

Intraluminal Behavior of Various Transporter Substrates in the Rat Gastrointestinal Tract

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Received, September 3, 2021; Revised, November 15, 2021; Accepted, November 18, 2021; Published, November 21, 2021

ABSTRACT -- Purpose: The aim of this study was to evaluate the intraluminal behavior of various transporter substrates in different regions of the gastrointestinal (GI) tract. **Methods:** Drug solutions containing non-absorbable FITC-dextran 4000 (FD-4), were orally administered to rats. Residual water was sampled from the GI regions to measure the luminal drug concentration. **Results:** Cephalexin (CEX), a substrate of the proton-coupled oligopeptide transporter, was absorbed rapidly, and no drug was detected in the lower small intestine. Saquinavir (SQV) was primarily absorbed in the upper region. However, unlike CEX, SQV was detected even in the lower segment probably due to the efflux of SQV via P-glycoprotein (P-gp). The concentration of methotrexate (MTX) showed a similar pattern to that of non-absorbable FD-4. The low absorption of MTX was probably due to efflux via several efflux transporters, and the limited expression of proton-coupled folate transporter, an absorptive transporter for MTX, in the upper region. **Conclusion:** This study revealed that the luminal concentration pattern of each drug differed considerably depending on the site because of the different absorption properties and luminal volumes. Although further investigation using a specific transporter inhibitor or transporter-knockout animals are necessary to clarify the actual contribution of each transporter to the drug absorption, this information will be valuable in evaluating transporter-mediated drug absorption in *in vitro* transport studies for ensuring optimal drug concentrations.

INTRODUCTION

A variety of influx and efflux transporters are expressed in the small intestine (1-3). Intestinal permeability of transporter substrates changes depending on intraluminal drug concentration (4,5), sometimes leading non-linear bioavailability (6,7). Total intestinal fluid volume was often used to estimate average luminal drug concentration for evaluation of transporter-mediated drug uptake (8,9). In our previous report, however, the luminal volume was drastically different depending on the segments of the gastrointestinal (GI) tract due to fluid absorption and secretion (10,11), indicating that the use of total intestinal volume to estimate average luminal drug concentration may have a risk of failure in the evaluation of drug absorption, especially via transporters with site-specific expression [e.g. Proton-coupled folate transporter (PCFT) and Multidrug resistance-associated protein 2 (MRP2) that are primarily expressed in proximal small intestine (2,12) and P-glycoprotein (P-gp) that is primarily expressed in distal small intestine (2)]. Thus, understanding the behavior of transporter

substrates in various segments of the GI tract is very important to evaluate and predict carrier-mediated absorption more accurately. Intraluminal drug behaviors in humans have been reported by several groups (13-15). However, since an intubation method was typically used for luminal fluid sampling, it is technically difficult to obtain a sample from the lower segment. Therefore, relevant information, especially for luminal behavior in the lower region (e.g., lower jejunum and ileum) is still limited. In our previous study, intraluminal concentrations of several drugs that are passively absorbed were measured by direct sampling of residual fluid from individual GI segments including lower regions (11,16), and it is elucidated that intraluminal behavior differed depending on physicochemical properties of drugs (e.g., solubility and permeability). It is considered that our methodology is useful to elucidate concentration patterns of transporter substrates in different GI segments.

In this study, to quantitatively measure the luminal concentrations as a function of time in individual GI segments, various transporter substrates were orally administered to rats. In

addition, non-absorbable FITC-dextran (FD-4) was co-administered as a control, and the drug absorption behavior was accurately determined by comparing the luminal concentrations of FD-4 and other drugs. Cephalexin (CEX), a substrate of the peptide-transporter 1 (PEPT1) (17); saquinavir (SQV), a substrate of P-gp (18) and MRP2 (2); and methotrexate (MTX), a substrate of PCFT (12), reduced folate carrier 1 (RFC1) (19), breast cancer resistance protein (BCRP) (20), MRP2 (12), and P-gp (21,22) were selected as model drugs to evaluate the impact of absorption behavior via various transporters on luminal concentration pattern of drugs.

ABBREVIATION. BA: bioavailability; BCRP: breast cancer resistance protein; CEX: Cephalexin ; FD-4 : FITC-dextran ; GI : gastrointestinal ; MTX: Methotrexate; MRP2: Multidrug resistance-associated protein 2; P-gp: P-glycoprotein; PCFT: proton-coupled folate transporter; PEPT1: peptide-transporter 1; SQV: Saquinavir

MATERIALS AND METHODS

Materials

FD-4 was purchased from Sigma-Aldrich (St. Louis, MO, USA). CEX, ritonavir, and metoprolol tartrate were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Saquinavir mesylate, MTX, and antipyrine were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). All other reagents were analytical-grade commercial products.

Luminal concentrations of FD-4 and drugs, and the plasma concentrations of drugs after oral administration

All experimental procedures were approved by the Animal Experimentation Ethics Committee of the Hiroshima International University and were in accordance with the Guide to the Care and Use of Experimental Animal Care. Male Sprague Dawley rats (244-298 g) were sourced from Japan SLC (Hamamatsu, Japan). Rats were fasted overnight prior to the experiments; water was provided ad libitum but limited to 2 h prior to administration.

The luminal concentration of FD-4 and drugs was measured as described previously (10,11). CEX and MTX were dissolved in Milli-Q water at concentrations of 1.0 and 0.025 mg/mL, respectively. Saquinavir mesylate was dissolved to a concentration of 0.2 mg/mL as free form in HCl solution (pH 2.4) instead of Milli-Q water due to solubility problems. Subsequently, FD-4 was solubilized in the respective drug solutions to a final concentration of 200 μ M.

Each solution (1 mL) was orally administered

to rats. The doses were 0.2 μ mol for FD-4 and 1.0, 0.2 and 0.025 mg for CEX, SQV and MTX, respectively. After 5, 10, 20, 30, 60, 90, 120, and 150 min, blood samples were collected from the right jugular vein, and rats were euthanized by injecting a potassium chloride solution (22.5 mg/rat) into the left jugular vein under anesthesia with isoflurane. Subsequently, each GI segment was dissected immediately to collect residual water from the stomach, duodenum (approximately 2-cm from the stomach), upper jejunum (approximately 20-cm from the stomach), lower jejunum (approximately 60-cm from the stomach), and ileum (approximately 10-cm from the cecum). Three and four rats were used in each time point for CEX and for SQV and MTX, respectively. The samples were diluted with Milli-Q water for CEX and MTX, and with dimethyl sulfoxide for SQV. The samples were further diluted using appropriate solvents: 50 mM Tris buffer (pH 8.0) for FD-4, Milli-Q water for CEX and MTX, and 50% methanol for SQV determination. Furthermore, CEX samples were mixed with an internal standard (1.0 μ g/mL antipyrine in 50% methanol) for LC-MS/MS analysis. SQV and MTX in the samples were analyzed by HPLC.

Collecting and Processing blood samples

CEX and MTX were solubilized in saline at 1.0 and 0.025 mg/mL, respectively, and saquinavir mesylate was dissolved at 0.2 mg/mL as free SQV in saline containing polyethylene glycol 400 at 30% v/v. Subsequently, each solution (0.5 mL) was administered into the jugular vein of rats. Thereafter, blood samples were taken from the cannulated femoral artery at 5, 10, 20, 30, 40, 50, 60, 75, and 90 min.

Blood samples collected after oral and intravenous administration of drugs were centrifuged and the obtained plasma (50 μ L) was mixed with 50 μ L of methanol and 50 μ L of an internal standard (1.0 μ g/mL antipyrine, 1.0 μ g/mL metoprolol and 0.1 μ g/mL ritonavir in methanol for CEX, MTX and SQV, respectively.). Precipitated proteins were removed by centrifugation, and the supernatant was quantified by LC-MS/MS.

Oral bioavailability (BA)

As described in a previous report (11), oral BA-time profiles of various drugs were calculated using decon.xls, a Microsoft excel incorporating a program for deconvolution. This program is currently

available at <http://www4.tokai.or.jp/DMPK/program.html>. The plasma concentration–time courses after oral administration of the three drugs were used as an output function, and those after the intravenous administration were used as an input function (23).

Quantification of FD-4 and drugs

FD-4 in the luminal samples was quantified using a multilabel luminescence counter (ARVO-MX, PerkinElmer Japan, Kanagawa, Japan), at wavelengths of 492 and 535 nm for excitation and emission, respectively.

MTX and SQV in luminal samples were determined using a Shimadzu HPLC system with a UV detector (SPD-20A; Shimadzu Corporation, Kyoto, Japan). An analytical column (YMC-Pack Pro C18, 150×6.0 mm I.D., YMC Co., Ltd. Japan) was used at 40°C; the mobile phase consisted of a 50 mM phosphate buffer (pH 2.5) and acetonitrile (85:15 v/v) or 50 mM phosphate buffer (pH 6.0) and acetonitrile (35:65 v/v) were run isocratically at a flow rate of 1 mL/min for MTX and SQV, respectively. MTX and SQV were detected at wavelengths of 304 and 240 nm, respectively.

Drugs in plasma samples and CEX in luminal samples were measured using an LC-MS/MS (LCMS-8040, Shimadzu, Kyoto, Japan). A Zorbax Eclipse XDB-C18 column (2.1 × 50 mm, I.D., 5 μm) was used at 40°C. The mobile phases consisted of 0.1% formic acid in Milli-Q water and methanol at 4:1 and 17:3 v/v were used for CEX and antipyrine (IS) and for MTX and metoprolol (IS) quantifications, respectively. For SQV and ritonavir (IS) determination, 10 mM ammonium formate containing 0.1% formic acid in Milli-Q water and methanol (7:13 v/v) was used. The transition monitored for quantification was m/z 348.0 > 157.9 for CEX, 189.0 > 56.1 for antipyrine, 455.1 > 308.1 for MTX, 268.2 > 116.1 for metoprolol, 671.3 > 570.2 for SQV, and 721.1 > 296.2 for ritonavir.

RESULTS

Luminal concentration–time profiles of FD-4 and experimental drugs

The luminal concentrations of FD-4 and experimental drugs are shown in Figures 1-3. The vertical axis displays concentration as the percentage in the initial dosing concentration of FD-4 (200 μM)

and drugs (1.0, 0.2, and 0.025 mg/mL for CEX, SQV, and MTX, respectively).

The gastric concentrations of FD-4 and CEX at 5 min were $54.3 \pm 15.6\%$ and $51.5 \pm 17.8\%$, respectively (Figure 1). Thereafter, the gastric CEX concentration decreased parallelly with the FD-4 concentration over time. In the duodenum and upper jejunum, the concentrations of CEX at 5 and 10 min were lower than those of non-absorbable FD-4, indicating that CEX was absorbed to some extent in these segments. In the lower jejunum and ileum, no CEX was detected, indicating that CEX was completely absorbed before it reached these regions. For FD-4, the maximum concentrations in the upper jejunum, lower jejunum and ileum (136.3 ± 93.9 , 245.8 ± 284.6 and $359.5 \pm 255.0\%$, respectively) were above the dosing concentration. This was attributed to the condensation of FD-4 by water absorption transitioned to the lower segment, which is consistent with our previous study (10).

The luminal SQV concentration-time courses are shown in Figure 2. SQV is known as a substrate of P-gp and MRP2, efflux transporters (2). After oral administration, SQV rapidly passed through the stomach and duodenum. In the upper jejunum, SQV was absorbed to some extent until it reached this region, leading lower concentration of SQV than that of FD-4. In the lower jejunum and ileum, some SQV was detected (the C_{max} was 61.8 ± 33.9 and $86.1 \pm 55.2\%$ in the lower jejunum and ileum, respectively), unlike in the case of CEX. In our previous study, a simulated intestinal fluid was produced based on concentrations of bile acids (51 mM) and phospholipids (3.7 mM) in the jejunum of fasted rats (24), which values are much higher than those in the human small intestine (25). The solubility of SQV in the rat simulated intestinal fluid was 264.2 ± 8.6 μg/mL (26). Although SQV is a lipophilic base, the concentrations of SQV in each segment of the small intestine were below the solubility values, and thus it is expected that SQV was present in a solubilized state in the small intestine.

The concentrations of FD-4 and MTX in the stomach at 5 min were $54.5 \pm 21.0\%$ and $50.2 \pm 24.9\%$, respectively, and decreased in the same manner (Figure 3). In the small intestine, the C_{max} values of MTX were 30.6 ± 35.5 , 165.4 ± 46.3 , 246.7 ± 155.3 and $232.3 \pm 152.1\%$, in the duodenum, upper jejunum, lower jejunum and ileum, respectively. These concentration values of MTX were only slightly lower than those of FD-4, indicating low absorption of MTX from the GI tract.

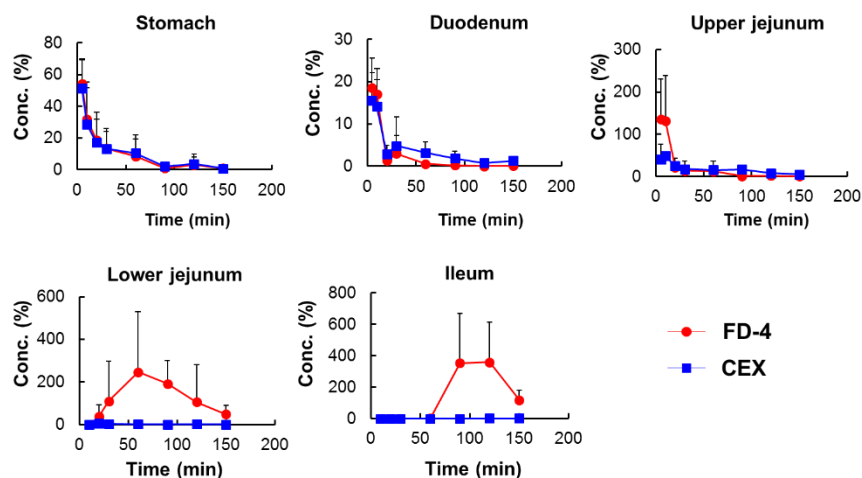


Figure 1. Luminal concentration-time profiles in different intestinal segments of FD-4 and CEX after oral administration. Results are expressed as mean values, with vertical bars showing S.D. of three experiments. The vertical axes are expressed as the percentage of the respective dosing concentrations (i.e., FD-4: 200 μ M or CEX: 1.0 mg/mL).

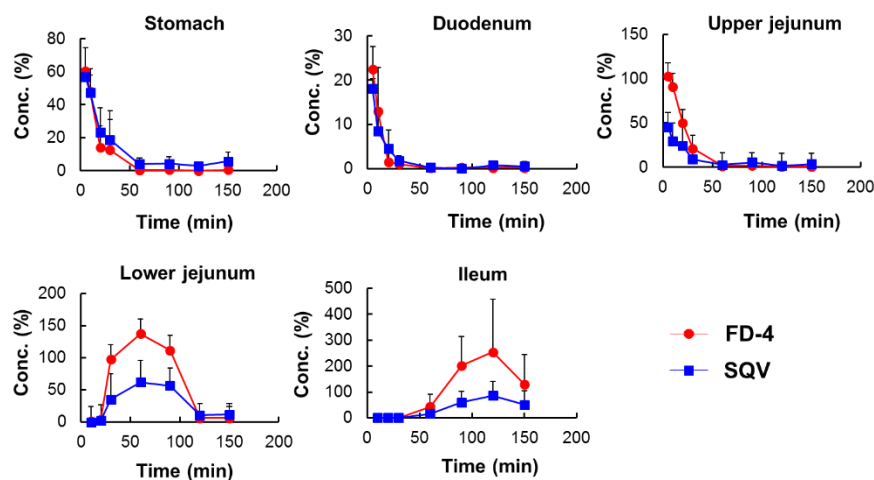


Figure 2. Luminal concentration-time profiles in different intestinal segments of FD-4 and SQV after oral administration. Results are expressed as mean values, with vertical bars showing S.D. of four experiments. The vertical axes are expressed as the percentage of the respective dosing concentrations (i.e., FD-4: 200 μ M or SQV: 0.2 mg/mL).

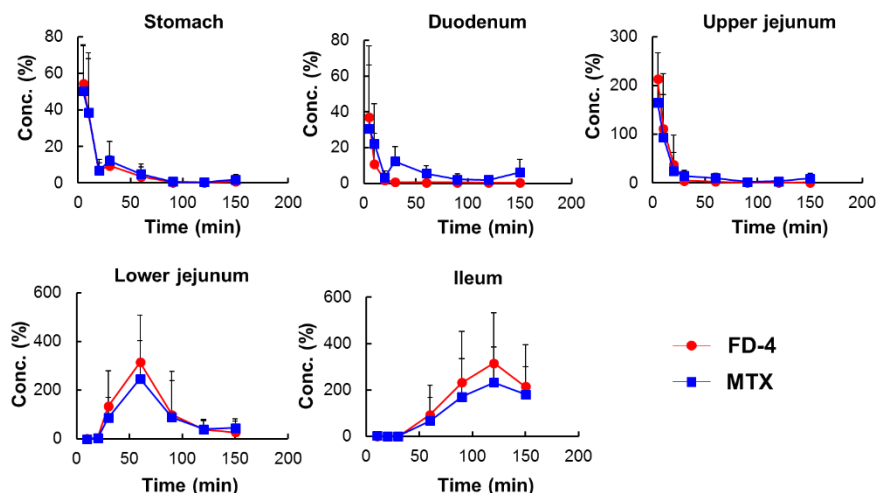


Figure 3. Luminal concentration-time profiles in different intestinal segments of FD-4 and MTX after oral administration. Results are expressed as mean values, with vertical bars showing S.D. of four experiments. The vertical axes are expressed as the percentage of the respective dosing concentrations (i.e., FD-4: 200 μ M or MTX: 0.025 mg/mL).

Time profiles of plasma concentrations and oral BA of experimental drugs

The plasma concentration-time profiles after oral and intravenous administrations of drugs were shown in Figure 4. The concentrations at 5 min after intravenous administrations were 4.44 ± 0.25 $\mu\text{g/mL}$, 88.6 ± 6.51 ng/mL and 80.7 ± 8.47 ng/mL for CEX, SQV and MTX, respectively, and decreased rapidly with time. In the oral administration groups, the plasma concentration reached the peak at 30, 5 and 10 min for CEX, SQV and MTX, respectively, and the C_{max} values were 2.39 ± 0.57 $\mu\text{g/mL}$ for CEX, 8.13 ± 4.12 ng/mL for SQV and 32.9 ± 49.9 ng/mL for MTX.

The time profiles of oral BA were calculated by the deconvolution method using the plasma concentration-time courses of drugs after oral and intravenous administrations (Figure 5). CEX was continuously absorbed, and the BA reached 85.2% at 150 min after oral administration. In the case of SQV, the BA rapidly increased to 4.7% at 20 min, and thereafter, the absorption rate decelerated, suggesting that SQV was primarily absorbed from the upper region. Similarly, the absorption rate of MTX was faster until 20 min and decreased in the later time period.

DISCUSSION

To accurately evaluate and predict carrier-mediated absorption, it is essential to understand the actual concentration pattern of transporter substrates in the GI tract and applying this information in the PBPK model and/or *in vitro* transport experiments. The present study therefore attempted to evaluate absorption behavior of various transporter substrates in the GI tract and its impact on the luminal drug concentration profiles.

After gastric emptying of CEX following oral administration, although the duodenal concentrations of CEX at 5 and 10 min were slightly lower than those of FD-4 due to CEX absorption, the duodenal CEX concentrations exceeded FD-4 concentrations at timescales longer than 20 min after oral administration (Figure 1). This may be due to biliary secretion of CEX into the duodenum (27). However, CEX did not exceed the FD-4 concentration in the upper jejunum, indicating that secreted CEX was rapidly re-absorbed. Ultimately, CEX was completely absorbed from the GI tract. Since PEPT1 is expressed throughout the rat small intestine (28), it is reasonable that CEX was efficiently absorbed from the small intestine. This observation is consistent

with the results of the BA-time profile that showed continuous absorption and high BA of CEX (85.2%) (Figure 5).

The fraction absorbed of SQV until it reached the upper jejunum and ileum was calculated based on the area under the luminal concentration-time curve of FD-4 ($\text{AUC}_{\text{GI}(\text{FD-4})}$) and SQV ($\text{AUC}_{\text{GI}(\text{SQV})}$) until 150 min according to our previous study (11). Since the difference between $\text{AUC}_{\text{GI}(\text{FD-4})}$ and $\text{AUC}_{\text{GI}(\text{SQV})}$ reflects to absorbed amount of SQV in these intestinal segments, the fraction absorbed of SQV (%) can be calculated as follows: Fraction absorbed of SQV (%) = $(1 - \text{AUC}_{\text{GI}(\text{SQV})} / \text{AUC}_{\text{GI}(\text{FD-4})}) \times 100$.

The estimation of the fraction absorbed revealed that 42.8% of administered SQV was absorbed until it reached upper jejunum, but only 20.1% of SQV was absorbed from the upper jejunum to ileum (The overall fraction absorbed of SQV was 62.9%). This is probably because that the absorption of SQV was impeded due to the efflux via P-gp in the distal region. The luminal concentration data was supported by the result of BA-time profile of SQV that showed rapid absorption of SQV at early time period (Figure 5). It has been reported that SQV is also a substrate for MRP2 that is expressed in the upper small intestine (2). However, since SQV was primarily absorbed from the upper region, contribution of MRP2 for SQV efflux may be low compared to P-gp.

The BA-time profile of MTX indicated that MTX was primarily absorbed in the upper region of the small intestine (Figure 5). Yokooji et al. have reported that MTX absorption in the proximal small intestine was higher than that found in the distal small intestine in rats (12). Their findings are consistent with our results. They also revealed that the site-specific absorption of MTX was primarily caused by the local expression of PCFT, an absorptive transporter for MTX, in the upper region of the small intestine (12), although other transporters (e.g., RFC1, MRP2, P-gp and BCRP) are also involved in MTX absorption. To clarify the actual contribution of each transporter to the absorption, further experiments using a specific transporter inhibitor or transporter-knockout animals are required.

The BA-time profile of MTX corresponded to the result of luminal concentration patterns of MTX (Figure 3). In the duodenum, the concentration of MTX exceeded the FD-4 concentration over the timeframe of 10-150 min (Figure 3). This was due to biliary secretion of MTX into the duodenal segment (29,30).

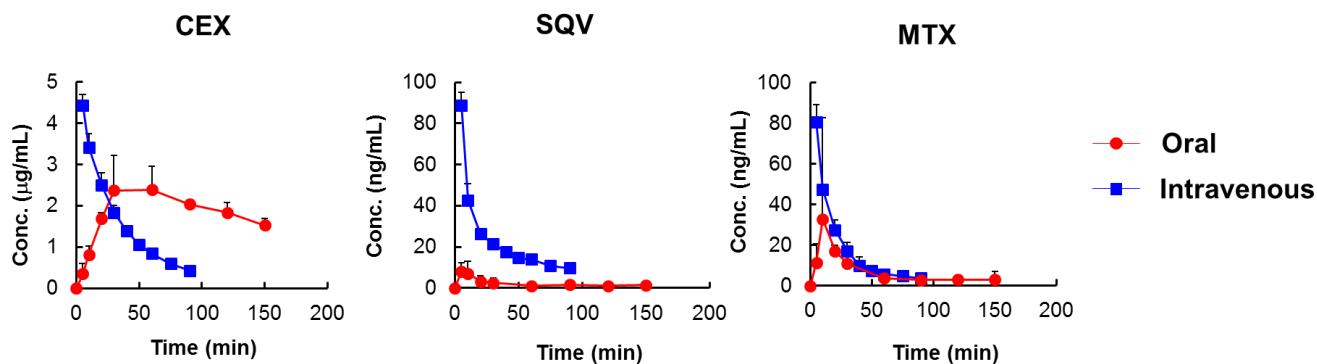


Figure 4. Time-courses of plasma concentration after oral and intravenous administration of various drugs. The doses of CEX, SQV, and MTX were 1.0, 0.2, and 0.025 mg/rat for oral administration and 0.5, 0.1, and 0.0125 mg/rat for intravenous administration, respectively. The data are expressed as the mean of each experiment with S.D. (n=3-4).

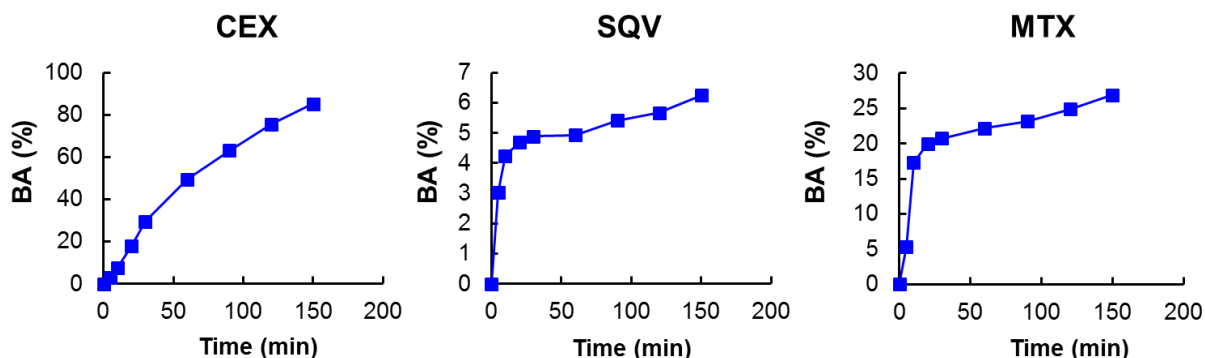


Figure 5. Time-course of BA after oral administration of various drugs, as calculated by the deconvolution method.

However, the MTX concentration lowered to the same extent as FD-4 concentration after 10 min in the upper jejunum. This indicated that the MTX was absorbed via PCFT and RFC1 (RFC1 is expressed in whole small intestine (19)) from the duodenum to upper jejunum. However, the difference in the concentration between FD-4 and MTX that reflects to fraction absorbed was almost unchanged from the upper jejunum to lower jejunum (i.e., MTX was almost not absorbed in these intestinal regions), probably due to efflux via P-gp and BCRP (BCRP is expressed in the whole small intestine (2)). Also, the concentration in the lower jejunum and ileum exceeded the dosing concentration, similar to FD-4, due to condensation by fluid absorption.

The guidelines for *in vitro* drug interaction studies from the FDA state that the investigated drug should be investigated to determine whether it is a substrate of P-gp and BCRP using *in vitro* transport experiments (31). For P-gp, since the main expression site is the lower region of the small

intestine, the drug concentration pattern in this region should be considered for accurate evaluation and prediction of P-gp-mediated drug interactions. The same is true for other transporters with specific expressions (e.g., PCFT). In addition, in the case of low-permeability drugs, attention should be paid to the condensation of drugs induced by fluid absorption.

CONCLUSIONS

The intraluminal behavior of drugs is often a black box. This study revealed the luminal concentration patterns of various transporter substrates after oral administration. We further examined the absorption behavior based on differences in the FD-4 and drug concentrations. The concentration-time profiles differed depending on the differences in the absorption properties at different sites for each drug and fluid volume in the individual GI segments. Since drug absorption pattern is determined by the

luminal drug concentration pattern, our findings are useful for setting the initial drug concentration for *in vitro* permeation studies to determine the actual effect of transporters on absorption and *in silico* predictions.

CONFLICT OF INTEREST. We have no financial or non-financial interests that represent conflicts of interest.

FUNDING. This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

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