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The Transport of a Hepatoprotective Agent, Isopropryl 2-(1-3-dithiethane-2-ylidene)-2[*N*-(4-methyl-thiazole-2-yl) carbamoyl] Acetate (YH439), across Caco-2 Cell Monolayers

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Isopropryl 2-(1-3-dithiethane-2-ylidene)-2[N-(4-methyl-thiazole-2-yl) carbamoyl] acetate (YH439) is currently under phase II clinical trials by the Yuhan Research Center for use as a hepatoprotective agent. Unfortunately, the oral bioavailbility of YH439, which is sparingly soluble in water (i.e., 0.3 μg/ml or 0.91 μM at room temperature), reportedly, is negligible regardless of the dose administered to rats in the 10-300 mg/kg range. The bioavailability of the compound increased up to 24%, when administered in the form of a micellar solution (700 μg/ml or 2.1 mM for YH439) at a dose of 10 mg/kg, suggesting that its limited solubility is associated with its negligible bioavailability. In order to obtain additional information concerning the bioavailability of YH439, the mechanism(s) involved in gastrointestinal (GI) absorption were investigated in the present study. For this purpose, the transport of YH430 across a Caco-2 cell monolayer was measured in a Transwell®. A permeability of 4.07×10^{-5} cm/s was obtained for the absorptive (i.e., apical to basolateral direction) transport of 0.42 μM YH439, implicating that the in vivo GI absorption is nearly complete. The absorptive transport exhibited a slight concentration-dependency with an intrinsic clearance (CL_i) of 0.38 μL/cm^2/sec, which accounted for 28.1% of the total intrinsic clearance (i.e., CL_i plus the intrinsic clearance for the linear component) of the transport. Thus, saturation of the absorption process appears to be a minor factor in limiting the bioavailability of the compound. The apparent permeability of YH439 from the basolateral to the apical direction (i.e., efflux, 6.67×10^{-5} cm/s) was comparable to that for absorptive transport, but, interestingly, a more distinct concentration-dependency was observed for this transport. However, the efflux does not appear to influence the bioavailability of the compound, as evidenced by the sufficiently high permeability in the absorption direction. Rather, a reportedly extensive first-pass hepatic metabolism appears to be a principal factor in limiting the bioavailability. In this respect, reducing the first-pass metabolism by some means would lead to a higher bioavailability of the compound. Thus, elevation of the absorption rate of YH439 becomes a necessity. From a practical point of view, increasing the concentration of YH439 in the GI fluid appears to be a feasible way to increase the absorption rate, because the compound is primarily absorbed via a linear mechanism. In summary, the solubilization of YH439, as previously demonstrated for a micellar solution of the compound, appears to be a practical way to increase the oral bioavailability of YH439.

Key words: YH439, Caco-2 cells, Transport; Bioavailability, Saturation, Solubilization.

INTRODUCTION

Isopropryl 2-(1-3-dithiethane-2-ylidene)-2[*N*-(4-methyl-thiazole-2-yl) carbamoyl] acetate (YH439, Mw 330.44) is currently under development for use as a hepatoprotective agent by the Research Center of Yuhan Co., and is being evaluated in phase II clinical trials. YH439 is known to protect the liver against chemical-induced hepatic injury probably by suppressing the expression of CYP450 2E1, the induction of which potentiates the toxicity of a number of xenobiotics via metabolic activation and/or

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the accumualtion of reactive metabolites. (Jeong *et al.*, 1996; Choi *et al.*, 1996). Unfortunately, the oral bioavailability of YH439 in rats was found to be negligible (< 1%) when the compound was administered in the form of a sodium carboxymethylcellulose suspension at a dose of 10 mg/kg (Kim *et al.*, 1996). The solubility of YH439 in water or in the form of a suspension was only 0.3 μg/ml (0.91 μM) at 25°C. This solubility could be increased up to 700 μg/ml (2.1 mM) when solubilized in mixed micelles of phosphatidylcholine and taurocholate (Choi, 1994; Kim *et al.*, 1996). The oral bioavailability of YH439 from the micellar solution was increased to 24 (Choi, 1994; Kim *et al.*, 1996) and 21.2 % (Yoon *et al.*, 1998) for a 10 mg/kg YH439 dose, suggesting the potential contribution of the solubility of YH439 to the oral bioavailability.

However, the absorption characteristics in the gastrointestinal (GI) tract and first-pass hepatic metabolism might influence the bioavailability of a compound as well. In fact, a bioavailability of only 0.9-3.7% was obtained for intact YH439 for oral doses of 50-500 mg/kg, despite the fact that approximately 40% of the doses, in terms of total radioactivity, were absorbed from the GI tract of rats, strongly suggesting an extensive first-pass hepatic metabolism for the compound (Kim *et al.*, 1998; Yoon *et al.*, 1998) in addition to degradation in the gastric juice (Yoon *et al.*, 1996). In terms of the absorption characteristics of YH439, however, no extensive investigation(s) aimed at elucidating the relevant mechanism has been carried out. Thus, the purpose of the present study is to investigate the mechanism of GI absorption of YH439, using a Caco-2 cell monolayer system as a model for GI absorption. The relevance of the absorption characteristics to the oral bioavailability of the compound were also examined.

MATERIALS AND METHODS

Materials

[^{14}C]YH439 (specific activity of 38.4 μCi/mg), unlabeled YH439, and its structural analogs (YH437, YH440) were generously donated by the Yuhan Research Center. Their structures and the position of labeling are shown in Fig. 1. [^{14}C]Mannitol (50 mCi/mmol, New England Nuclear, Boston, MA), fetal bovine serum (Hyclone Labortories, Logan, UT), trypsin-EDTA (Life Technologies. Inc., Gaithersburg, MD), Dulbecco's modified eagles's medium, a nonessential amino acid solution, penicillin, streptomycin, Hanks balanced salt solution (HBSS), N-[2-hydroxyethyl] piperazine-N-[2-ethanesulfonic acid] (HEPES), and 2-(N-morpholino)ethane sulfonic acids (MES) were used as purchased from Sigma Chemical Co.(St. Louis, MO). All other reagents were of analytical grade.

Caco-2 cell culture

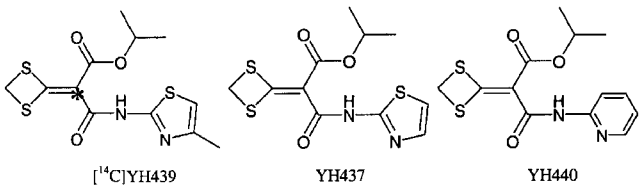

Fig. 1. Structures of YH439 and analogs thereof, YH437 and YH440. The asterisk indicates the position of ^{14}C labeling.

The human colon adenocarcinoma cell line, Caco-2 (American Type Culture Collection, Rockville, MD), was grown as monolayers in Dulbecco's modified Eagles' medium, 10% fetal bovine serum, 1% non-essential amino acid solution, 100 units/ml penicillin, and 0.1 mg/ml streptomycin at 37°C in an atmosphere of 5% CO_2 and 90% relative humidity. Stock cultures were grown in 75-cm^2 tissue culture flasks and were split 1:3 at 80 to 90% confluency using 0.02% EDTA and 0.05% trypsin. The Caco-2 cells from passage numbers of 46 to 55 were seeded on permeable polycarbonate inserts (1-cm^2, 0.4-mm pore size; Corning Costar Corp., Cambridge, MA) in 12 Transwell® plates (Corning Costar Corp.) at a density of 2.5×10^5 to 3.0×10^5 cells/cm^2. The inserts were fed by complete media every 2 days for the first week and then daily until they were used for the transport experiments. The integrity of the cell monolayers was evaluated by measuring transepithelial electrical resistance (TEER) values with an EVOM™ epithelial volt/ohm-meter (World Precision Instruments, Sarasota, FL). When the TEER value reached 300 to 700 Ω cm^2, the transport of [^{14}C] mannitol (5.4 mM) became <0.25% of the dose/h, corresponding to an apparent permeability (P_{app}) value of 5.6×10^{-7}cm/sec. Cell inserts which showed such TEER values were used for the transport experiments.

Transepithelial transport

Prior to the transport experiments, the cell monolayers were washed twice with the incubation medium (pH 7.4, HBSS containing 25 mM HEPES and 25 mM glucose). After each wash, the plates were incubated in the incubation medium for 30 min at 37°C, and the TEER value was measured. The incubation medium on both sides of the cell monolayers was then removed by aspiration (Augustijns *et al.*, 1993).

For measurement of the apical to basolateral transport, 0.5 ml of the incubation medium containing [^{14}C]YH439 (0.2~17.2 μM) and DMSO (0.5%) was added on the apical side, and 1.5 ml of the incubation medium without YH439 was added to the baso-lateral side. The purpose of the addition of DMSO to the donor and receiver sides was to increase the solubility of YH439 in water (0.3 μg/ml at 25°C). The addition of DMSO up to 1% was confirmed to have no effect on the permeability of [^{14}C]

mannitol across the cell monolayers (i.e., $P_{app}=5.6\times10^{-7}$ cm/sec for 5.4 μM mannitol) in our preliminary studies. The inserts were moved, every 15 min for 1 h, to new Transwells® to which fresh incubation medium was added. In each transport experiment, three inserts were used. After moving the inserts to new wells, 200 μl of DMSO was added to the basolateral side of each remaining well to give a total DMSO concentration of 14.3% (v/v), and the well was incubated for 2 h at 37°C. By this addition of DMSO and subsequent incubation for 2 h, the adsorption of YH439 to the well surface could be reduced to less than 5% when examined using 0.5 nM and 0.65 μM YH439, which was as high as 16.4% in the absence of DMSO. After the incubation, 1 ml of the medium in the basolateral side was transferred, using a disposable pipet, into a scintillation vial which contained 6 ml of scintillation cocktail (Ultima Gold, Packard, Meriden, CT) for liquid scintillation counting (LSC). The pipet tips were also placed in the vials in order to count the YH439 that had adsorbed to their surface.

For the measurement of the basolateral to apical transport, 1.5 ml of the incubation medium containing [^{14}C]YH439 (0.2~17.2 μM) and DMSO (0.5%) was added in the basolateral side, and 0.5 ml of the incubation medium containing 0.5% DMSO without the drug was added to the apical side. In each experi-ment, three inserts were used. The inserts were then incubated at 37°C, and 300 μl aliquot of the incubation medium in the apical side was replaced by 300 μl of fresh medium at 15-min intervals for 1 h. The radioactivity contained by the 300 μl aliquot of each 15-min sample was determined by LSC as described above.

In order to examine the concentration dependency of the transport of YH439, incubation medium containing varying concentrations of YH439 (0.2~17.2 μM) and 0.5 % DMSO was loaded to the apical or basal side of the insert. The transport of YH439 was then measured as described above. The effect of structural analogs of YH439, YH437 and YH440 on the transport of [^{14}C] YH 439 was also investigated, by adding the unlabelled analogs at 50 to 100-fold higher concentrations to the incubation medium (i.e., the concentration of [^{14}C]YH 439 was 0.55 μM, and those of unlabelled compounds were 50, 25 and 50 μM for YH439, YH437 and YH440, respectively, in each case, the maximum concentration available in terms of solubility for each analog compound). The concentration of DMSO in the incubation medium was elevated to 1% in this case in order to achieve above mentioned concentrations for YH439 and the structural analogs. The 1% DMSO was confirmed to have no effect on the leakage of mannitol from the cell monolayers.

In addition, the net flux of YH439 across the cell monolayers was determined as follows: The incubation medium containing 0.5% DMSO and 0.41 μM YH439 was loaded to both the apical (0.5 ml) and basolateral (1.5 ml) sides, and incubated as described above. During the incubation, 200 and 600 μl aliquots were collected respectively from the apical and basolateral sides at 30, 45, 60 and 120 min. After each sampling, equivalent volumes of fresh media were placed to both sides. The concentration of YH439 in each sample was measured by LSC, and the concentration ratio of YH439 between the apical and basal sides were then calculated.

RESULTS AND DISCUSSION

The transport of YH439 across the caco-2 cell monolayers

The apical to basolateral and basolateral to apical fluxes of YH439 across the Caco-2 cell monolayers are shown in Fig. 2. The apparent permeability (P_{app}) of YH439 from the apical to the basolateral direction was calculated to be 4.07×10^{-5} cm/s for 0.42 μM YH439, which is sufficiently high for complete GI absorption based on the previously established correlations between P_{app} and oral bioavailability in man (Artursson and Karlsson, 1991; Rubas et al., 1996; Yee, 1997). The high permeability is consistent with the high lipophilicity of the compound (i.e., log D = 4 for the partition between n-octanol/water, Choi., 1994). In spite of the high P_{app}, the actual bioavailability of YH439 in rats has been reported to be negligible when the compound was administered orally in the form of a sodium CMC suspension at YH439 doses of 10, 50,

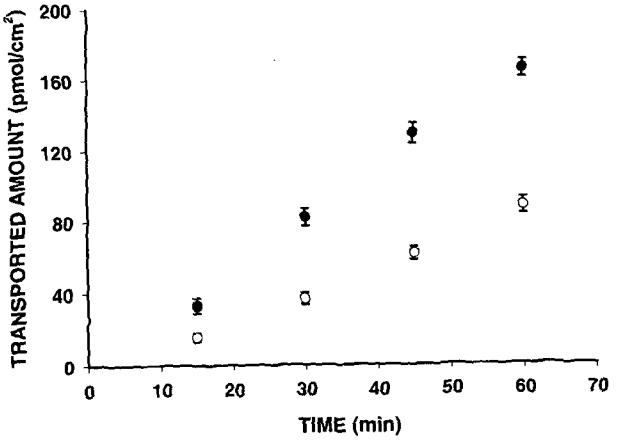

Fig. 2. Time courses for transepithelial transport of YH439 by Caco-2 cell monolayers. For the apical to basolateral flux (●), 0.5 ml of incubation medium (pH 7.4) containing dimethylsulfoxide (0.5%) and YH439 (0.42 μM) was added to the apical side, and 1.5 ml of the incubation medium (pH 7.4) containing dimethylsulfoxide (0.5%) without YH439 was added to the basolateral side. For the basolateral to apical flux (○), 1.5 ml of incubation medium (pH 7.4) containing dimethylsulfoxide (0.5%) and YH439 (0.42 μM) was added to the basolateral side, and 0.5 ml of the incubation medium (pH 7.4) containing dimethylsulfoxide without YH439 was added to the apical side. Each point represents the mean ± SD of six experiments.

100, 300 and 500 mg/kg (Choi., 1994; Kim *et al.*, 1996; Yoon *et al.*, 1998). This can be explained based on the assumption that saturation of the absorption process occurs at a given YH439 concentration in the GI fluid (i.e., its solubility), and also assuming that a carrier mediated transport predominates in the overall absorption process of the compound.

Interestingly, YH439 was transported in the basolateral to apical direction with an apparent permeability (P_{app}) of 6.67×10^{-5} cm/s for 0.42 μM YH439, which is 1.6-fold larger than that in the apical to basolateral direction, suggesting the presence of a transport system in the direction of efflux. In an attempt to confirm this issue, the net flux of YH439 was measured by determining the temporal concentration ratio of YH439 between the apical /basolateral sides after loading YH439 to both sides at an identical concentration (i.e., 0.41 μM). Consistent with the results from Fig. 2, the concentration ratio of YH439 exceeded unity and increased as a function of time (Fig. 3), thus confirming the presence of a carrier-mediated, possibly active, transport mechanism(s) in the efflux (i.e., basolateral to apical flux) for this compound.

Stimulated by the involvement of a carrier-mediated transport in the efflux, the effect of YH439 concentration on the flux of the compound in both directions was then examined. A concentration range of 0.2 17.2 μM YH439 was used in this examination. The apical to basal flux exhibited a nearly linear increase as a function of the concentration (Fig. 4). However, an Eadie-Hofstee plot (data not shown) of the data indicates the presence of saturable component in the flux. On the other hand, a more clear concentration dependency was observed for the basal to apical flux (Fig. 4), which was confirmed by the Eadie-Hofstee plot (data not shown). Despite of the presence of saturable components, the linear components still appeared to contribute substantially to the fluxes of both directions (Fig. 4). Therefore, it seems reasonable to assume that the fluxes of YH439 in both directions are mediated both by saturable and nonsaturable processes. Thus, the flux-concentration data (Fig. 4) were analyzed according to the following equation. A nonlinear regression analysis was performed in the fitting of the data to the equation.

$$V = \frac{V_{max} \times C}{K_m + C} + K \times C$$

In this equation, V, V_{max}, K_m, K and C represent flux, maximum flux, Michaelis constant, linear rate constant and the concentration of YH439 in the donor compartment, respectively. Using the regression, V_{max} , K_m and K for the apical to basal flux were calculated to be 1.5 pmol/cm^2/sec, 3.9 μM and 0.97 μL/cm^2/sec, respectively. The intrinsic clearance (CL_i) for the saturable component (V_{max}/K_m) was calculated to be 0.38 μL/cm^2/sec (Table I), which explains 28.1% of the total intrinsic clearance (i.e., CL_i plus K). For the basolateral to apical flux, V_{max}, K_m, K and CL_i were calculated to be 10.5 pmol/cm^2/sec, 2.8 μM, 0.31 μL/cm^2/sec and 3.8 μL/cm^2/sec, respectively (Table I). Contrary to the case of the apical to basolateral flux, the contribution of the saturable component (i.e., CL_i) to the overall efflux was determined to be dominant (92%).

Based on the above observations, it seems reasonable to hypothesize the involvement of carrier mediated transport systems in the saturable component and passive diffusion in the linear component of the flux, respectively. An analysis of saturable components of the flux in both

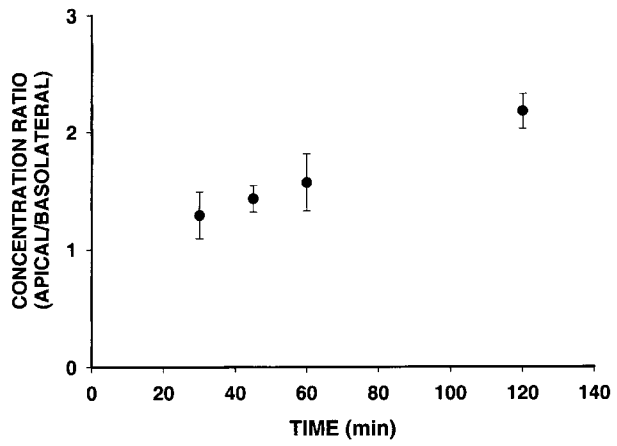

Fig. 3. Time course of the concentration ratio of YH439 between the apical/basolateral side following addition of the incubation medium containing dimethylsulfoxide (0.5%) and 0.42 μM YH439 to both apical (0.5 ml) and basolateral side (1.5 ml). Each point represents the mean ± SD of six experiments.

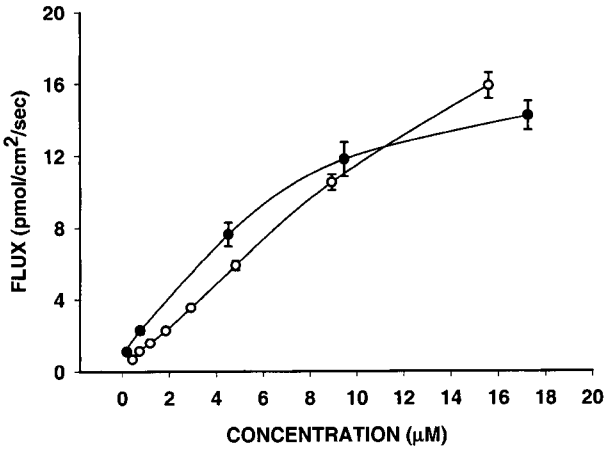

Fig. 4. Concentration dependency of YH439 transport across Caco-2 cell monolayers. Apical to basolateral (○) and the basolateral to apical (●) fluxes were measured as described in Fig. 2, and plotted against the concentration of YH439. Each point represents the mean ± SD of three experiments.

Table I. Summary of Kinetic parameters based on the Michaelis-Menten model and linear components

Parameter	Apical to basolateral	Basolateral to apical
V_{max} (pmol/cm^2/sec)	1.5	10.5
K_m (μM)	3.9	2.8
K (μL/cm^2/sec)	0.97	0.31
CL_i (μL/cm^2/sec)	0.38	3.8

CL_i was calculated by V_{max}/K_m

directions reveals that the affinity of YH439 to both carriers is comparable, while the intrinsic clearance (CL_i) is 10-fold larger for the efflux direction compared to the absorption direction. This analysis is consistent with the larger P_{app} for the efflux direction than for the absorption direction (Fig. 2), and the presence of a net efflux of the compound across the Caco-2 cell monolayers (Fig. 3).

Since above results suggested carrier-mediated transport for both directions, the effect of structural analogs, YH437 and YH440, on the flux in both directions was investigated. The P_{app} of [^{14}C]YH439 (0.55 μM) from the apical to basolateral side was decreased significantly (37% decrease, $P<0.01$) by the presence of 50 μM unlabeled YH439, but not by the presence of 25 μM YH437 and 50 μM YH440 (Fig. 5). The P_{app} of [^{14}C]YH439 (0.55 μM) from the basolateral to apical side, on the other hand, was inhibited by the presence of 50 μM unlabeled YH439 (30% decrease, $P<0.05$), 25 μM YH437 (20% decrease, $P<0.05$) and 50 μM YH440 (38% decrease, $P<0.05$) (Fig. 5), suggesting the involvement of identical carrier system(s) in the efflux of these compounds. However, the inhibition of the flux by the presence of unlabeled YH439

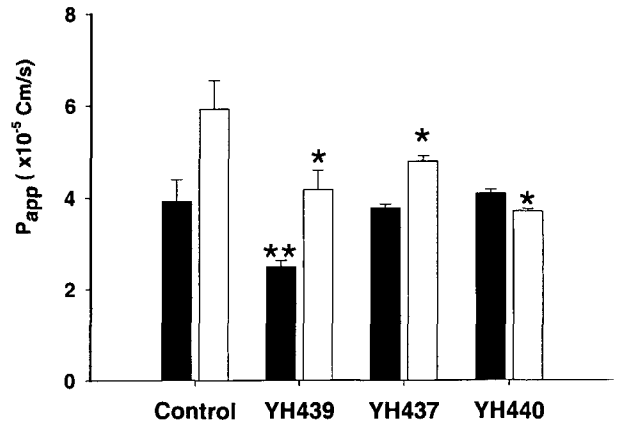

Fig. 5. Effect of unlabeled YH439 (50 μM), YH437 (25 μM) and YH440 (50 μM) on the apical to basolateral (■) and basolateral to apical flux (□) of [^{14}C]YH439 (0.55 mM). The incubation medium (pH 7.4) in each experiment contained 1% (v/v) dimethylsulfoxide. Each bar represents the mean ± SD of three experiments. *$P<0.05$, **$P<0.01$ compared with the respective control values.

and structural analogs was not profound for either direction. This might be due to a larger contribution of the linear component, probably passive diffusion, to overall transport of YH439. The contribution of the carrier-mediated component to the overall transport was larger for the efflux direction compared to the absorption direction, based on a comparison of CL_i and K (Table I). This may explain the efflux-specific inhibition by the structural analogs (Fig. 5). The issue of whether the carrier-mediated systems in both directions are identical or not was not pursued in the this study. However, the results obtained here indicate that YH439 has a comparable affinity (K_m) to the carriers of both directions, and that the compound is more efficiently transported in the efflux direction compared to the absorption direction (Table I).

Despite the presence of an efflux system for YH439, the contribution of this system to the limited bioavailability of the compound would be predicted to be minimal, as evidenced by the fairly large P_{app} for the apical to basolateral flux. Therefore, it can be concluded that the efflux is not a governing mechanism for the limited bioavailability of YH439. The saturation of the absorption process at a given GI concentration was then examined as a possible source of the limited bioavailability. Although absorptive transport exhibited a slight concentration-dependency (Fig. 4), the CL_i for the saturable component (0.38 μL/cm^2/sec, from Table I) accounted for only 28.1% of the total intrinsic clearance (i.e., CL_i plus intrinsic clearance for the linear component) of the compound. Thus, the saturation of the absorption process can be eliminated from the principal mechanism of the limited bioavail-ability of the compound. The above results indicate that the limited bioavailability of YH439 is not related to aspects of the absorption of the compound. In other words, considering the nearly linear characteristics of the absorption, increasing the concentration of YH439 in the GI fluid represents an effective way to achieve higher bioavailability.

However, it should be noted that YH439 undergoes extensive first-pass hepatic metabolism (Yoon *et al.*, 1996; 1998). In this respect, an appropriate reduction in first-pass metabolism would likely to lead to an increased bioavailability of the compound. The metabolism would be reduced if the absorption rate of the compound is elevated. An increase in absorption would be possible by increasing the concentration of YH439 in the GI fluid, since the compound is rapidly absorbed from the GI fluid with a sufficiently high permeability of 4.07×10^{-5} cm/s without exhibiting a significant concentration-dependency for absorption (Table I). In summary, the solubilization of YH439, as previously demonstrated for a micellar solution of the compound (Choi, 1994; Kim *et al.*, 1996), appears to represent a practical way to increase the oral bioavailability of YH439.

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