentration of solubilizing agents		
	1%	2%	5%
D's PBS alone[a]	16.8 ± 0.4	14.9 ± 0.4	19.5 ± 0.4
+Methanol	16.5 ± 0.3	16.9 ± 0.3**	23.0 ± 0.3**
+DMSO	25.3 ± 0.3**	18.9 ± 0.3**	28.3 ± 0.5**
+DMAA	18.3 ± 0.6	21.3 ± 0.3**	34.1 ± 0.2**
+PEG 400	17.6 ± 0.2	18.3 ± 0.2**	16.8 ± 1.1*
+Tween 80	78.1 ± 0.1**	100.2 ± 1.0**	100.9 ± 0.4**
+β-CD	100.2 ± 0.4**	100.9 ± 0.4**	100.7 ± 0.7**
+HP-β-CD	93.3 ± 1.1**	100.4 ± 0.0**	–

Phenytoin was sonicated for 30 min in D's PBS (pH 7.4) containing each concentration of the solubilizing agents. Phenytoin concentration was targeted at 1 mM. The suspension or clear solution obtained was let stand for 30 min at room temperature and then centrifuged at 27,000 × *g* for 30 min at 25 °C. The phenytoin concentration in the supernatant was determined by HPLC. Each value represents the percent dissolved in the test medium (mean ± S.E. of three determinations). $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from D's PBS alone.
[a] Data were collected in every experiment using different concentrations of solubilizing agents.

cytotoxicity and/or modification of intestinal absorption (Anderberg et al., 1992; Sugiyama et al., 1997). Table 1 shows the dissolution of phenytoin in D's PBS containing each of the solubilizing agents at concentrations of 1, 2 or 5% (v/v or w/v). The final concentration of phenytoin was targeted at 1 mM when it dissolved in a test medium completely. At concentrations of 1 and 2%, the solubilizing capacities of methanol, DMSO, DMAA and PEG400 were low and almost comparable to that of D's PBS alone. Even at a concentration of 5%, they dissolved only 20–30% of the phenytoin although they exhibited slightly greater solubilizing capacities compared with D's PBS alone. On the other hand, Tween 80, β-CD, and HP-β-CD exerted relatively high-solubilizing capacities. They dissolved phenytoin completely at concentrations of 2 and 5%. Observation with the naked eye indicated that phenytoin was completely dissolved in 1% Tween 80 and HP-β-CD media, respectively. After high-speed centrifugation, however, it was shown that the concentration of phenytoin in the supernatant was approximately 80 and 95% that of the control, respectively. These results implied that observation with the naked eye did not always verify the complete dissolution of a compound in a medium. In order to further examine the solubilizing capacity of Tween 80 and β-CD, phenytoin dissolution was compared at the targeted concentrations of 1, 2 and 5 mM (Fig. 1). Except 5% β-CD, the solubilizing capacities decreased with the increase in phenytoin concentration. Since the solubility of β-CD itself is low, it did not completely dissolve in D's PBS at a concentration of 5%. However, the 5% β-CD dispersion maintained a relatively high-solubilizing capacity and completely dissolved phenytoin, irrespective of the phenytoin concentrations. Previously, Tsuruoka et al. (1981) demonstrated that β-CD significantly increased the oral bioavailability of phenytoin by increasing its solubility. Our present results are supportive of the previous findings.

These results suggest that β-CD is one of the most potent agents for solubilizing poorly water-soluble compounds such as phenytoin and that even at a low concentration of 1% (ca. 8.8 mM) β-CD exerts enough effect to accomplish it. Although HP-β-CD is a possible alternative of β-CD, a higher concentration might be required for it.

3.2. Effect of β-CD on the integrity of the intestinal epithelium

β-CD produces the greatest cell damage to erythrocytes among natural cyclodextrins, which was attributed to the considerable β-CD-induced release of cholesterol from the erythrocyte membrane (Irie et al., 1982). Moreover, a modified form of β-CD such as DM-β-CD is harmful to the Caco-2 cell membranes (Totterman et al., 1997; Tanino et al., 1999). Thus, the possible modulating effect of β-CD on the integrity of the rat intestinal membrane during in situ experiments should be examined carefully. In order to do this, changes in the disappearance of FD-4 as a marker of

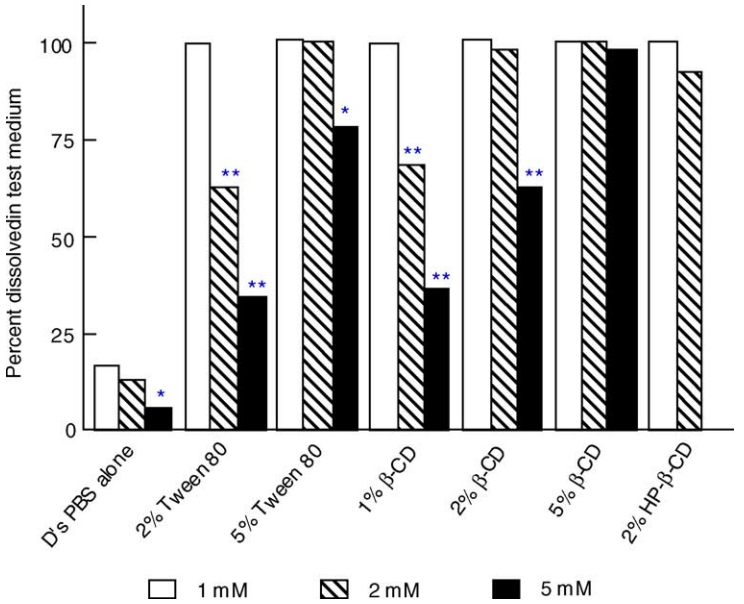

Fig. 1. Comparison of the solubilizing capacities of Tween 80, β-CD, and HP-β-CD for the dissolution of phenytoin at different concentrations. Phenytoin was sonicated for 30 min in D's PBS (pH 7.4) containing each concentration of the solubilizing agents. Phenytoin concentration was targeted at 1, 2, or 5 mM. The suspension or clear solution obtained was let stand for 30 min at room temperature and then centrifuged at 27,000 × *g* for 30 min at 25 °C. The phenytoin concentration in the supernatant was determined by HPLC. Each column represents the mean with S.E. of three determinations, although the bars of S.E. are hidden by column. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from 1 mM.

paracellular permeation were evaluated in the presence of 1% β-CD. Protein concentration and LDH activity in the loop contents recovered after the absorption experiments of FD-4 with or without 1% β-CD were also determined. This investigation was done using in situ loop technique, according to our previous reports (Saitoh et al., 1998; Nakayama et al., 2000). As summarized in Table 2, the disappearance of FD-4 in the control experiment was region-dependent; that is, it disappeared in the duodenum at a relatively greater rate than expected. In spite of an observation that protein concentration was almost identical in three regions in control experiments, LDH activity was much greater in the duodenum than in the jejunum and ileum. However, no significant alterations were observed in these three parameters with or without 1% β-CD, suggesting that 1% β-CD induced no or only a very limited impact on the rat intestinal membrane in the present studies. A recent paper shows that β-CD failed to induce any symptoms of cytotoxicity in

Table 2
Effect of 1% β-CD on the disappearance of FD-4 and the release of protein and LDH from the mucosa during 30-min absorption experiments using in situ rat intestinal loops

	FD-4 disappeared[a]		Protein released (mg/ml)		LDH released (IU/l) at 25 °C	
	Control	+1% β-CD	Control	+1% β-CD	Control	+1% β-CD
Duodenum	51.7 ± 2.7	49.0 ± 8.7	0.11 ± 0.01	0.09 ± 0.03	56.6 ± 7.2	63.9 ± 10.3
Jejunum	31.8 ± 1.6	39.0 ± 4.5	0.09 ± 0.02	0.09 ± 0.00	8.8 ± 5.7	5.6 ± 4.1
lleum	12.3 ± 8.3	12.5 ± 2.7	0.08 ± 0.02	0.09 ± 0.01	0.0 ± 0.0	0.0 ± 0.0

The concentration of FD-4 was 0.1 mM. Each value represents the mean ± S.E. of three experiments. Protein and LDH activity were determined using bop contents recovered into a 10-ml volumetric flask after absorption experiments using FD-4.

[a] Percent of dose that disappeared during 30 min.

Table 3
Effect of β-CD and HP-β-CD on the disappearance of caffeine and ceftibuten from rat jejunal loops

	Percent disappeared during 10 min	
	Caffeine	Ceftibuten
Control	73.6 ± 0.9	62.4 ± 3.2
+1% β-CD	70.4 ± 1.8	61.3 ± 1.9
+1% HP-β-CD	73.2 ± 2.1	60.4 ± 1.9

The concentration of caffeine and ceftibuten was 0.1 mM. Each value represents the mean ± S.E. of three experiments.

Caco-2 cell monolayers at a concentration of 15 mM (Ono et al., 2001). Our present results obtained with 1% (ca. 8.8 mM) β-CD closely support this result.

In this study, we did not investigate the possible modulating effect of Tween 80 and HP-β-CD on the integrity of the rat intestinal membrane in situ. Takahashi et al. (2002) demonstrated that 5% Tween 80 and HP-β-CD are appropriate agents for in vitro permeability studies using 21-day cultured Caco-2 cell monolayers. According to their observations, it seems that 2% Tween 80 and HP-β-CD are also useful for in situ absorption experiments.

3.3. Effect of β-CD on the absorption of three model compounds from the rat intestine

Previously, it was shown that 15 mM β-CD enhanced passive apical-to-basolateral permeation of rhodamine 123 in Caco-2 cell monolayers without affecting P-gp-mediated basolateral-to-apical permeation of rhodamine 123 (Ono et al., 2001). However, whether β-CD and its derivatives are capable of influencing the intrinsic passive and active intestinal absorption systems is not fully understood. In order to clarify whether 1% β-CD could alter intestinal absorption from in situ rat jejunal loops, the disappearance of caffeine, ceftibuten, and rhodamine 123 was evaluated in the presence of 1% β-CD. Caffeine is considered to permeate the intestinal membrane by passive diffusion. Ceftibuten and rhodamine 123 are potent substrates of PEPT1 (Tsuji and Tamai, 1996) and P-gp (Yumoto et al., 1999), respectively. One percent HP-β-CD was used for comparison, confirming that it dissolved these three compounds completely. As shown in Table 3, neither 1% β-CD nor 1% HP-β-CD exhibited any significant impact on the disappearance of caffeine and ceftibuten for 10 min, possibly suggesting that these two cyclodextrins have a negligible effect on passive and PEPT1-mediated absorption processes in the rat intestine under the present experimental conditions. It is general understanding that poorly water-soluble compounds such as phenytoin and caffeine differently interact with β-CDs. There is a paper describing that β-CD complexation improved the permeation of phenytoin through cellophane membrane (Tsuruoka et al., 1981). In this study, 0.1 mM caffeine completely dissolved in D's PBS without

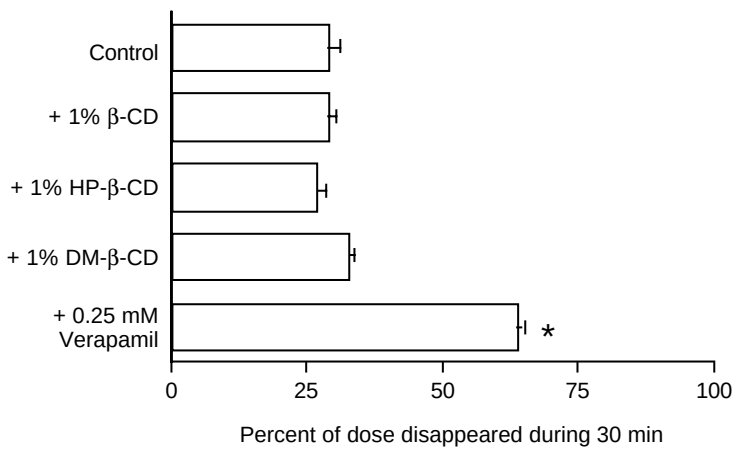

Fig. 2. Effect of three β-CDs and verapamil on the disappearance of rhodamine 123 from rat jejunal loops. The concentration of rhodamine 123 was 0.1 mM. Each column represents the mean with S.E. of six experiments for the control and three experiments for the other. *$P < 0.05$, significantly different from the control.

adding 1% β-CD and, therefore, the interaction between 0.1 mM caffeine and 1% β-CD might be less significant. It should be further elucidated whether a greater magnitude of complexation between β-CD and a water-insoluble drug could modulate the passive membrane permeation of the drug significantly.

One percent β-CD and 1% HP-β-CD also failed to modify the disappearance of rhodamine 123 for 30 min from intestinal loops (Fig. 2). Recently, it has been reported that DM-β-CD is capable of inhibiting the P-gp function in Caco-2 cells (Arima et al., 2001; Yunomae et al., 2003). Thus, for comparison, we investigated the enhancing effect of 1% DM-β-CD on the disappearance of rhodamine 123, but could not find any significant alteration in it. In order to clarify how much P-gp restricts the disappearance of rhodamine 123 in the rat jejunum, 0.25 mM verapamil, a potent P-gp inhibitor, was added to the test solutions. The treatment enhanced the disappearance of rhodamine 123 more than two-fold compared with the control, confirming that the absorption of rhodamine 123 from the rat jejunum is strongly affected by P-gp. Accordingly, it seems unlikely that β-CD, HP-β-CD, and DM-β-CD significantly modify the function of P-gp in situ at the low concentration of 1%. The inability of 1% β-CD to alter the disappearance of caffeine, ceftibuten, and rhodamine 123 suggests that 1% β-CD fulfills the requisites for the desired solubilizing agent.

4. Conclusion

The results from this study suggest that β-CD is considered as one of the most suitable solubilizing agents for evaluating poorly water-soluble drugs using the in situ loop and perfusion techniques. It was because even at a low concentration of 1% β-CD exhibited greater solubilizing capacity and because 1% β-CD did not have any significant impact on the membrane integrity and passive and several carrier-mediated absorption systems in situ. In the recent developmental stages, drug concentrations applied to in situ absorption experiments must be much less than 1 mM. Accordingly, 1% β-CD is expected to be widely applicable to many drugs with very limited water solubility in in situ absorption studies. Its suitability for in vitro and in vivo absorption studies is currently unclear. Further study is now underway to clarify the limits of 1% β-CD in dissolving various water-insoluble compounds.

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