Electronic Supplementary Material (ESI) for Lab on a Chip.

Electronic supplementary information

Kinetic analysis of sequential metabolism of triazolam and its extrapolation to humans using an entero-hepatic two-organ microphysiological system

Hiroshi Arakawa,[a] Shinji Sugiura,[b] Takumi Kawanishi,[a] Kazumi Shin,[b] Hiroko Toyoda,[b,c] Taku Satoh,[b,c] Yasuyuki Sakai,[d] Toshiyuki Kanamori,[b] and Yukio Kato[a]

a. Faculty of Pharmacy, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kanazawa, Japan

b. Biotechnology Research Institute for Drug Discovery, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan

c. Stem Cell Evaluation Technology Research Association, Tsukuba, Japan

d. Department of Chemical System Engineering, Graduate School of Engineering, The University of Tokyo.

Materials and Methods

Mass-balance equations and definition of parameters in mechanism-based pharmacokinetic models

Mass-balance equations according to pharmacokinetic models (Fig. 2 and S1) were shown as follows:

Two-organ MPS (Fig. 2A):

$$\frac{dC_1}{dt} = (-PS \times (C_1 - C_2) - CL_{ads3} \times C_1 + k_3 \times X_4)/V_a \quad (1)$$

$$\frac{dC_2}{dt} = \left(PS \times (C_1 - C_2) - (CL_{int,\alpha-OH} + CL_{int,4-OH} + CL_{ads3}) \times C_2 + k_3 \times X_5\right)/V_s \quad (2)$$

$$\frac{dC_3}{dt} = \left(CL_{int,\alpha-OH} \times C_2 - (PS_{\alpha-OH} + CL_{int,\alpha-OH-Glu}) \times C_3\right)/V_s \quad (3)$$

$$\frac{dC_4}{dt} = \left(CL_{int,4-OH} \times C_2 - (PS_{4-OH} + CL_{int,4-OH-Glu}) \times C_4\right)/V_s \quad (4)$$

$$\frac{dC_5}{dt} = \left(CL_{int,\alpha-OH-Glu} \times C_3 - PS_{\alpha-OH-Glu} \times (C_5 - C_9)\right)/V_s \quad (5)$$

$$\frac{dC_6}{dt} = \left(CL_{int,4-OH-Glu} \times C_4 - PS_{4-OH-Glu} \times (C_6 - C_{10})\right)/V_s \quad (6)$$

$$\frac{dC_7}{dt} = \left(PS_{\alpha-OH-glu} \times C_3\right)/V_a \quad (7)$$

$$\frac{dC_8}{dt} = \left(PS_{4-OH-glu} \times C_4\right)/V_a \quad (8)$$

$$\frac{dC_9}{dt} = (-PS_{\alpha-OH-Glu} \times (C_9 - C_5))/V_a \quad (9)$$

$$\frac{dC_{10}}{dt} = (-PS_{4-OH-Glu} \times (C_{10} - C_6))/V_a \quad (10)$$

$$\frac{dX_4}{dt} = CL_{ads3} \times C_1 - k_3 \times X_4 \quad (11)$$

$$\frac{dX_5}{dt} = CL_{ads3} \times C_2 - k_3 \times X_5 \quad (12)$$

Caco-2 single culture (Fig. 2B):

$$\frac{dC_1}{dt} = (-PS \times (C_1 - C_2) - CL_{ads1} \times C_1 + k_1 \times X_1)/V_a \quad (13)$$

$$\frac{dC_2}{dt} = (PS \times (C_1 - C_2) - CL_{ads1} \times C_2 + k_1 \times X_2)/V_s \quad (14)$$

$$\frac{dX_1}{dt} = CL_{ads1} \times C_1 - k_1 \times X_1 \quad (15)$$

$$\frac{dX_2}{dt} = CL_{ads1} \times C_2 - k_1 \times X_2 \quad (16)$$

HepaRG single culture (Fig. 2C):

$$\frac{dC_2}{dt} = \left(-(CL_{int,\alpha-OH} + CL_{int,4-OH} + CL_{ads2}) \times C_2 + k_2 \times X_3\right)/V_s \quad (17)$$

$$\frac{dC_3}{dt} = \left(CL_{int,\alpha-OH} \times C_2 - CL_{int,\alpha-OH-Glu} \times C_3\right)/V_s \quad (18)$$

$$\frac{dC_4}{dt} = \left(CL_{int,4-OH} \times C_2 - CL_{int,4-OH-Glu} \times C_4\right)/V_s \quad (19)$$

$$\frac{dC_5}{dt} = \left(CL_{int,\alpha-OH-Glu} \times C_3\right)/V_s \quad (20)$$

$$\frac{dC_6}{dt} = \left(CL_{int,4-OH-Glu} \times C_4\right)/V_s \quad (21)$$

$$\frac{dX_3}{dt} = CL_{ads2} \times C_2 - k_2 \times X_3 \quad (22)$$

Two-organ MPS with medium circulation (Fig. S1):

$$\frac{dC_1}{dt} = (-PS \times (C_1 - C_{11}) - CL_{ads3} \times C_1 + k_3 \times X_4)/V_a \quad (23)$$

$$\frac{dC_2}{dt} = \left(-Q \times (C_2 - C_{11}) - (CL_{int,\alpha-OH} + CL_{int,4-OH}) \times C_2\right)/V_{s/2} \quad (24)$$

$$\frac{dC_3}{dt} = \left(CL_{int,\alpha-OH} \times C_2 - CL_{int,\alpha-OH-Glu} \times C_3 - Q \times (C_3 - C_{12})\right)/V_{s/2} \quad (25)$$

$$\frac{dC_4}{dt} = \left(CL_{int,4-OH} \times C_2 - CL_{int,4-OH-Glu} \times C_4 - Q \times (C_4 - C_{13})\right)/V_{s/2} \quad (26)$$

$$\frac{dC_5}{dt} = \left(CL_{int,\alpha-OH-Glu} \times C_3 - Q \times (C_5 - C_{14})\right)/V_{s/2} \quad (27)$$

$$\frac{dC_6}{dt} = \left(CL_{int,4-OH-Glu} \times C_4 - Q \times (C_6 - C_{15})\right)/V_{s/2} \quad (28)$$

$$\frac{dC_7}{dt} = (PS_{\alpha-OH} \times C_{12})/V_a \quad (29)$$

$$\frac{dC_8}{dt} = (PS_{4-OH} \times C_{13})/V_a \quad (30)$$

$$\frac{dC_9}{dt} = (-\mathrm{PS}_{\alpha-\mathrm{OH-Glu}} \times (C_9 - C_{14}))/V_a \quad (31)$$

$$\frac{dC_{10}}{dt} = (-\mathrm{PS}_{4-\mathrm{OH-Glu}} \times (C_{10} - C_{15}))/V_a \quad (32)$$

$$\frac{dC_{11}}{dt} = (-\mathrm{PS} \times (C_{11} - C_1) - Q \times (C_{11} - C_2) - \mathrm{CL}_{ads3} \times C_{11} + k_3 \times X_5)/V_{s/2} \quad (33)$$

$$\frac{dC_{12}}{dt} = (Q \times (C_3 - C_{12}) - \mathrm{PS}_{\alpha-\mathrm{OH}} \times C_{12})/V_{s/2} \quad (34)$$

$$\frac{dC_{13}}{dt} = (Q \times (C_4 - C_{13}) - \mathrm{PS}_{4-\mathrm{OH}} \times C_{13})/V_{s/2} \quad (35)$$

$$\frac{dC_{14}}{dt} = (Q \times (C_5 - C_{14}) - \mathrm{PS}_{\alpha-\mathrm{OH-Glu}} \times (C_{14} - C_9))/V_{s/2} \quad (36)$$

$$\frac{dC_{15}}{dt} = (Q \times (C_6 - C_{15}) - \mathrm{PS}_{4-\mathrm{OH-Glu}} \times (C_{10} - C_6))/V_{s/2} \quad (37)$$

$$\frac{dX_4}{dt} = \mathrm{CL}_{ads3} \times C_1 - k_3 \times X_4 \quad (38)$$

$$\frac{dX_5}{dt} = \mathrm{CL}_{ads3} \times C_{11} - k_3 \times X_5 \quad (39)$$

where C_1 to C_{15} are the concentrations of TRZ or its metabolites in the respective compartment (see Fig. 2 for details), X_1 to X_5 are the amounts of TRZ adsorbed to culture apparatus (see Fig. 2 for details), V_a and V_s are distribution volumes of the apical and systemic compartments, respectively, CL_{ads} is the adsorption clearance in the two-organ MPS, k is the desorption rate constant from culture apparatus, PS is intrinsic clearance of permeability through intestinal membranes, CL_{int} is the hepatic intrinsic clearance of metabolism, Q is flow rate of medium circulation and was set to be the average of estimated values (34 mL/h). Note that Vs was divided into two compartments with a distribution volume ($V_{s/2}$) half of V_s in pharmacokinetic model with medium circulation.

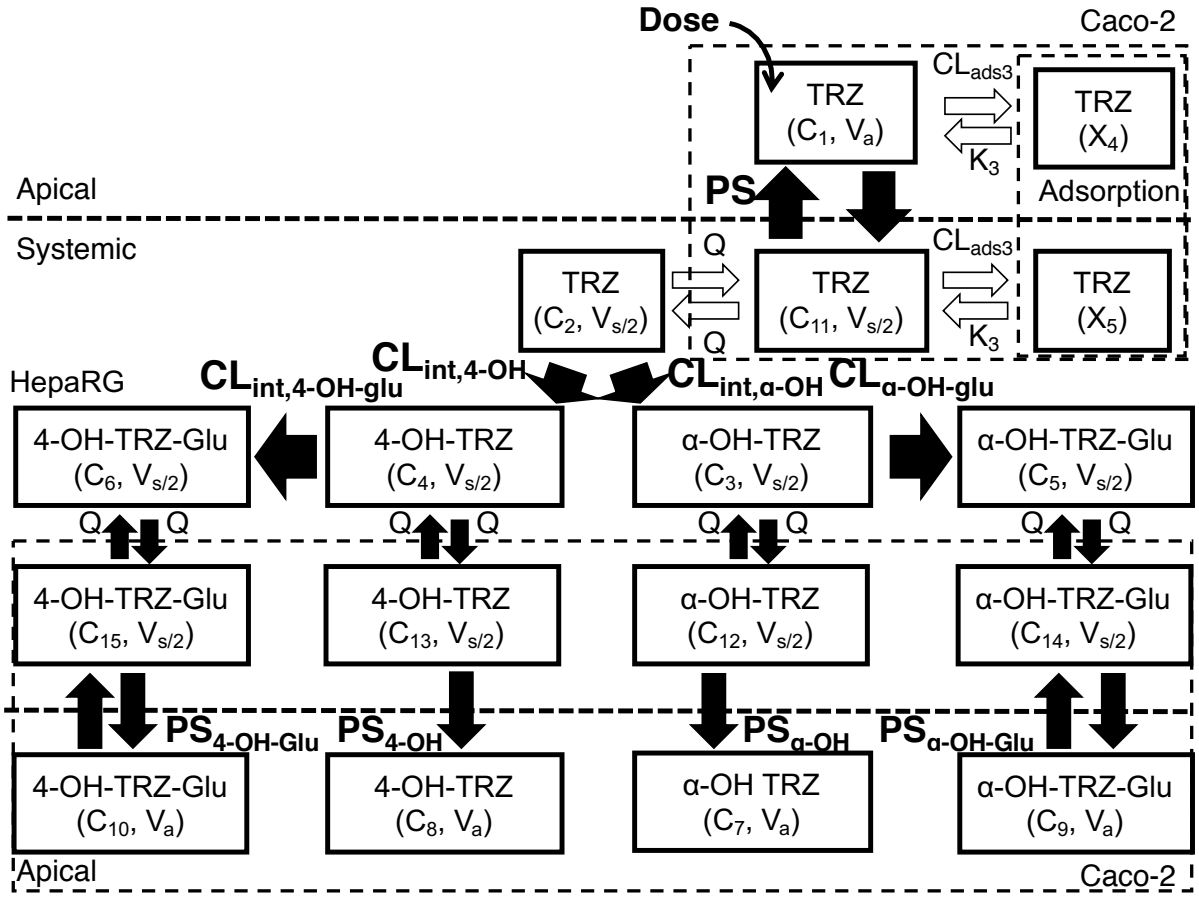

Figure S1. Structure of pharmacokinetic model incorporating circulation flow for two-organ MPS.

The Q represents flow rate of medium circulation. C_1-C_{15} represent concentration of TRZ or its metabolites, whereas X_1-X_5 represent amount of adsorption of TRZ in each compartment. PS, intrinsic clearance by passive diffusion across plasma membranes; CL_{int}, intrinsic clearance of production of metabolites; V_a and V_s, distribution volume; CL_{ads}, intrinsic clearance of adsorption; k, desorption rate constant.

Extrapolation to plasma concentration-time profile of TRZ, and α- and 4-OH-TRZ in humans

First, mass-balance equations for the extrapolation were constructed as follows: These equations were principally derived from the pharmacokinetic model for two-organ MPS (Fig. 2A) except that (i) several scaling factors (SFs) were also included, (ii) plasma protein binding of TRZ was also considered, (iii) adsorption compartment was not included, (iv) intestinal secretion of OH-TRZ was not included, because TRZ was excreted into urine as a form of glucuronides[1].

$$\frac{dC_1}{dt} = \left(-PS \times SF_{PS} \times SF_{intestine} \times \left(C_1 - C_2 \times f_{p,TRZ}\right)\right)/(V_a \times SF_{Va}) \quad (40)$$

$$\frac{dC_2}{dt} = \left(PS \times SF_{PS} \times SF_{intestine} \times (C_1 - C_2 \times f_{p,TRZ}) - (CL_{int,\alpha-OH} \times SF_{\alpha-OH} \times SF_{hepatocyte} + CL_{int,4-OH} \times SF_{4-OH} \times SF_{hepatocyte}) \times C_2 \times f_{p,TRZ}\right)/(V_s \times SF_{Vs}) \quad (41)$$

$$\frac{dC_3}{dt} = \left(CL_{int,\alpha-OH} \times SF_{\alpha-OH} \times SF_{hepatocyte} \times C_2 \times f_{p,TRZ} - (PS_{\alpha-OH-glu} + CL_{int,\alpha-OH-Glu} \times SF_{\alpha-OH-Glu} \times SF_{hepatocyte}) \times C_3\right)/(V_s \times SF_{Vs}) \quad (42)$$

$$\frac{dC_4}{dt} = \left(CL_{int,4-OH} \times SF_{4-OH} \times SF_{hepatocyte} \times C_2 \times f_{p,TRZ} - (PS_{4-OH-Glu} + CL_{int,4-OH-Glu} \times SF_{4-OH-Glu} \times SF_{hepatocyte}) \times C_4\right)/(V_s \times SF_{Vs}) \quad (43)$$

$$\frac{dC_5}{dt} = \left(CL_{int,\alpha-OH-Glu} \times SF_{\alpha-OH-Glu} \times SF_{hepatocyte} \times C_3 - PS_{\alpha-OH-glu} \times (C_5 - C_9)\right)/(V_s \times SF_{Vs}) \quad (44)$$

$$\frac{dC_6}{dt} = \left(CL_{int,4-OH-Glu} \times SF_{4-OH-Glu} \times SF_{hepatocyte} \times C_4 - PS_{4-OH-Glu} \times (C_6 - C_{10})\right)/(V_s \times SF_{Vs}) \quad (45)$$

$$\frac{dC_7}{dt} = (-PS_{\alpha-OH} \times C_3)/V_a \quad (46)$$

$$\frac{dC_8}{dt} = (-PS_{4-OH} \times C_4)/(V_a \times SF_{Va}) \quad (47)$$

$$\frac{dC_9}{dt} = \left(-PS_{\alpha-OH-glu} \times (C_9 - C_5)\right)/(V_a \times SF_{Va}) \quad (48)$$

$$\frac{dC_{10}}{dt} = \left(-PS_{4-OH-glu} \times (C_{10} - C_6)\right)/(V_a \times SF_{Va}) \quad (49)$$

where $f_{p,TRZ}$ is plasma unbound fraction for TRZ, $SF_{intestine}$ is SF for intestinal surface area, $SF_{hepatocyte}$ is SF for numbers of hepatocytes, SF_{Va} is SF for volume of intestinal fluid, and SF_{Vs} is SF for the volume of systemic compartment. The $f_{p,TRZ}$ was set to be 0.1 according to the literature[2]. The plasma unbound fraction of the TRZ metabolites was assumed to be unity, as no information in literature was available. The values of physiological SFs are shown in Table S1. $SF_{intestine}$ was calculated as the ratio of surface area of human small intestine (2.0×10^6 cm^2)[3] to that of Caco-2 cell chamber used in the present study (0.33 cm^2). $SF_{hepatocyte}$ was calculated as the ratio of the number of hepatocytes in human liver (1.2×10^8 cells/g liver)[4] to that of HepaRG cells in the present study (6.4×10^4 cells), assuming 1,800 g liver for a human with a body weight of 70 kg[5]. SF_{Va} was calculated as the ratio of the volume of a cup of water (250 mL) to that of medium of apical side in the two-organ MPS (0.2 mL). SF_{Vs} was calculated by dividing the sum of volume of human plasma (3,000 mL)[5] and liver (1,690 mL)[5] by that of medium on

the systemic side, in the two-organ MPS (0.6 mL). On the other hand, empirical SFs were also defined and were included in the above mass-balance equations, where SF_{PS}, $SF_{\alpha\text{-OH}}$, $SF_{4\text{-OH}}$, $SF_{\alpha\text{-OH-Glu}}$, and $SF_{4\text{-OH-Glu}}$ were the ratio of PS, $CL_{int,\alpha\text{-OH}}$, $CL_{int,4\text{-OH}}$, $CL_{int,\alpha\text{-OH-Glu}}$, and $CL_{int,4\text{-OH-Glu}}$ values in humans *in vivo*, to those in the two-organ MPS. These SFs values were estimated by fitting of the literature information on plasma concentration-time profile of TRZ, and α- and 4-OH-TRZ in human volunteers[6] to Eqs. (40) – (49).

Table S1 Physiological SFs

SFs	Values[a]
$SF_{intestine}$	6.06×10^6
$SF_{hepatocyte}$	3.38×10^6
SF_{Va}	1250
SF_{Vs}	7.82×10^3

[a]These values were calculated based on literature information according to Materials and Methods in ESI.

Cell culture

Human colon carcinoma Caco-2 cells were obtained from the RIKEN Cell Bank, and maintained in E-MEM with L-glutamine and phenol red (FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan) with 20% FBS, 1% non-essential amino acids, and 1% penicillin-streptomycin mixed solution (Nakalai Tesque, Kyoto, Japan). Human hepatoma HepaRG cells were obtained from KAC (Kyoto, Japan) as pre-differentiated cryopreserved vials and cultured in Medium 640, Medium 670, and MIL502 obtained from KAC.

Medium change in the two-organ MPS and single cultures for analysis of concentration-time profiles

Medium change for the analysis of concentration-time profiles of TRZ and its metabolites on Day 0 was carried out as follows: In the two-organ culture, the circulation channels, whose volume was 50 μL in total, were first filled with the test medium without TRZ before the start of circulation. The medium on the systemic side of the Caco-2 chamber was removed, with 50 μL of medium remaining in the culture well. The medium on the apical side of the Caco-2 chamber was removed, and 205 μL of test medium containing 20 μM TRZ was added to the apical side of the Caco-2 chamber. The medium in the HepaRG chamber was removed, with 50 μL medium remaining in the culture well, and 455 μL of test medium without TRZ was added to the HepaRG chamber. In total, 605 μL of the test medium without TRZ remained on the systemic side, in the two-organ culture.

In the Caco-2 single culture, the medium on the systemic side of the Caco-2 chamber was removed, with 50 μL medium remaining in the culture well, and 455 μL of test medium without TRZ was added to the systemic side of the Caco-2 chamber. In total, 505 μL of test medium without TRZ

remained on the systemic side in the Caco-2 chamber. The medium on the apical side of the Caco-2 chamber was removed, and 205 μL of test medium containing 17.5 μM TRZ was added to the apical side of the Caco-2 chamber.

In the HepaRG single culture, the medium on the HepaRG chamber was removed with 50 μL medium remaining in the culture well, and 255 μL of 5.98 μM test medium was added to the HepaRG chamber. In total, 305 μL of test medium containing 5.0 μM TRZ remained in the HepaRG chamber.

Adsorption of TRZ in cell-free culture device

Adsorption of TRZ in cell-free culture device (cell-free system) was evaluated in the two-organ MPS without cells, Caco-2 single culture without cells, and HepaRG single culture without cells. The medium in the cell-free system was replaced with the test medium containing TRZ, in the same manner as the analysis of the concentration-time profile of TRZ in each culture condition with cells, on Day 0. Immediately after replacing the medium, 5 μL of the medium was collected (t = 0), the culture devices were placed in the incubator with 5% CO_2 atmosphere at 37 °C, and the medium was circulated in the cell-free system. The medium in the cell-free system was collected in the same manner as the analysis of concentration-time profile of TRZ in each culture condition with cells, at 2, 4, 24, 29, and 48 h after the replacement of the medium. The collected medium was stored at -20 °C until the liquid chromatography-mass spectrometry (LC-MS/MS) analysis.

Linearity in the metabolism of TRZ in HepaRG cells

The linearity of the time course and concentration dependence of the metabolism of TRZ and its hydroxy metabolites, in HepaRG cells was assessed as follows. HepaRG cells were inoculated into the collagen coated 96-well plate (Corning Inc., Corning, NY) at 7.2×10^4 cells/well in 100 μL of Medium 670, and cultured for 3 days. HepaRG cells were further cultured in induction medium, Medium 640, for 2 days with the medium changed after 1 day. For the time course experiment, the test medium containing 1 μM of TRZ, α-OH-TRZ, and 4-OH-TRZ were prepared by mixing each stock solution with MIL502 to give a final volume and DMSO concentration of 100 μL and 0.2%, respectively. The medium was removed at 15, 30, 60, and 120 min after the start of incubation under 5% CO_2 atmosphere at 37 °C. For the concentration dependence experiment, the test medium containing 1, 2, 5, and 10 μM of TRZ, α-OH, and 4-OH were prepared by mixing each stock solution with MIL502 to give a final volume and DMSO concentration of 100 μL and 0.2%, respectively. The medium was removed at 60 min after start of the incubation under 5% CO_2 atmosphere at 37 °C. All the medium samples were stored at -30 °C until LC-MS/MS analysis.

Sample treatment for LC-MS/MS analysis

Medium sample (10 μL) was mixed with 30 μL of water containing an internal standard

(midazolam) and 110 μL of methanol, followed by centrifugation at 21,500 g for 10 min at 4°C. The supernatants were subjected to LC-MS/MS analysis. To perform the deglucuronidation assay, 40 μL of medium samples reacted with β-glucuronidase were mixed with 110 μL of methanol, followed by centrifugation at 21,500 g for 10 min at 4°C. The supernatants were subjected to LC-MS/MS analysis.

Gene expression profiling

RNA was extracted by using a RNeasy mini Kit (QIAGEN, Valencia, CA) from HepaRG cells at 48 h after the start of medium circulation with Caco-2 cells, in the two-organ MPS and HepaRG single culture. Reverse transcription was then performed to synthesize cDNA by using a QuantiTect Reverse Transcription Kit (QIAGEN). Quantitative real-time polymerase chain reaction (qPCR) analysis was carried out by using TaqMan probes and primer sets from TaqMan gene expression assays (Table S2, Applied Biosystems, Foster City, CA), Light Cycler 480 Probes master and Light Cycler 480 (Roche Applied Science, Penzberg, Germany). Expression level of mRNA was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The relative expression level in two-organ MPS, to HepaRG single culture, was evaluated by the second derivative maximum and standard curve method. qPCR was carried out for four samples in each culture condition, and the mean value and standard deviation was calculated. Statistical comparisons were performed by two-way ANOVA, followed by the Bonferroni multiple comparisons using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). *P* values less than 0.05 were considered as statistically significant.

Table S2 List of TaqMan probe sets used in the qPCR

Gene	Catalog No.
CYP3A4	Hs00604506_m1
CYP 3A5	Hs02511768_s1
UGT1A4	Hs01655285_s1
UGT2B4	Hs02383831_s1
UGT2B7	Hs00426592_m1
GAPDH	Hs02786624_g1

Results

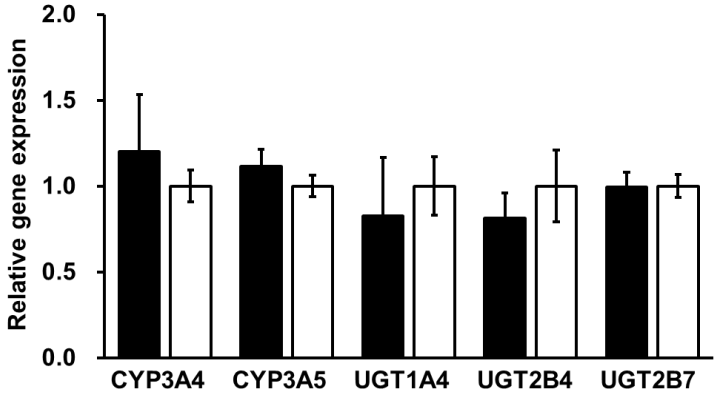

Figure S2. Gene expression of metabolic enzymes in HepaRG cells.

Expression of mRNA for each metabolic enzyme in HepaRG cells in two-organ MPS (closed bars) and single culture (open bars) was measured by qPCR. Total RNA was extracted at day 0 shown in Fig. 1 and S1. The expression level was normalized to GAPDH and calculated relative to that in the HepaRG single culture. Data are shown as mean ± S.D., (n=4). No significant difference was detected between two-organ MPS and HepaRG single culture in the expression of analyzed genes.

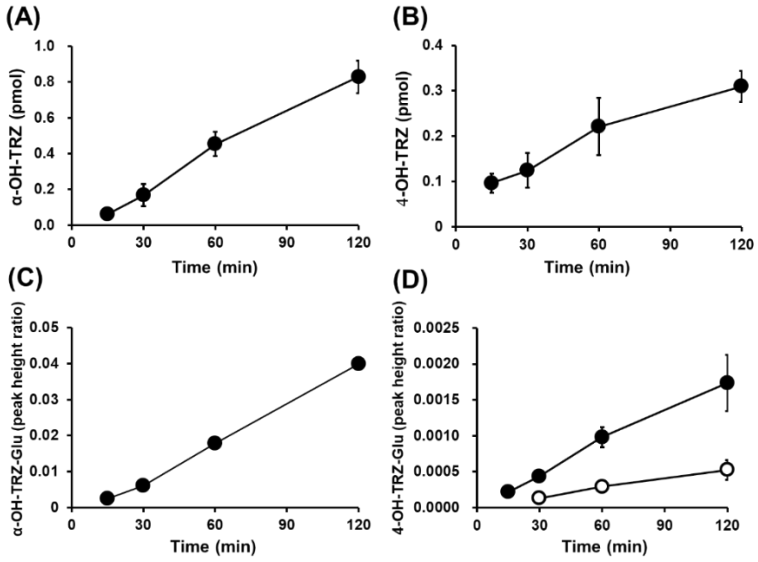

Figure S3. Time-dependent formation of TRZ metabolites in HepaRG cells.

Formation of α-OH-TRZ (A) and 4-OH-TRZ (B) in HepaRG cells was measured at 15, 30, 60, and 120 min after adding 1 μM TRZ, whereas that of α-OH-TRZ-Glu (C) and 4-OH-TRZ-Glu (D) in HepaRG cells was measured at 15, 30, 60, and 120 min after adding 1 μM α-OH-TRZ and 4-OH-TRZ, respectively. Amount of α-OH-TRZ-Glu and 4-OH-TRZ-Glu was measured as the ratio of their peak height to that of internal standard (IS). Closed and open symbols represent 4-OH-TRZ-Glu observed at retention time of 4.61 and 4.96 min, respectively. Data are shown as mean ± S.D., (n=4).

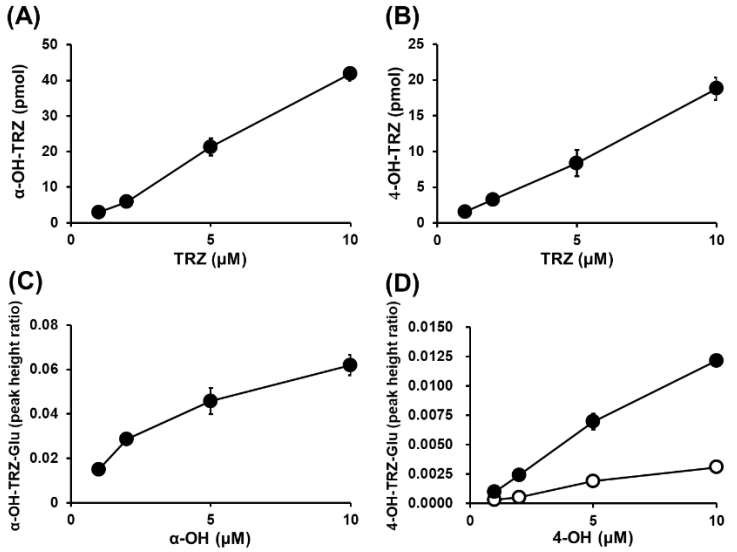

Figure S4. Concentration-dependent formation of TRZ metabolites in HepaRG cells.

Production of α-OH-TRZ (A) and 4-OH-TRZ (B) in HepaRG cells was measured at 60 min after adding 1, 2, 5, and 10 μM TRZ, whereas that of α-OH-TRZ-Glu (C) and 4-OH-TRZ-Glu (D) in HepaRG cells was measured at 60 min after adding 1, 2, 5, and 10 μM α-OH-TRZ and 4-OH-TRZ, respectively. Amount of α-OH-TRZ-Glu and 4-OH-TRZ-Glu was measured as the ratio of their peak height to that of internal standard (IS). Closed and open symbols represent 4-OH-TRZ-Glu observed at retention time of 4.61 and 4.96 min, respectively. Data are shown as mean ± S.D., (n=4).

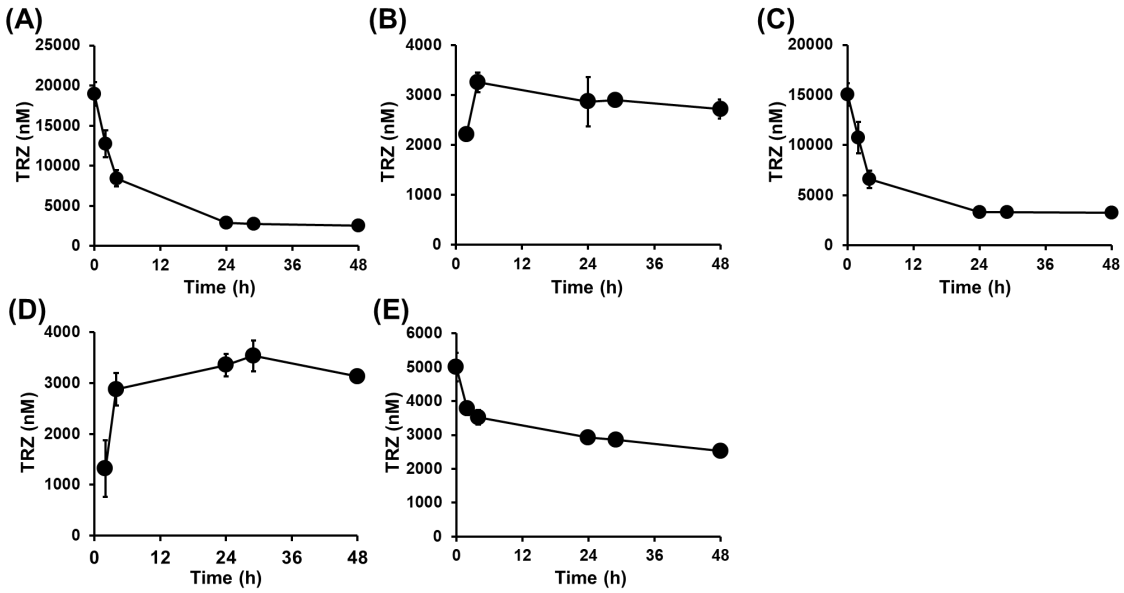

Figure S5. Adsorption of TRZ in cell-free system

Concentration-time profile of TRZ was measured after replacing the medium with that containing TRZ on apical (A) and systemic (B) side of the cell-free system corresponding to two-organ culture, apical (C) and systemic (D) side of cell-free system corresponding to Caco-2 single culture, and medium of cell-free system corresponding to HepaRG single culture (E). Each symbol indicates mean ± S.D., (n=4).

Table S3 Parameters in the mechanism-based pharmacokinetic model with or without medium circulation

Parameters	Medium circulation	
	(+)	(-)[c]
Dose (nmol)	4	4
$CL_{int,a\text{-}OH}$ (μL/h)[a]	1.43 ± 0.92	1.43 ± 0.89
$CL_{int,4\text{-}OH}$ (μL/h)[a]	0.208 ± 0.315	0.207 ± 0.306
$CL_{int,a\text{-}OH\text{-}Glu}$ (μL/h)[a]	131 ± 181	131 ± 176
$CL_{int,4\text{-}OH\text{-}Glu}$ (μL/h)[a]	66.1 ± 203.6	65.9 ± 198.0
V_a (mL)[b]	0.2	0.2
V_s (mL)[b]	0.6	0.6[e]
PS (μL/h)	27.7[d]	27.7[d]
$PS_{a\text{-}OH}$ (μL/h)[a]	4.56 ± 9.29	4.55 ± 9.05
$PS_{4\text{-}OH}$ (μL/h)[a]	10.9 ± 35.5	11.0 ± 34.6
$PS_{a\text{-}OH\text{-}Glu}$ (μL/h)[a]	19.2 ± 89.6	19.2 ± 87.1
$PS_{4\text{-}OH\text{-}Glu}$ (μL/h)[a]	2.09×10^{8} ± 3.01×10^{13}	348 ± 2158
CL_{ads1} (μL/h)[a]	–	–
CL_{ads2} (μL/h)[a]	–	–
CL_{ads3} (μL/h)[a]	16.4 ± 7.0	16.4 ± 6.8
k_1 (/h)[a]	–	–
k_2 (/h)[a]	–	–
k_3 (/h)[a]	0.0626 ± 0.0349	0.0626 ± 0.0340

[a]Obtained by fitting and shown as parameter estimate ± parameter S.D.

[b]Fixed to the volume of the medium.

[c]Parameters estimated were also shown in Table 4.

[d]Fixed to the value obtained in Caco-2 single culture.

[e]V_s was calculated as $2 \times V_{s/2}$.

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