Intestinal First-Pass Metabolism of Eperisone in the Rat

Kiyoshi Mihara,[1,3] Mina Matsumura,[1] Eiji Yoshioka,[1] Kazuhiko Hanada,[1] Hiromitsu Nakasa,[2] Shigeru Ohmori,[2] Mitsukazu Kitada,[2] and Hiroyasu Ogata[1]

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Purpose. The purpose of this study was to clarify quantitatively the contribution of the intestine to the first-pass metabolism of eperisone in rats.

Methods. The systemic availabilities of eperisone were estimated by administering the drug into the duodenum, portal vein, and femoral vein in rats *in vivo*. The first-pass metabolism of eperisone was confirmed in the perfused rat small intestine *in situ*. Metabolism of eperisone to an ω-1-hydroxylated metabolite (HMO), the first step of eperisone metabolism, was studied using rat intestinal microsomes *in vitro*.

Results. The bioavailabilities in the intestine were 0.176 and 0.0879 at administration rates of 100 and 25 mg/h/kg, respectively, whereas those in the liver were 0.532 and 0.486, respectively. In the intestinal perfusion experiment, the appearance clearance to the portal vein from the intestinal lumen was much lower than the elimination clearance from the intestinal lumen, resulting in high metabolic clearance of eperisone in the small intestine. Eperisone was biotransformed to HMO by rat intestinal microsomes, and this was inhibited by α-naphthoflavone and an anti-rat CYP1A antibody.

Conclusions. Those data strongly suggest that eperisone may be metabolized to HMO by CYP1A in rat intestinal microsomes during the first-pass through the epithelium of the small intestine.

KEY WORDS: bioavailability; metabolism; intestine; eperisone; microsomes; rat.

INTRODUCTION

In general, drugs administered orally are absorbed from the gastrointestinal (GI) tract through the epithelium, appear in the portal vein, and then enter the circulation after first passing through the liver. Although the first-pass effect in the liver after oral administration has been well studied by many investigators, there have been few direct demonstrations of the GI contribution to the first-pass effect because of the difficulty in estimating it. Especially, *in vivo* experiments cannot distinguish easily between the extent of the absorption and the avoidance of first-pass disposition in GI tissues (1–3).

Various techniques for isolation of hepatic cytochrome P450 (CYP) of many species have already been established, and many CYPs have been identified as the main enzymes associated with the metabolism of some drugs. On the other hand, there have been no efficient techniques for isolation of intestinal CYPs, except for those of humans (4), because of their very low activity (5–9). This may account for the paucity of information on drug metabolism by intestinal CYPs, in comparison with phase two reactions in the GI, such as sulfate conjugation, glucuronidation and *N*-acetylation, which have been well described (10). Recently, the metabolism of cyclosporine, protease inhibitors and midazolam by intestinal CYP3A4 was reported (1,11,12). The role of intestinal CYP in carcinogenesis has also been reviewed, the enterocytes at the tips of intestinal villi showing sufficiently high CYP activities, suggesting the importance of drug transformation in the intestine (13). Therefore, it seems important to identify the major CYPs associated with intestinal drug metabolism to predict the oral bioavailability and carcinogenicity of clinically used drugs.

Eperisone hydrochloride (4′-ethyl-2-methyl-3-piperidinopropiophenone hydrochloride; EMPP) is a synthesized antispastic agent (14) that has been shown to have very low bioavailability after oral administration in rats (15) and extensively high oral clearance in humans. These data suggest extensive first-pass metabolism of EMPP after oral administration in rats and humans because absorption of the drug is very high (15).

In the present study, we determined the bioavailability of EMPP after oral administration and estimated quantitatively the contribution of intestinal metabolism to the first-pass disposition of EMPP in rats *in vivo* and also in the perfused rat intestine. In addition, as EMPP was expected to be biotransformed mainly by CYPs (16) in rat microsomes, we defined the main subfamily of CYP associated with biotransformation to an ω-1-hydroxylated metabolite (HMO), first step of EMPP metabolism (16), in an *in vitro* study.

MATERIALS AND METHODS

Materials

EMPP and its ω-1 hydroxylated metabolite, 1-[4′-(1-hydroxyethyl)phenyl]-2-methyl-3-(1-piperidinyl)-1-oxopropane (HMO), were kindly supplied by Eisai Co. (Tokyo, Japan). Cinnarizine, as an internal standard substance for high-performance liquid chromatography (HPLC), was also a gift from Eisai Co. (Tokyo, Japan). AEC Chromogen Kit was purchased from Sigma Chemical Co. (St. Louis, MO). Vectastain® ABC Kit was from Vector Loboratorys Inc. (Burlingame, CA). Anti-Rat CYP1A1, 2B1, 3A2, and 4A1 goat antibodis were obtained from Daiichi Pure Chemicals, Tokyo, Japan. All other chemicals were reagent grade products obtained commercially.

Instrumentation

For measurement of EMPP concentration in plasma, a HPLC system (Shimadzu, Kyoto, Japan) consisted of a LC-9A pump, a SPD-9A variable wavelength UV detector, and C-R6A integrator was used. We used a Nucleosil-7C8 reversed-phase column (4.6 × 150 mm; Macherey-Nagel, Düren, Germany) and a Bensill-5C8 reversed-phase column (4.6 × 150 mm; Bentech Ltd., Chiba, Japan) for measurement of

[1] Department of Biopharmaceutics, Meiji Pharmaceutical University, 2-522-1 Noshio, Kiyose, Tokyo 204-8588, Japan.

[2] Division of Pharmacy, Chiba University Hospital, Faculty of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-0856, Japan.

[3] To whom correspondence should be addressed. (e-mail: miharak@my-pharm.ac.jp)

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EMPP and HMO, respectively. The absorbance was monitored at 260 nm for EMPP and 254 nm for HMO.

Animals

Male Wistar rats were purchased from a commercial source and housed in a light- and humidity-controlled animal facility. All the procedures involving animals were consistent with the guidelines (NIH publication #85–23, revised 1985) and approved by the animal ethics committee at Meiji Pharmaceutical University.

In Vivo Experiments

The drug was administered via three different routes, i.e., infusion into the femoral vein (i.v.), into the portal vein (p.v.), and into the duodenum in place of per-oral (p.o.) administration. Male Wistar rats (230–250 g) maintained on standard food were fasted 18 h before the performance of the experiment. The rat was anesthetized with pentobarbital (50 mg/kg, i.p.) and abdominal incision was performed to expose the distal ileum. A heparinized polyethylene tube (PE-10), which was used for the intra-p.v. drug administration, was inserted into the mesenteric vein. Then, the femoral vein was cannulated with a PE-10, which was used for the i.v. drug administration. The proximal duodenum at 2 cm from pylorus was also cannulated with a PE-10 for the intra-duodenum drug administration (p.o. administration). Three cannulations were carried out in all rats for drug administration or the shame operation.

EMPP solution (50 mg/mL, 1.0 mL/kg) was infused for 30 or 120 min through the femoral cannula in the i.v. administration and was infused for 2, 30, or 120 min through the mesenteric cannula in the intra-portal vein administration. In both cases, about 200 μL of the blood samples were obtained from jugular vein at 5, 15, 30, 60, 90, 120, and 180 min. In the intra-duodenum administration, the solution was injected bolusly or infused for 15, 30, or 120 min through the duodenum cannula and about 200 μL of the blood samples were obtained from jugular vein at 15, 30, 60, 90, 120, 180, and 240 min. All blood samples were heparinized and were immediately centrifuged for 2 min (2,500 × g). The supernatant plasma samples were then frozen at –20°C in other tubes before measurement.

In Situ Perfusion into Rat Small Intestinal Tract

Male Wistar rats (200 g) fed commercial chow were fasted 18 h before the performance of the experiment. Intestinal lumen and mesenteric vein were perfused at the same time with saline and heparinized rat blood, respectively, but EMPP was perfused only into the intestinal lumen. The perfused small intestine was prepared according to the method reported previously (17). Briefly, after anesthetizing the rat with pentobarbital (50 mg/kg, i.p.), the abdominal organs were exposed by incision. A catheter (PE-10) filled with heparinized (800 unit/mL) rat blood was inserted into the artery. The portal vein was catheterized with a heparinized PE-10. Then, the blood perfusion was started with a flow rate of 0.15 mL/min at 37°C. Immediately, the jejunum was cannulated with polyethylene tubes (i.d., 2 mm; o.d., 4 mm) near the duodenum and the jejunum at the 20 cm from the prior cannula to construct an intestinal loop. Saline containing EMPP (20 μg/mL) was perfused (1.0 mL/min) in the loop at 37°C and then venous blood sampling was started. Because about 100 μg of EMPP would be infused into the rat duodenum for a minute in the *in vivo* experiment (25 mg/h/kg), we assumed the intestinal concentration of EMPP to be around 20 μg/mL. Blood was collected continuously for every 10 min for 60 min. The luminal perfusate passing through the intestinal loop was obtained at 5, 15, 25, 35, 45, and 55 min for 1 min. The blood collected was weighed and the volume was estimated from the weight assuming the density to be 1.0. The blood was hemolyzed by the same volume of ethanol. The perfusate sampled was centrifuged for 2 min (2,500 × g). The hemolyzed blood and the supernatant perfusate samples were then stocked at –20°C before measurement.

In Vitro Experiment Using Intestinal Microsomes

Preparation of Intestinal and Hepatic Microsomes

Each 30 cm of upper jejunum was removed from two male Wistar rats (~9–11 weeks) and immediately placed into cold saline at 4°C. After washing inside, these were cut open lengthwise. The mucosa was obtained by slicing off with the edge of a slide glass and suspended in 50 mM cold phosphate buffer (pH 7.4). The suspension was sonicated for 5 s in an ice-cold bath, and this process was repeated three times. The solution was homogenized immediately after 600 μL of phenylmethylsulfonyl fluoride (PMSF) solution (1 mg of PMSF dissolved in 10 ml of acetone) was added. The suspension was centrifuged (10,000 × g) for 30 min at 4°C and the supernatant was centrifuged (105,000 × g) for 60 min at 4°C. The microsomal pellet was resuspended in 1.2 mL of cold 50 mM phosphate buffer (pH 7.4) and was used immediately. Hepatic microsomes were prepared according to the method previously reported (18).

The content of CYP was determined by the reported method (19). Protein concentration was measured according to the previous method (20). The assay of NADPH cytochrome P450 reductase activity was carried out using the reported method (21). Enzymatic activity of CYP in the microsomes was estimated by measuring initial velocity of aminopyrine *N*-demethylation (22), 4-nitroanisole *O*-deethylation (23), and 7-ethoxycoumarine *O*-deethylation (24).

Immunoblot Analysis

Immunoblot of the liver and intestinal microsomes was performed as described previously (25). In brief, microsome proteins (50–100 μg) were separated by electrophoresis in 10% sodium dodecyl sulfate-polyacrylanlide gels. Separated proteins were electrophoretically transferred to polyvinylidene fluoride sheets and the remaining protein binding sites were blocked. The sheets were incubated with anti-CYP antibodies and stood for 2.0 h at room temperature. Then, the sheets were incubated with secondary antibody, biotin-conjugated rabbit affinity purified antibody to goat IgG (Organon Teknika Corporation, Durham, NC) for 45 min. The immunoreactive proteins were visualized by avidin-biotin-peroxidase complex method (Vectastain® ABC Kit) followed by addition of the enzyme substrate, 3-amino-9-ethyl

carbazole (AEC Chromogen Kit). Each stained band was scanned and the optical density was determined by a computer program, NIH Image (National Institutes of Health, Bethesda, MD). The amount of isoenzyme in the microsomes were calculated by comparing with the pure isoenzyme as a standard.

EMPP Metabolism and Its Inhibition

Eight hundred microliters of 50 mM phosphate buffer, 10 μL of EMPP solutions (5, 10, 20, and 50 mM), and 100 μL of the microsomal suspension were mixed in an ice cold glass tube. The metabolic reaction was started after 100 μL of NADPH-generating system containing 8 mM NADP, 80 mM glucose-6-phosphate, 10 units/mL glucose-6-phosphate dehydrogenase, and 60 mM $MgCl_2$ was added. After incubation for 10 min at 37°C, the reaction was stopped by adding 1 mL of 5% trichloroacetic acid (TCA) and the concentration of HMO formed was measured by HPLC.

The effect of metabolic inhibitors and antibodies to rat CYPs on the rate of HMO formation from EMPP was studied. Alpha-NF (2 mM in acetone), TAO (10 mM in acetone), *n*-octylamine (10 mM in Dimethyl sulfoxide), SKF 525-A (10 mM in water), quinidine (0.5 mM in methanol), or cimetidine (100 mM in methanol) were used. Ten microliters of the inhibitor solution except for TAO was added to 790 μL of 50 mM phosphate buffer, and the buffer solution was mixed with the EMPP solution (100 μM) and microsomal suspension according to the procedure described above. In the case of TAO as an inhibitor, 10 μL of TAO solution was added to 790 μL of 50 mM phosphate buffer followed by addition of 100 μL of the microsomal suspension. The solution was mixed with 100 μL of the NADPH-generating system and preincubated for 15 min at 37°C. Then, the reaction was started by addition of 10 μL of the EMPP solution (100 μM). Solvents of the inhibitors were used for the control studies. Rabbit antiserum against rat CYP1A, CYP2B, CYP2C, and CYP3A, were used to inhibit the reaction according to the method reported (26). Five or ten microliters of each antiserum was mixed with 100 μL of the microsomal suspension. The suspension was incubated for 10 min at 37°C followed by addition to 780 μL of 50 mM phosphate buffer and the EMPP solution (100 μM). Normal rabbit serum was used for the control.

Measurements

EMPP Determination

EMPP in the samples were determined using a HPLC system after extraction, where the mobile phase was prepared by mixing 5% of acetate buffer (pH 3.15) with acetonitrile in the volume ratio of 45 to 55. One hundred microliters of plasma or 200 μL of hemolyzed blood sample was taken into a glass tube followed by spiking of 100 μL of internal standard solution (1 mg of cinnarizine dissolved in 1 mL of mobile phase) and 0.5 M NaOH solution. The aqueous mixture was shaken with 6 mL of hexane for 10 min. After centrifugation (1,000 × g) at 4°C for 10 min, 5 mL of the organic phase was transferred into another glass tube and evaporated at 50°C with a gentle stream of nitrogen following addition of 50 μL of 0.01% of Tween80 in ethanol. The residue was dissolved with 100 μL of mobile phase by vortex mixing and then 50 μL of the solution was injected on the column.

HMO Determination

HMO formed in the microsomal suspension was also determined using HPLC system. The mobile phase was prepared by mixing 0.01 M of phosphate buffer (pH 7.5) with acetonitrile in the volume ratio of 72 to 28. The suspension added with TCA was centrifuged (1,000 μg) at 4°C for 5 min and 1 mL of the supernatant was put into a glass tube followed by spiking of 200 μL of 1 M NaOH solution. The aqueous mixture was shaken with 5 mL of diethylether for 10 min. After centrifugation (1,000 × g) at 4°C for 5 min, 4 mL of the organic phase was transferred into another glass tube and evaporated at 40°C with a gentle stream of nitrogen. The residue was dissolved with 100 μL of mobile phase by vortex mixing and then 50 μL of the solution was injected on the column.

Data Analysis

In Vivo *Experiment*

The area under the plasma concentration versus time curve after drug administration into the femoral vein ($AUC_{i.v.}$), the portal vein ($AUC_{p.v.}$), and the duodenum ($AUC_{p.o.}$) were calculated using the trapezoidal and log-trapezoidal rules for ascending and decreasing plasma concentrations. Clearance after drug administration into the femoral vein ($CL_{i.v.}$), into the portal vein ($CL_{p.v.}$), and into the duodenum ($CL_{p.o.}$) were given as follows:

$$CL_{iv} = Dose/AUC_{iv}$$

$$CL_{pv} = Dose/AUC_{pv}$$

$$CL_{po} = Dose/AUC_{po}$$

where Dose denotes the administration dose.

Total bioavailability (F_t), hepatic bioavailability (F_h), and gastrointestinal bioavailability (F_g) were given as follows:

$$F_t = AUC_{po}/AUC_{iv}$$

$$F_h = AUC_{pv}/AUC_{iv}$$

$$F_a \cdot F_g = AUC_{po}/AUC_{pv} = F_t/F_h$$

where F_a is an absorption fraction and assumed to be 1.0.

Total first-pass elimination and first-pass eliminations in the GI tract and liver were estimated as follows:

$$\text{Total first-pass elimination fraction} = 1 - F_t$$

$$\text{First-pass elimination fraction of GI tract} = 1 - F_g$$

$$\text{First-pass elimination fraction of liver} = (1 - F_t) - (1 - F_g) = F_g - F_t$$

In Situ *Experiment*

At a steady-state condition in EMPP perfusion into intestinal tract, usually 30 min after beginning of the perfusion, elimination clearance from intestinal lumen (CL_e), appear-

ance clearance to portal vein from intestinal lumen (CL_a), and metabolic clearance (CL_m) were calculated as follows:

$$CL_e = \frac{Q_p \cdot (C_{in} - C_{out})}{C_{in}}$$

$$CL_a = \frac{Q_v \cdot C_v}{C_{in}}$$

$$CL_m = CL_e - CL_a$$

where Q_p is the perfusion flow rate into the intestinal loop, C_{in} and C_{out} denote EMPP concentration in the perfusate before and after passing through the intestinal loop, respectively, Q_v is the blood flow rate of the mesenteric vein, and C_v is EMPP concentration in the mesenteric blood. Those clearances were calculated in the samples after 30 min and were averaged.

In Vitro *Experiment*

A reciprocal form of the velocity of HMO generation (1/V) and that of the initial EMPP concentration (1/S) profile was fitted to the following equation by use of a linear iterative least squares method:

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \cdot \frac{1}{S}$$

and the maximum velocity (V_{max}) and Michaelis constant (K_m) were estimated.

Statistics

In the *in vivo* and *in situ* experiment, clearances were compared by an one-way ANOVA followed by multiple range test, Scheffe test. Statistical significance was assumed when the corresponding *P* values were lower than $\alpha = 0.05$.

RESULTS

In Vivo Experiments

Plasma concentration versus time curves for EMPP are shown in Fig. 1 when EMPP was infused at a rate of 25 mg/h/kg from the femoral vein, portal vein and duodenum. The concentrations in the administration phase were about 10 times lower for duodenal administration than for femoral vein administration. On the other hand, infusion from the portal vein resulted in a decrease in the plasma concentration by only one half compared with femoral vein infusion. Within this plasma concentration range, each EMPP elimination profile after the peak concentration was parallel to the others, showing linear disposition characteristics in the systemic circulation. Figure 2 shows the relationship between the infusion rate and clearance of EMPP administered into the duodenum ($CL_{p.o.}$), portal vein ($CL_{p.v.}$), and femoral vein ($CL_{i.v.}$) at a dose of 50 mg/kg. In the case, the bolus injection represented a very rapid administration rate. Although $CL_{p.v.}$ and $CL_{i.v.}$ showed little change at any of the infusion rates, $CL_{p.o.}$ was clearly decreased as the rate increased, showing a non-linear elimination process. At infusion rates of 25 and 100 mg/h/kg, $CL_{p.o.}$ was significantly ($P < 0.05$) larger than $CL_{p.v.}$ and $CL_{i.v.}$. There was no difference between $CL_{p.v.}$ and $CL_{p.o.}$ when EMPP was administered as a bolus. Table I demonstrates the bioavailabilities of EMPP after infusion of 50 mg/kg at rates of 25 and 100 mg/h/kg into the intestinal tract (total bioavailability; F_t) and portal vein (hepatic bioavailability; F_h), and also shows the GI tract bioavailability (F_g) calculated by dividing F_t by F_h. The F_t value was very low, being 0.043 and 0.094 at infusion rates of 25 and 100 mg/h/kg, respectively. At each infusion rate, the calculated F_g was obviously lower than F_h. In addition, F_g at the lower infusion rate was only about half the value of that at the higher infusion rate. The first-pass elimination fractions of EMPP are also shown in Table I. Contribution to the first-pass elimination of the GI tract was 20 and 10 times larger than that of the liver at infusion rates of 25 and 100 mg/h/kg, respectively.

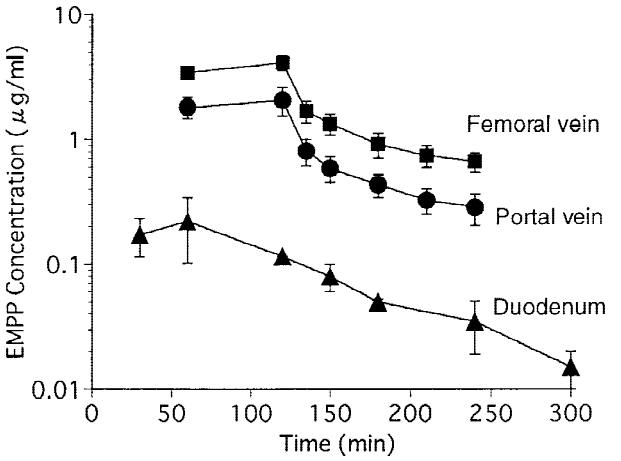

Fig. 1. Plasma concentration versus time curves of EMPP administered from the femoral vein (■), portal vein (●), and duodenum (▲) in rats at the infused rate of 25 mg/h/kg. The data are expressed as mean ± SD (n = 5).

In Situ Perfusion into Rat Small Intestinal Tract

Elimination of EMPP in the intestine was demonstrated directly and quantitatively by *in situ* perfusion into the rat small-intestinal tract. The value of CL_e (0.112 ± 0.071 mL/

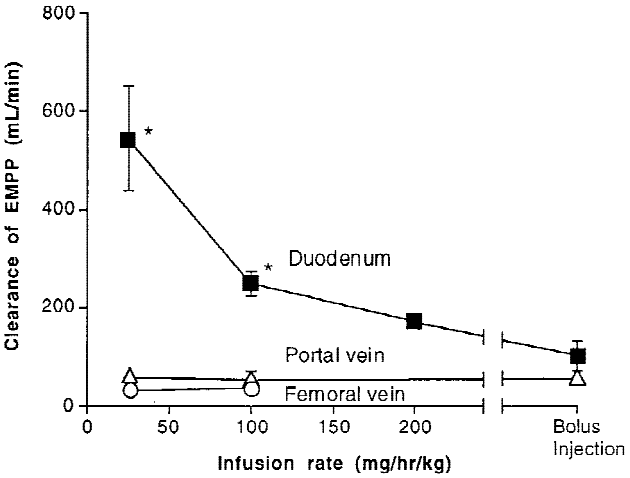

Fig. 2. Influence of infusion rate on clearance of EMPP administered to rats (50 mg/kg) from various roots. Administration route: The duodenum (■), portal vein (△), femoral vein (○). *Significantly different ($P < 0.05$) from those of the other routes. Each point shows mean ± SD (n = 5).

Table I. Total, Hepatic, and GI Tract Bioavailability and First-Pass Elimination Fraction of EMPP Administered to the Rats by Different Infusion Rates[a]

	Infusion rate (mg/h/kg)[b]	
	25	100
Bioavailability		
Total	0.043 ± 0.009	0.094 ± 0.010
Hepatic	0.486 ± 0.091	0.532 ± 0.163
GI tract[c]	0.0879	0.176
First-pass elimination fraction		
Total	0.957	0.906
Hepatic	0.045	0.082
GI tract	0.912	0.824

[a] The data are expressed as mean ± SD (n = 5).
[b] Administration dose was 50 mg/kg.
[c] Those are calculated by dividing total bioavailability by hepatic one.

min) was much lower than the luminal perfusion rate (1.0 mL/min). CL_a was also significantly lower (0.004 ± 0.001 mL/min) than the blood perfusion rate (0.15 mL/min). The value of CL_m (0.108 ± 0.07 mL/min) was apparently (about 27 times) larger than that of CL_a, although the difference was not statistically significant because of the high variance of CL_m.

In Vitro Experiment Using Intestinal Microsomes

The microsomal content of CYP from the intestine was about 30 pmol/mg protein, which was comparable to values reported previously (13) and was 5% that of hepatic microsomes. The NADPH cytochrome P450 reductase activity was about 0.02 unit/mg protein in the intestinal microsomes and 0.09 unit/mg protein in the hepatic microsomes. Enzymatic activities of CYP in the microsomes for aminopyrine *N*-demethylation, 4-nitroanisole *O*-deethylation and 7-ethoxycoumarine *O*-deethylation were 0.362, 0.287, and 0.193 nmol/min/mg protein, respectively, being much higher than those reported previously (5–9). Immunoblot analysis revealed that the intestinal microsomes had a small but significant content of CYP1A1, 1A2 and 3A2 compared with liver microsomes (Table II).

Effects of cofactors (NADPH and NADH) and carbon monoxide on the formation of HMO were then examined. The enzymatic activity with NADPH alone was three times higher than that with NADH alone, and was similar to that with both cofactors. Carbon monoxide prevented the formation of HMO. These data confirmed the contribution of CYP to HMO formation.

We determined the metabolic kinetic parameters of HMO formation from EMPP in both intestinal and hepatic microsomes. V_{max} of the intestinal microsomes was 0.057 nmol/min/mg protein, being much lower than that of the hepatic microsomes (5.008), and the degree of difference was comparable with that of the CYP content of the microsomes. On the other hand, the intestinal K_m was 0.243 mM, which was similar to the hepatic value (0.117).

Table II. Immunoquantification of Cytochrome P450 Isoenzymes in the Rat Intestinal and Hepatic Microsomes[a]

	CYP1A1 (pmol/mg protein)	CYP1A2 (pmol/mg protein)	CYP3A2 (pmol/mg protein)
Intestine	4.69	3.23	3.03
Liver	2.10	36.1	46.4

[a] The data are expressed as mean (n = 2).

Inhibition of EMPP transformation to HMO was performed with various inhibitors of CYP components (Fig. 3). The inhibitory fraction of α-NF (a specific inhibitor of CYP1A) was the largest. Nonspecific inhibitors, n-octylamine and SKF 525-A, showed little effect. Cimetidine, a nonspecific inhibitor but affecting mainly CYP3A, had a very small effect. No effect was shown by TAO (a specific inhibitor of CYP3A) and quinidine (a specific inhibitor of CYP2D). Figure 4 demonstrates the effect of rabbit antibodies against rat CYP1A, CYP2B, CYP2C, and CYP3A. Addition of the antibody against rat CYP1A resulted in a significant decrease of enzymatic activity for HMO formation. In the inhibition studies, the values were averaged for two experiments, although the differences between the two values were around 10% on average.

DISCUSSION

Recently, many investigators have reported the oxidative metabolism mediated by CYP3A4 in the gut (1–3), concluding that the low and variable oral availability of drugs would be due to gastrointestinal first-pass metabolism. However, little has been known about the gastrointestinal metabolism caused by other CYPs. Therefore, in the present study using EMPP as a model drug that is known to be transformed to an oxidative metabolite (HMO), we evaluated the contribution of intestinal metabolism to the first-pass disposition of EMPP and clarified the main CYPs associated with its oxidative metabolism.

Gastrointestinal bioavailability (F_g) consists of an absorption fraction (F_a) and a fraction that escapes intestinal metabolism (F_{gm}). Therefore, it is impossible to know the extent of drug metabolism in the intestine without data on the extent of drug absorption from the intestinal lumen after oral administration. Because EMPP was given as a solution and is known to be well absorbed, it was considered to be absorbed almost completely when administered into the GI tract, sig-

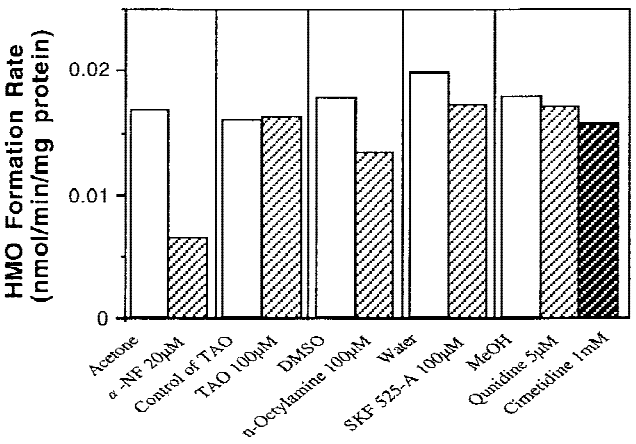

Fig. 3. Effect of inhibitors on HMO formation from EMPP in rat intestinal microsomes. The data are expressed as mean (n = 2).

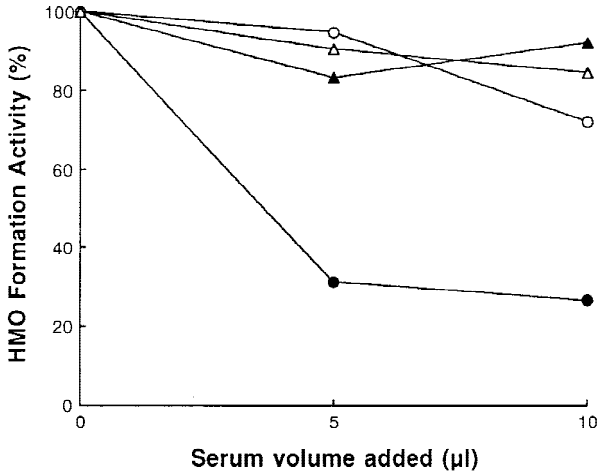

Fig. 4. Effect of antibodies on HMO formation from EMPP in rat intestinal microsomes. Symbols: Anti-CYP1A (●), Anti-CYP2B (○), Anti-CYP2C (▲), and Anti-CYP3A (△). The data are expressed as mean (n = 2).

nifying that the absorption fraction (F_a) would be equivalent to one. Thus, we were able to assume F_g ($= F_a \times F_{gm}$), to be F_{gm} and considered it possible to estimate the extent of the gastrointestinal and hepatic contributions to the first-pass metabolism of orally administered EMPP quantitatively from the route-dependent differences in bioavailability. To prevent the concentration becoming high in the portal vein, the EMPP solution was infused at various rates, since a high portal level of EMPP would result in a low hepatic extraction ratio and consequently lead to underestimation of the hepatic contribution and overestimation of the gastrointestinal contribution. It was also important that each elimination profile after the peak concentration was parallel to the others (Fig. 1) and that clearance of EMPP after intravenous infusion was constant at rates of 25 and 100 mg/h/kg (Fig. 2). Furthermore, the clearance values for intravenous infusion were similar to those for bolus injection at a dose of 5 mg/kg, one-tenth of the dose used in this study (data not shown). These phenomena suggested that around these administration rates EMPP has linear disposition kinetics after reaching the general circulation, thus enabling the contribution of the liver and gastrointestine to be evaluated accurately.

The total clearance of EMPP after intravenous infusion was about 30 mL/min, which is much larger than the rat liver plasma flow rate (8 mL/min). The reason for this is unclear, but may due to distribution to blood cells and/or elimination in extrahepatic sites such as the lungs.

As shown in Table I, the total bioavailability (F_t) was exceedingly low. The hepatic first-pass availability (F_h) was effectively small (about one-half) but larger than the calculated gastrointestinal bioavailability (F_g). The contribution of the GI tract to first-pass elimination is 10 to 20 times greater than that of the liver. We also found that F_g decreased as the infusion rate was reduced, whereas F_h did not change regardless of the infusion rate. These data suggest that the gastrointestine, and not the liver, is the main organ involved in EMPP elimination after oral administration. The lower bioavailability in the gastrointestine is caused by gastrointestinal metabolism because transporters such as P-glycoprotein may not participate to any great extent in the absorption of such a well permeating drug in the gut (27).

In the experiment using perfused rat intestine, we maintained steady-state conditions to obtain a simple mass balance equation. Both of the perfusion rates we employed were much larger than the clearance of elimination (CL_e) and the clearance of appearance (CL_a), showing they were not rate-limiting in the process of drug absorption and permeation, and therefore that CL_e and CL_a were intrinsic phenomena for EMPP in the intestine. The fact that the clearance of metabolism (CL_m) was 27 times larger than that of CL_a agreed with the *in vivo* results and confirmed the hypothesis that a significant fraction of absorbed EMPP was eliminated during the first passage through the gastrointestine.

EMPP was transformed to the ω-1 hydroxylated metabolite of HMO by the intestinal microsomes, in association with NADPH, and the reaction was inhibited markedly by carbon monoxide. These results would represent the transformation to HMO by CYPs. Only the antibody against rat CYP1A, and not other antibodies, decreased enzymatic activity for HMO formation significantly. Alpha-NF, a specific inhibitor of CYP1A, also had the largest inhibitory effect on MHO formation among all the inhibitors tested. These data suggest that CYP1A in intestinal microsomes is the main type of CYP associated with the biotransformation of EMPP to HMO.

Although a number of investigators have reported that CYP3A4 is probably associated with the metabolism of many drugs in the human gastrointestine (1,28), there have been few reports describing drug metabolism by other CYP components. Only CYP3A4 was found to be present in the human gastrointestine in significant amounts, and therefore believed to play a noteworthy role in gastrointestinal drug metabolism. There may be no reason to rule out other CYP components because the small amount of enzyme present would possibly eliminate drugs significantly in the gastrointestine. Therefore, our results suggest that CYP1A is also an important CYP isoenzyme for biotransforming drugs or xenobiotics administered orally in the human gastrointestine (9), even though the present data were derived from rats. Because propranolol and imipramine, substrates of CYP1A (29), show very small bioavailability, the gut cells may eliminate these drugs to a significant degree.

It is also notable that the intestine has a larger contribution than the liver, even though the former has a lower enzyme content. All absorbed drug molecules must pass through the gastrointestinal cells, but only a proportion of the molecules in the portal vein can be taken up into hepatocytes. Because plasma protein binding and the plasma membranes of the liver may prevent drugs from moving into hepatocytes, they would be rate-limiting in the hepatic elimination of some drugs. Therefore, it is likely that the contribution of the liver is lower than that of the gastrointestine to the first-pass metabolism of EMPP, which shows very high (about 90%) plasma protein binding.

In the present *in vitro* study without using any inducer, we were able to obtain rat intestinal microsomes, which had a significantly higher protein content and enzymatic activity than those in other reports (5–9). Immunoblot analysis also demonstrated that the microsomes contained significant amounts of CYP1A and 3A (Table II). These results were considered due to two improvements in the preparation process. One was sonication before homogenization, and the other was the use of PMSF, a protease inhibitor, as a substitute for trypsin inhibitor. The higher protein content would

have been due mainly to the sonication, which completely disrupted all of the mucosal cells. If only homogenization had been used, enterocytes might have remained attached to mucus, and eliminated along with it by centrifugation. The sonication would have been effective for degrading the structure of the mucus and dispersing the intestinal mucosal suspension. Although PMSF might have been useful for obtaining high enzymatic activity in the microsomes, there might have been other reasons, such as a high protein concentration in the microsomal suspension due to the sonication, which would have decreased the ratio of degradation by protease.

In conclusion, after oral administration of EMPP, the contribution of the GI tract to the first-pass effect is much higher than that of the liver. It is suggested that CYP1A in intestinal microsomes is the main component associated with the first-step metabolism of EMPP.

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