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Stereoselective Disposition of Flurbiprofen From a Mutual Prodrug With a Histamine H_2-Antagonist to Reduce Gastrointestinal Lesions in the Rat

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ABSTRACT The in vitro and in vivo stereoselective hydrolysis characteristics of the mutual prodrug FP-PPA, which is a conjugate of flurbiprofen (FP) with the histamine H_2-antagonist PPA, to reduce gastrointestinal lesions induced by FP were investigated and compared with those of FP methyl ester (*rac*-FP-Me) and FP ethyleneglycol ester (*rac*-FP-EG). The *rac*-FP derivatives were hydrolyzed preferentially to the (+)-S-isomer in plasma and to the (−)-R-isomer in liver and small intestinal mucosa. Interestingly, in the gastric mucosa, the stereoselectivity of hydrolysis of (−)-R-FP-PPA was opposite from that of *rac*-FP-Me and *rac*-FP-EG, which suggested that the stereoselective hydrolysis of FP-PPA was helpful in reducing gastric damage induced by (+)-S-FP. However, hydrolysis of all *rac*-FP derivatives was found to be catalyzed by carboxylesterases in the gastric mucosa. The stereoselective disposition of FP enantiomers early after intravenous administration of *rac*-FP-PPA could be explained by the stereoselective formation of (−)-R-FP from *rac*-FP-PPA in the liver. (−)-R-FP-PPA was completely hydrolyzed to form (−)-R-FP in vivo, while 78% of (+)-S-FP-PPA was hydrolyzed to (+)-S-FP, with a corresponding decrease in the area under the curve. Twenty-five percent of (+)-S-FP-PPA might be eliminated as the intact prodrug or its metabolites other than FP. The most important bioconversion of FP-PPA occurred in plasma, and additional hydrolysis of the R-enantiomer in liver resulted in the stereoselectivity observed following both i.v. and p.o. administration.

KEY WORDS: hydrolysis, carboxylesterase, bioconversion, plasma concentration, pharmacokinetics

The biological activity of chiral compounds is often restricted to one of the enantiomers because binding sites of enzymes and receptor proteins preferentially interact with one stereoisomer. Esterases play important roles in the biotransformation of toxic environmental chemicals such as organophosphorous insecticides and some ester drugs and are known to have stereochemical requirements. Moreover, in recent years several esterases[1,2] as well as lipases[3,4] have successfully been exploited for the resolution of racemic mixtures using their stereospecific hydrolytic characteristics to obtain optically pure compounds. A previous toxicological investigation of isomer-specific differential cytotoxicity of cocaine in hepatocytes was shown to be related to stereoselective differences in the rate of hydrolytic inactivation by hepatic carboxylesterases.[5] Despite the common use of esters as "prodrugs" and the stereoselectivity of esterases, there have been few reports on stereochemical differences in the enzymatic hydrolysis of such prodrugs.

The nonsteroidal antiinflammatory drugs (NSAIDs) are of great clinical importance, and numerous 2-arylpropionic acid prodrugs have been investigated as promising means of reducing or abolishing gastrointestinal toxicity.[6,7] The 2-arylpropionic acids possess a chiral center at the carbon atom α to the carboxyl function, and it is well known that the enantiomers differ in their pharmacodynamic properties, therapeutic effects[8–10], adverse reactions,[11] and disposition kinetics.[12,13] However, the stereochemistry of hydrolysis of these prodrugs and its effect on the disposition kinetics of their parent enantiomers have not been thoroughly studied.

Recently, we developed a mutual prodrug, FP-PPA (Fig. 1A), by combining flurbiprofen (FP), an antiinflammatory drug, with *N*-{3-[3-(1-piperidinyl-methyl)phenoxy]propyl}-2-(2-hydroxyethylthio)acetamide (PPA), a novel histamine H_2-antagonist which retained the individual pharmacological effects of its parent compounds, and demonstrated that FP-PPA caused little gastric damage following oral administration while showing the same antiinflammatory effect as intact FP.[14] Furthermore, it was reported that the dose of PPA in FP-PPA was too small to give a clinical antiulcer effect, so there was no disadvantage arising from released PPA after multiple dosing.[15] FP-PPA, like other 2-arylpropionic acid prodrugs, has a single chiral center in its chemical structure due to the FP moiety, and the drug conjugate must be hydrolyzed to FP to show its

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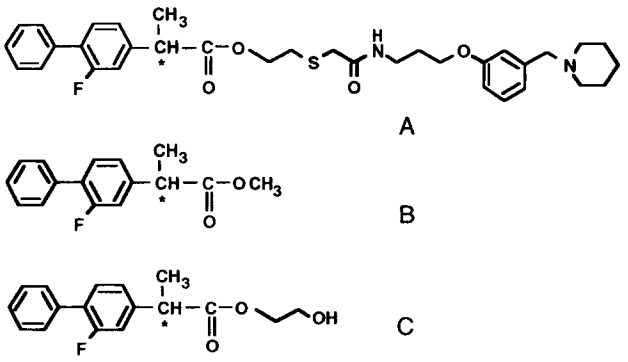

Fig. 1. Chemical structures of FP-PPA (A), FP-Me (B), and FP-EG (C).

antiinflammatory effect. The antiinflammatory activity[16,17] and adverse effects[18] of FP are due to its (+)-S-enantiomer. Therefore, it is interesting that release of (+)-S-FP from *rac*-FP-PPA occurs in the gastric mucosa and systemic circulation.

In this study, we investigated the stereoselective hydrolysis of FP-PPA, which might contribute to the overall enantioselectivity in its pharmacokinetics and pharmacological profile, including the reduction of gastric damage in rats.

MATERIALS AND METHODS

Materials

FP was purchased from Recerca and Sintesi (Bergamo, Italy). All other materials were of analytical grade. Deionized distilled water was used throughout the study. FP esters were prepared by reaction of 2-(2-fluoro-4-biphenylyl) propionyl chloride with the corresponding alcohol according to the previously reported method.[14]

Subcellular Fractionation

Male Wistar rats (weighing 200–250 g) were used in this study. Blood was withdrawn into a heparinized syringe and then centrifuged at 1500g to obtain plasma. The liver was perfused with 1.15% (w/v) KCl, excised, and placed in ice-cold 0.01 *M* phosphate buffer (pH 7.4) containing 1.15% (w/v) KCl. All subsequent procedures were carried out at 4°C. The liver was blotted, minced, and homogenized 1:4 (w/w) in 0.01 *M* phosphate buffer (pH 7.4) containing 1.15% (w/v) KCl. Homogenates were centrifuged for 20 min at 10,000 *g*, giving the supernatant (S10) fraction, which was further centrifuged at 100,000 *g* for 1 h. The resulting supernatant was used as the cytosolic fraction, and the pellets were washed and resuspended in appropriate buffers and used as microsomes.

The stomach was removed, opened along the greater curvature, and washed with cold 1.15% (w/v) KCl. The entire length of the small intestine (ca 5 cm from the proximal end of the duodenum to ca 5 cm from the distal end of the ileum) was obtained from each rat. After extrusion of its contents, the intestine was gently cleaned in cold 1.15% (w/v) KCl. The stomach and/or opened intestine were placed on a chilled glass plate, and the mucosa was scraped from the underlying muscle with a glass microscope slide. The scraped mucosa (about 2.5–3.0 g) was homogenized with 5 ml of 0.01 *M* phosphate buffer (pH 7.4) containing 1.15% (w/v) KCl. Homogenates were centrifuged for 20 min at 10,000 *g* to obtain the S10 fraction, which was further centrifuged at 100,000 *g* for 1 h to obtain microsomal and cytosolic fractions. For each preparation of small intestine and gastric mucosal esterase, five rats were used. Protein contents of each fraction were determined by the method of Lowry et al.,[19] with bovine serum albumin as a standard.

In Vitro Hydrolysis Studies

All in vitro hydrolysis studies were performed in 1 ml of tissue preparations, diluted with 0.01 *M* phosphate buffer (pH 7.4) containing 1.15% (w/v) KCl to give 100 μg/ml protein. After preincubation at 37°C for 10 min in a shaking water bath, the reaction was initiated by addition of 10 μl acetonitrile solutions of the *rac*-FP derivatives (1 m*M*). Aliquots (100 μl) of reaction mixtures were taken out at appropriate time intervals. In inhibition experiments, the S10 fraction of gastric mucosa was preincubated at 37°C with various esterase inhibitors for 10 min prior to addition of substrates, and 100 μl aliquots of reaction mixtures were taken out 30 min after addition of the substrates. The reaction was stopped by addition of 100 μl of acetonitrile containing 1 μg/ml of 4-biphenylcarboxylic acid as an internal standard. The FP produced from FP-PPA was extracted in 6 ml of benzene after addition of 1 ml of 1 *N* HCl. In the case of FP-Me and FP-EG, 100 μl of reaction sample were added to 100 μl of acetonitrile containing 8μg/ml of 4-biphenylcarboxylic acid and to 3 ml of pH 10 phosphate buffer; FP esters were then removed in 6 ml of chloroform. 2.5 ml of aqueous phase were added to 1 ml of 2 *N* HCl, and FP was extracted in 6 ml of benzene. FP enantiomers were determined with chiral stationary phase high-performance liquid chromatography (HPLC). Degradation of FP derivatives during extraction of FP from samples was negligible since the FP derivatives were stable at low pH and relatively stable at pH 10 ($t_{1/2} > 14$ h).

The first-order rate constants for enzymatic hydrolysis were calculated from the slopes of semilogarithmic plots of the FP concentration ($C_0 - C_t$) vs. time, where C_0 is the initial concentration of the (+)-S- and (−)-R-isomeric ester derivatives equivalent to FP and C_t is the concentration of liberated FP enantiomers at time t.

In Vivo Absorption Studies

Plasma concentration of FP enantiomers after administration of FP and/or FP-PPA to rats Male Wistar rats (weighing 200–250 g) were used after 24 h fasting with water ad libitum. *Rac*-FP(3 mg/kg) and *rac*-FP-PPA (equivalent to 3 mg/kg of *rac*-FP) were intravenously administered as a saline solution containing 0.1% ethanol or orally administered as an aqueous suspension in 5% (w/v) gum arabic. At appropriate time intervals after administration of drugs, 0.2 ml of blood was withdrawn into a heparinized syringe from the jugular vein and then centrifuged at 1500g to obtain plasma samples. Aliquots of plasma (0.1 ml) were treated to extract FP by the same method as hydrolysis experiment, and FP was analyzed by HPLC.

Pharmacokinetic methods Pharmacokinetic analysis after i.v. administration was performed based on conformity with one- and two-compartment models for intact FP-PPA and FP.[15] For i.v. administration of FP, biexponential equations

were applied to express the plasma concentration time course with the parameters described in the model as follows:

$$C^{FP}_{FPiv} = \frac{Dose}{V_2}\left\{\frac{(k_{32}-\alpha)}{(\beta-\alpha)}e^{-\alpha t} + \frac{(k_{32}-\beta)}{(\alpha-\beta)}e^{-\beta t}\right\} \quad (1)$$

In Equation 1, $\alpha > \beta$

$$\alpha = \frac{1}{2}\left\{(k_{20}+k_{23}+k_{32}) + \sqrt{(k_{20}+k_{23}+k_{32})^2 - 4k_{20}k_{32}}\right\}$$

$$\beta = \frac{1}{2}\left\{(k_{20}+k_{23}+k_{32}) - \sqrt{(k_{20}+k_{23}+k_{32})^2 - 4k_{20}k_{32}}\right\}$$

where V_2 is the distribution volume of the central compartment of FP, k_{20} is the first-order elimination rate constant for FP, and k_{23} and k_{32} are first-order kinetic constants for the distribution between central and peripheral compartments of FP. When FP-PPA was administered i.v., the plasma concentration time course of the FP formed was as follows:

$$C^{FP}_{FP\text{-}PPAi.v.} = \frac{Dose\ k_{12}}{V_2}\left\{\frac{(k_{32}-\alpha)}{(\beta-\alpha)(k_{10}+k_{12}-\alpha)}e^{-\alpha t} + \frac{(k_{32}-\beta)}{(\alpha-\beta)(k_{10}+k_{12}-\beta)}e^{-\beta t} + \frac{(k_{32}-k_{10}-k_{12})}{(\alpha-k_{10}-k_{12})(\beta-k_{10}-k_{12})}e^{-(k_{10}+k_{12})t}\right\} \quad (2)$$

where k_{12} is the first-order conversion rate constant of FP-PPA to FP in systemic circulation and k_{10} is the first-order elimination rate constant of FP-PPA except for k_{12}. The mean plasma concentration time course data after i.v. administration of *rac*-FP were simultaneously fitted with plasma time course data in each rat after i.v. administration of *rac*-FP-PPA. The best line fitting for each FP enantiomer was performed using the nonlinear least-squares regression program MULTI[20] with weighting factors equal to 1 and the Damping Gauss Newton algorithm. Initial values of the parameters were determined with the Simplex method.

The area under the plasma concentration time curve (AUC) was calculated by the trapezoidal method until infinite time. The percentage hydrolysis in vivo was determined by the deconvolution method. The calculation was performed using the plasma concentration of FP as an input function and that of FP-PPA as an output function.

HPLC Analysis

Reversed-phase HPLC was performed on a chiral ULTRON ES-OVM column (2.0 mm i.d. × 15 cm; Shinwa Chemical Industries, Kyoto, Japan). The eluent was pH 3.0 phosphate buffer/acetonitrile (100/15 v/v, flow rate 0.1 ml/min) and monitored at 247 nm. In all HPLC analyses, not more than 5 μl of samples was injected. Retention times were 12.7, 16.7, and 19.6 min for internal standard, (+)-S-, and (−)-R-FP, respectively. No interfering endogenous peaks were observed in chromatograms. The detection limit for each enantiomer was 50 ng/ml in plasma. The calibration curve obtained from 1–20 μg/ml was constructed by plotting the ratio of peak area of FP and internal standard vs. FP concentration ($r = 0.998$). The coefficient of variation and relative error in range from 1 to 20 μg/ml of each enantiomer of FP were 1.51–6.93% and 1.94–3.93% for the (+)-S-enantiomer and 0.74–3.23% and 1.93–10.71% for the (−)-R-enantiomer, respectively. Attempts were made to separate the enantiomers of FP derivatives using a chiral column, but these failed.

Statistical Analysis

Results were expressed as means ± SD. Student's *t* test was employed to test the statistical significance of differences between (+)-S- and (−)-R-FP for the various parameters.

RESULTS AND DISCUSSION

Stereoselective Hydrolysis of FP Derivatives

Stereoselectivity of esterase activity in plasma and S10 fraction Some stereoisomeric chiral ester prodrugs are known to be hydrolyzed at different rates by esterases.[21] However, there have been few reports of stereochemical differences in enzymatic hydrolysis of ester prodrugs of 2-arylpropionic acid.[22,23] In this study, the stereoselective hydrolysis of *rac*-FP-Me (Fig. 1B) and *rac*-FP-EG (Fig. 1C) as prodrugs was compared with that of *rac*-FP-PPA. FP esters were hydrolyzed according to pseudo-first-order kinetics in all of the tissue preparations examined. The apparent first-order hydrolytic rate constants are listed in Table 1. FP derivatives were stereoselectively hydrolyzed in plasma and all tissue homogenates. Hydrolysis of FP derivatives was slow and not stereoselective in pH 7.4 phosphate buffer (k_{obs}: FP-PPA 0.054 h^{-1}, FP-Me 0.043 h^{-1}, FP-EG 0.017 h^{-1}) and, interestingly, FP derivatives were not hydrolyzed in the presence of 4% human serum albumin until 5 h, suggesting a protective effect of albumin against hydrolysis of FP derivatives in plasma. Therefore, it was clear that stereoselective hydrolysis in tissue preparations was enzymatic. The (+)-S-isomers of FP derivatives were preferentially hydrolyzed in plasma, but the R-isomer was hydrolyzed predominantly in the small intestinal mucosa and liver S10 fraction. Interestingly, the stereopreference of hydrolysis for FP-PPA in the gastric mucosa was opposite that of the other prodrugs. In our previous report, we demonstrated that *rac*-FP-Me and *rac*-FP-EG reduced gastric damage (69.4% and 61.5% inhibition, respectively) to a much lesser degree than *rac*-FP-PPA (93.6% inhibition).[14] It was concluded that the reduction of gastric damage by FP-PPA was due to masking of the carboxylic group of FP and the coexistence of the histamine H_2-antagonist PPA, which represses the secretion of gastric acid and protects the mucous membranes, with FP in the gastric mucosa after hydrolysis[14] because gastric damage by FP was thought to depend on the first pass of intact FP in the gastric mucosa, due to the lack of enterohepatic recirculation of FP[24] and the limited damage caused by intravenous administration of FP.[25] However, taking into consideration the observation that orally administered optically pure (−)-R-FP caused very little gastric irritation and no ileal or jejunal toxicity,[18] it was also suggested that the stereopreferential disposition of (−)-R-FP (100–1000 times less potent than (+)-S-FP in inhibition of prostaglandin synthetase) into the gastric mucosa after oral administration

TABLE 1. In vitro apparent first-order rate constants for stereoselective hydrolysis of racemic FP-PPA, racemic FP-Me, and racemic FP-EG in plasma and several tissue homogenates of rats

Tissues	Rate constant (h^{-1})					
	rac-FP-PPA	S/R	*rac*-FP-Me	S/R	*rac*-FP-EG	S/R
Plasma						
S	0.16 ± 0.02	1.30	2.51 ± 0.37	3.64	1.03 ± 1.95	4.70
R	0.12 ± 0.03[a]		0.69 ± 0.07[a]		0.22 ± 0.04[a]	
Gastric mucosa (S10)						
S	0.05 ± 0.00	0.09	0.48 ± 0.07	4.57	0.09 ± 0.01	1.80
R	0.55 ± 0.11[a]		0.11 ± 0.00[a]		0.05 ± 0.00[a]	
Small intestinal mucosa (S10)						
S	0.23 ± 0.20	0.17	0.50 ± 0.10	0.63	0.08 ± 0.01	0.32
R	1.37 ± 0.39[a]		0.79 ± 0.16[a]		0.26 ± 0.01[a]	
Liver (S10)						
S	0.16 ± 0.01	0.25	1.53 ± 0.20	0.59	0.33 ± 0.03	0.28
R	0.61 ± 0.18[a]		2.61 ± 0.45[a]		1.16 ± 0.13[a]	

Data from plasma and liver represent means ± SD for five rats. Data from gastric and small intestinal mucosa were obtained from triplicate samples. The final concentrations of drugs and protein were 10 μ*M* as racemates and 100 μg/ml, respectively.
[a] Values significantly different from corresponding enantiomer at $P < 0.05$.

of *rac*-FP-PPA contributed to the prevention of gastric mucosal damage compared with *rac*-FP-Me and *rac*-FP-EG.

The apparent first-order hydrolytic rate constants of *rac*-FP-PPA in plasma and liver S10 fraction were smaller than those of both enantiomers of *rac*-FP-Me and *rac*-FP-EG, suggesting that the systemic hydrolysis of FP-PPA in vivo was slower than that of FP-Me or FP-EG. However, even for FP-PPA, hydrolysis in intact (nondiluted) plasma was fast, with $t_{1/2}$ of 40 sec, indicating the rapid bioconversion of FP derivatives, especially the S-enantiomer, in plasma. A recent study indicated that the various ester prodrugs of ibuprofen and FP were hydrolyzed stereoselectively, yielding the (−)-R-enantiomer preferentially in human plasma independent of their basic or neutral ester moieties.[22] It was suggested that there might be species differences between humans and rats in the stereoselective hydrolysis characteristics of 2-arylpropionic acid derivatives in plasma. Species differences in stereoselective hydrolysis by blood esterases have been demonstrated for (±)-esmolol, an ultrashort-acting β-blocker ester.[26] Human plasma contains cholinesterase but little carboxylesterase, in contrast to rat plasma which has high carboxylesterase levels.[27,28] So, carboxylesterase and/or cholinesterase in plasma might show opposite stereopreferences to 2-arylpropionic acid derivatives. Furthermore, the S/R ratio for hydrolysis of FP-PPA was three- to fourfold smaller than those of general prodrugs, suggesting that the contribution of esterases might be different toward FP-PPA and other prodrugs.

Stereoselectivity of microsomal and cytosolic esterase activity Table 2 summarizes the apparent first-order rate constants of stereoselective hydrolysis of the FP derivatives in the microsomal and cytosolic fractions. In the liver, the hydrolytic activities of microsomal fractions were much greater than those of cytosolic fractions, indicating that microsomal esterases contribute to the overall hepatic hydrolytic activities of the FP derivatives. Although *rac*-FP-PPA and *rac*-FP-EG showed similar selectivity to esterases in both cytosolic and microsomal fractions, *rac*-FP-Me showed the opposite selectivity. This suggested that the cytoplasmic enzyme can be distinguished from the microsomal enzyme with respect to its difference in stereoselectivity.

Interestingly, the microsomal esterases of the gastric mucosa did not hydrolyze the FP derivatives under these conditions. However, cytosolic fractions from small intestinal mucosa showed higher and/or comparable enzyme activity of S- and R-enantiomers in comparison with those of microsomal fraction for all FP derivatives. Although the subcellular fraction showing the greatest enzyme activity for FP derivatives differed between liver and gastrointestinal mucosa, the S/R ratio in each subfraction of intestinal mucosa was similar to that in liver. Recent studies have focused on stereoselective hydrolysis in the intestinal mucosa: Yang et al.[21] used oxazepam 3-acetate as a substrate and demonstrated opposite stereoselectivity between microsomal and cytosolic esterases. The different 2-arylpropionic acid derivatives may be hydrolyzed with different stereoselectivities in each subcellular fraction of the small intestine, as in the liver. Although there have been a few reports concerning hydrolysis in the gastric mucosa, as the stomach is the target organ of toxicity of acetylsalicylate, some studies of the properties of purified aspirin esterase of gastric mucosal origin have been performed. Acetylsalicylate hydrolase (ASA hydrolase) activity of rabbit gastric fundic mucosa was found predominantly in the 100,000 *g* supernatant fraction,[29] consistent with localization of the hydrolases for *rac*-FP derivatives.

Since the volume of the cytosolic fraction was much greater than that of the microsomal fraction, it seemed that the dominant hydrolytic activity in the gastrointestinal mucosa was in the cytosolic fraction. The higher hydrolytic activity in the cytosolic fraction indicates that ester-type drugs are readily hydrolyzed during absorption because drugs are transported across a mucous membrane with a long transit time. Therefore, it is considered that stereoselective hydrolysis of FP derivatives in the gastrointestinal membrane during absorption plays an important role in induction of mucosal damage and the first-pass effect. In fact, the gastric mucosal concentrations of (−)-R- and (+)-S-FP enantiomers were 12.15 ± 2.58 μg/g and

TABLE 2. In vitro apparent first-order rate constants for stereoselective hydrolysis of racemic FP-PPA, racemic FP-Me, and racemic FP-EG by microsomal and cytosolic esterases in several tissues of rats

Tissues	Subcellular fraction	Rate constant (h^{-1})					
		rac-FP-PPA	S/R	*rac*-FP-Me	S/R	*rac*-FP-EG	S/R
Gastric mucosa	Cytosol						
	S	0.15 ± 0.05	0.22	0.63 ± 0.16	3.95	0.34 ± 0.47	3.06
	R	0.69 ± 0.12[a]		0.16 ± 0.04[a]		0.13 ± 0.03[a]	
	Microsomes						
	S	—		—		—	
	R	—		—		—	
Small intestinal mucosa	Cytosol						
	S	0.69 ± 0.09	0.42	0.62 ± 0.03	1.00	0.23 ± 0.05	0.37
	R	1.64 ± 0.25[a]		0.62 ± 0.03		0.63 ± 0.01[a]	
	Microsomes						
	S	0.24 ± 0.02	0.17	0.09 ± 0.01	0.18	0.06 ± 0.02	0.25
	R	1.45 ± 0.25[a]		0.48 ± 0.04[a]		0.24 ± 0.02[a]	
Liver	Cytosol						
	S	0.04 ± 0.01	0.12	0.76 ± 0.04	1.17	0.08 ± 0.004	0.46
	R	0.38 ± 0.03[a]		0.65 ± 0.08[a]		0.17 ± 0.03[a]	
	Microsomes						
	S	1.90 ± 0.22	0.18	13.64 ± 5.05	0.36	12.03 ± 1.97	0.24
	R	10.90 ± 2.38[a]		38.01 ± 5.17[a]		50.41 ± 15.76[a]	

Data from liver represent means ± SD for five rats. Data from gastric and small intestinal mucosa were obtained from triplicate samples. The final concentrations of drugs and protein were 10 μ*M* as racemates and 100 μg/ml, respectively.
[a]Values significantly different from corresponding enantiomer at $P < 0.05$.

12.54 ± 3.36 μg/g for FP, 1.38 ± 0.20 μg/g and 1.26 ± 0.26 μg/g for FP-PPA, and 1.49 ± 0.33 μg/g and 1.74 ± 0.38 μg/g for FP-Me, respectively, at 1 h after oral administration. The concentrations of each enantiomer after administration of FP-PPA were comparable with those after FP-Me administration. The S/R ratios of the mucosal concentrations of FP after FP-PPA and FP-Me administration were 0.9 and 1.2, reflecting stereoselective hydrolysis in vitro. This slight stereoselectivity occurred because total concentrations of FP derivatives in the gastric mucosa were so high that the enzyme activity could be saturated (FP-PPA, 24 ± 3.8 μg/g; FP-Me, 10.8 ± 1.4 μg/g).[14] The S/R ratio of the gastric mucosal concentrations of FP after dosing of FP-Me was 2.26 at 2 h due to decreasing mucosal total concentration, while that of FP-PPA was 1.0 even at 2 h (data not shown). These data suggested that the reduction of gastric damage by FP-PPA was mainly due to the presence of PPA in the gastric mucosa, and the stereoselective hydrolysis toward the R-enantiomer was also helpful for reducing the gastric damage.

The Nature of Gastric Mucosal Esterases

To assess the nature of gastric mucosal esterases, the sensitivity of gastric mucosal homogenate preparations to inhibition by several esterase inhibitors was investigated. Paraoxon (PO) and diisopropylfluorophosphate (DFP) are strong inhibitors of cholinesterase and carboxylesterase (B-esterases). Bis (*p*-nitrophenyl) phosphate (BNPP) is a specific inhibitor of carboxylesterase isoenzymes. Eserine (ES) inhibits cholinesterase and, to some extent, carboxyesterase. Decamethonium bromide (DM) predominantly inhibits acetylcholinesterase. As shown in Figure 2, PO and DFP caused almost complete inhibition of FP-PPA and FP-Me hydrolyses by S10 fraction of the gastric mucosa, indicating involvement of B-esterases. Significant inhibition by BNPP, but the reduced effect of ES and DM, suggested the involvement of carboxylesterase. The inhibition patterns of various inhibitors against esterases for *rac*-FP-EG were fundamentally the same as those for *rac*-FP-Me. ASA hydrolase was inhibited by very low concentrations of DFP and by ES at much higher concentrations.[29] The majority of esterases hydrolyzing *rac*-FP derivatives in the gastric mucosa might be related to the ASA hydrolase.

Interestingly, the stereoselective hydrolytic characteristics of *rac*-FP-PPA were unusual compared with those of *rac*-FP-Me and *rac*-FP-EG in the rat gastric mucosa, though the hydrolases responsible were identical for all three substrates. PPA liberated from FP-PPA may have affected the stereoselective hydrolysis of *rac*-FP-PPA. However, the formation of FP enantiomers from *rac*-FP-Me was not affected by the addition of 100 m*M* PPA (data not shown), indicating that PPA had no effect on the stereoselective hydrolysis of FP derivatives. Toone et al.[30] reported that pig liver esterase (PLE) exhibited reversals in stereoselectivity, such as changing from *R* to *S* preference within structurally similar series of substrates that were governed by apparently trivial changes in substrate size or structure. Based on their proposed model, the active site of PLE has been suggested to be composed of two hydrophobic pockets (larger and smaller pockets) and two others that are more polar in character. Nonpolar functional groups of the substrate would bind to one of the two hydrophobic pockets, depending on its size and R or S ester locations in the serine sphere, which is the active site of serine hydrolases, and would trigger the size-induced stereoselectivity observed for PLE. So, the reversal of stereoselectivity in the hydrolysis of FP derivatives in rat gastric mucosal esterases might be explained by such

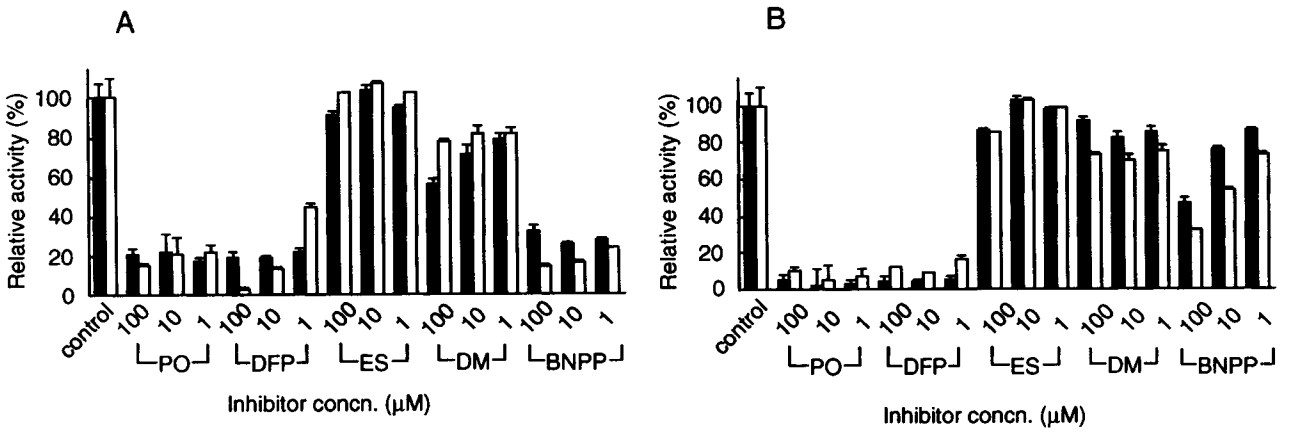

Fig. 2. Effects of several esterase inhibitors on the stereoselective properties of esterases in the S10 fraction from rat gastric mucosa. Data were obtained from triplicate samples. Final concentrations of FP derivatives [*rac*-FP-PPA **(A)** and *rac*-FP-Me **(B)**] and protein were 10 m*M* and 0.1 mg/ml, respectively. Open and closed columns represent the (−)-R- and (+)-S-enantiomers, respectively. "Control" indicates the absence of inhibitors. PO, paraoxon; DFP, diisopropylfluorophosphate; ES, eserine; DM, decamethonium bromide; BNPP, bis(*p*-nitrophenyl)phosphate.

an active site model of PLE based on size-induced reversal of stereoselectivity or by differences in their electric charge.

Stereoselective Disposition of FP Derivatives

Intravenous administration The plasma concentration of (+)-S- and (−)-R-FP after i.v. injection of *rac*-FP and/or *rac*-FP-PPA (equivalent to 3 mg/kg of *rac*-FP) are shown in Figure 3. The mean plasma concentration-time data for the FP enantiomer after *rac*-FP administration (Fig. 3A) could be described by a biexponential equation, and the stereoselective differences in the pharmacokinetics of the enantiomers of FP in rats were the same as those described by Jamali et al.[24] With the i.v. injection of *rac*-FP-PPA (Fig. 3B), both enantiomers of FP appeared rapidly in plasma, reflecting fast hydrolysis in plasma. Moreover, the (−)-R-isomer of FP appeared in plasma more rapidly than (+)-S-FP, and the peak level of the (−)-R-enantiomer was higher than that of the (+)-S-enantiomer. These data suggested that the stereoselective bioconversion of FP-PPA to FP was related to stereoselective hydrolysis in liver. Although the plasma concentrations of (+)-S-FP liberated from *rac*-FP-PPA were significantly lower than those from *rac*-FP, the elimination profiles of both enantiomers after *rac*-FP-PPA administration were similar to those after administration of *rac*-FP (Fig. 3B). FP is known to be converted from the (−)-R- to the (+)-S-enantiomer in rats only to a very limited extent, in contrast to ibuprofen and fenoprofen[8,31] because CoA thioester formation, which is essential for (−)-R- to (+)-S- inversion, does not occur.[32] Moreover, the biliary route is only a minor pathway of elimination in rats.[24] The lack of chiral inversion and enterohepatic recirculation of FP make it a simple two-compartment model for each enantiomer of FP. A one-compartment model comprising two simple elimination processes, conversion of FP-PPA into FP in the systemic circulation and other pathways (except hydrolysis), was assumed for the pharmacokinetics of FP-PPA.[15] Furthermore, the lack of chiral inversion of intact FP-PPA was assumed.

The plasma concentration-time data after i.v. administration of *rac*-FP and/or *rac*-FP-PPA were simultaneously fitted to multiexponential equations (Eqs. 1 and 2), and the disposition kinetics of the FP enantiomers are summarized in Table 3. The observed plasma concentrations fitted well to the calculated concentration curves using the parameters listed in Table 3, as shown in Figure 3. There were statistically significant differences between the enantiomers for the first-order elimination rate constant (k_{20}) in both treated groups but no stereoselective differences in the distribution volume (V_2). In particular, the stereoselective difference of k_{20} was accompanied by a significant difference in the plasma concentration-time AUC and total body clearance (CL_{tot}) between both enantiomers. The first-order bioconversion rate constant (k_{12}) of FP-PPA was large compared with k_{20} due to rapid hydrolysis in plasma. In particular, the significantly greater k_{12} of (−)-R-FP-PPA than (+)-S-FP-PPA reflects the in vitro hydrolysis in the liver rather than plasma. All parameters, except the AUC of both enantiomers after FP-PPA administration, were almost the same as those of FP administration. The plasma concentration profiles and pharmacokinetic parameters demonstrated that the liberated FP enantiomers from corresponding FP-PPA behaved in the same way when intact FP was administered and that PPA had no effect on the stereoselective disposition of FP.

The plasma AUC for (+)-S-FP after *rac*-FP-PPA administration was significantly decreased by 29.3% compared with that after *rac*-FP administration. However, AUCs for (−)-R-FP were the same between *rac*-FP- and *rac*-FP-PPA-administered groups. The elimination processes of FP-PPA represented both bioconversion to FP(k_{12}) and disappearance as intact FP-PPA and other metabolites (k_{10}). The fractions of hydrolysis to FP in total elimination ($k_{12}/(k_{10} + k_{12})$) were 0.75 and 0.9 for the (+)-S- and (−)-R-enantiomers, respectively. In addition, the cumulative amounts of FP enantiomers converted from *rac*-FP-PPA in the systemic circulation were estimated by the deconvolution method using the plasma levels of FP enantiomers after the i.v. administration of *rac*-FP-PPA as the output function and those after i.v. administration of *rac*-FP as the weighting function. The cumulative amount-time curves for (+)-S- and (−)-R-FP-PPA reached maximum values

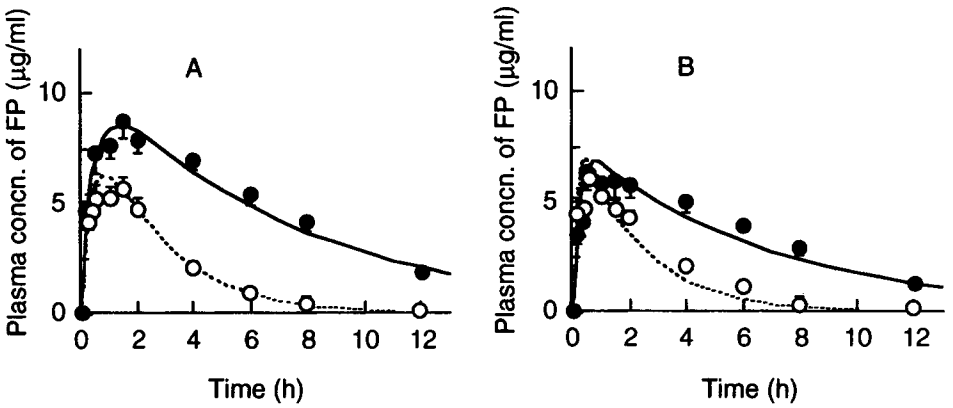

Fig. 3. Plasma concentrations of FP enantiomers after i.v. administration of racemic FP **(A)** and racemic FP-PPA **(B)** (equivalent to 3 mg/kg of racemic FP) to rats. Data represent means ± SE for six rats. Open and closed symbols represent (−)-R-FP and (+)-S-FP, respectively. Solid and broken lines are simulation curves for (+)-S- and (−)-R-FP, respectively, calculated with Eqs. 1 and 2 (see "Materials and Methods") and the parameters listed in Table 3.

TABLE 3. Pharmacokinetic parameters after i.v. administration of racemic FP and racemic FP-PPA (equivalent to 3 mg/kg of racemic FP) to rats

Parameters		*rac*-FP		*rac*-FP-PPA	
		S	R	S	R
k_{12}	(h^{-1})	—	—	4.89 ± 0.08	13.61 ± 0.25[a]
k_{10}	(h^{-1})	—	—	1.64 ± 0.26	1.50 ± 0.03[a]
k_{20}	(h^{-1})	0.27 ± 0.04	1.19 ± 0.12[a]	0.27 ± 0.02	0.91 ± 0.06[a]
k_{23}	(h^{-1})	1.68 ± 0.15	1.79 ± 0.21[a]	1.96 ± 0.26	2.30 ± 0.49[a,b]
k_{32}	(h^{-1})	2.13 ± 0.12	1.80 ± 0.24	3.20 ± 0.26[b]	3.30 ± 0.54[a,b]
V_2	(ml/kg)	71.85 ± 2.53	74.38 ± 3.81	70.65 ± 2.59	70.67 ± 5.98
AUC_{iv}^{FP}	(μg·h/ml)	87.45 ± 9.50	29.65 ± 1.27[a]	66.51 ± 0.28	28.75 ± 1.79
CL_{tot}	(ml/h/kg)	17.15 ± 0.52	50.59 ± 1.28[a]	22.55 ± 0.29[b]	52.17 ± 4.00[a]

All values are shown as means ± SE ($n = 6$).
[a] Values significantly different from corresponding antipode at $P < 0.05$.
[b] Values significantly different from *rac*-FP at $P < 0.05$.

at 1.50 and 0.50 h after dosing, respectively. Although (−)-R-FP-PPA was almost completely (97%) hydrolyzed to (−)-R-FP, (+)-S-FP-PPA was only 78% hydrolyzed to (+)-S-FP. These results suggested that a small amount of (+)-S-FP-PPA might remain in unhydrolyzed form in the plasma after administration of *rac*-FP-PPA. It has been reported that PPA was mainly metabolized to 3-(1-piperidinylmethyl)phenol, which, along with its conjugate, were excreted in urine in the rat.[33] When 3-(piperidinylmethyl)phenol was removed from FP-PPA before hydrolysis, the derived ester of FP with N-2-(2-hydroxyethyltio)acethyl propylamine was more stable than FP-PPA in plasma (k_{obs}: 0.05 h^{-1} for racemate in 0.1 mg/ml protein of plasma). Furthermore, FP is mainly metabolized to 4-hydroxy FP in rat, which we could not detect in our experimental conditions. One possibility of the fate of unhydrolyzed (+)-S-FP-PPA is that it might be eliminated as intact (+)-S-FP-PPA or as metabolites of FP-PPA. Although the significant decrease in the AUC of pharmacologically active (+)-S-FP might adversely affect the therapeutic potential of FP-PPA, we previously demonstrated that FP-PPA had an antiinflammatory effect equivalent to intact FP even at the dose of 3 mg/kg as a racemate (equivalent to 1 mg/kg of *rac*-FP).[14]

Oral administration Figure 4 shows the time course of the levels of FP enantiomers after oral administration of *rac*-FP and/or *rac*-FP-PPA (equivalent to 3 mg/kg of *rac*-FP) to rats. As shown in Figure 4A, (+)-S-FP showed higher concentrations than the (−)-R-enantiomer throughout the period after administration of *rac*-FP, similar to i.v. administration. After *rac*-FP-PPA administration (Fig. 4B), the time at which maximum plasma concentrations of both enantiomers were reached (T_{max}) was shorter than that in the *rac*-FP group due to the increased absorption rate in accordance with increasing hydrophobicity (log $PC_{n\text{-octanol/water}}$; FP:1.66, FP-PPA:3.2). The maximal values of plasma concentration and elimination phase of (−)-R-FP were similar between *rac*-FP-PPA and *rac*-FP administration groups. However, (+)-S-FP released from (+)-S-FP-PPA led to significantly low plasma concentration in comparison with that of the *rac*-FP group, similar to i.v. administration. Interestingly, plasma concentrations of (−)-R-FP were higher than its antipode at 10 and 20 min after administration, which suggested the fast hydrolysis of FP-PPA in presystemic and systemic circulation.

However, the metabolic clearances of FP-PPA calculated from the i.v. administration of FP-PPA ($k_{12} \cdot V_2$) were 5.7 and 16.0 ml/min/kg for (+)-S- and (−)-R-FP, respectively, smaller

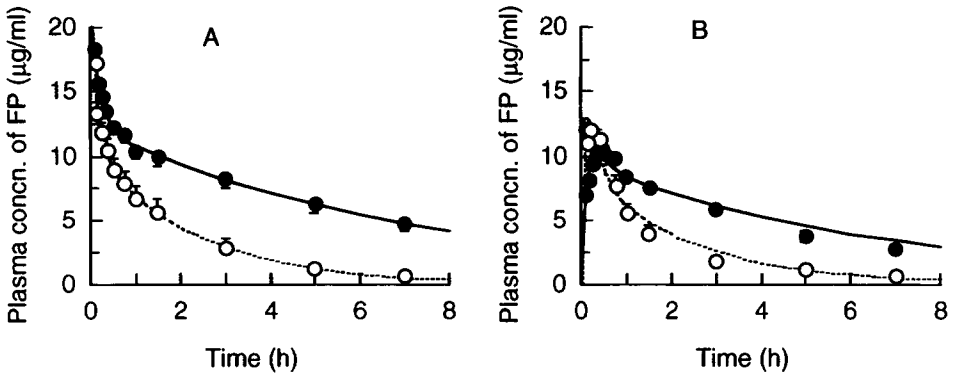

Fig. 4. Plasma concentrations of FP enantiomers after oral administration of racemic FP **(A)** and racemic FP-PPA **(B)** equivalent to 3 mg/kg of racemic FP) to rats. Data represent means ± SE for six rats. Open and closed symbols represent (−)-R-FP and (+)-S-FP, respectively. Solid and broken lines are the simulation curves for (+)-S- and (−)-R-FP, respectively, calculated using the parameters obtained from the compartment model without first-pass effect. Calculated values of *ka* and F were as follows: (+)-S- and (−)-R-FP after FP administration, 1.67 h^{-1}, 0.85 and 1.73 h^{-1}, 0.85; (+)-S- and (−)-R-FP after FP-PPA administration, 3.99 h^{-1}, 0.79 and 3.76 h^{-1}, 0.8.

than hepatic blood flow (58.8 ml/min/kg). In addition, to clarify the occurrence of first-pass hydrolysis, pharmacokinetic analysis was carried out using a compartmental model with and without first-pass hydrolysis. In the calculation of the pharmacokinetic parameters of the FP enantiomers after *rac*-FP-PPA administration, the mean plasma concentration-time course data obtained after i.v. administration of *rac*-FP and *rac*-FP-PPA were simultaneously fitted with the plasma-time course data of each rat which received *rac*-FP-PPA orally. However, when first-pass hydrolysis occurred, the calculation of pharmacokinetic parameters by the Damping Gauss Newton method for both enantiomers had failed. However, in the absence of first-pass hydrolysis, optimal parameters were obtained which were similar to those calculated from data following i.v. administration. Moreover, the plasma concentration calculated from the obtained parameters fitted well with the observed concentrations, as shown by the solid and dotted lines in Figure 4. These observations suggest that first-pass hydrolysis in gastrointestinal mucosa and liver are of little importance following oral administration of FP-PPA. This is supported by the gastric mucosal concentration of FP after administration of FP-PPA, being only 10% of total concentration, which was not stereoselective. The absorption rate constants were 3.99 ± 0.7 h^{-1} and 3.76 ± 1.36 h^{-1} for (+)-S- and (−)-R- FP-PPA, respectively, and 1.67 ± 0.32 h^{-1} and 1.73 ± 0.38 h^{-1} for (+)-S- and (−)-R-FP, respectively. The stereoselectivity of the absorption process was negligible, and fast absorption of FP-PPA compared with FP was explained by the difference in hydrophobicity between the two compounds. In conclusion, the higher plasma concentrations of (−)-R-FP than (+)-S-FP at early times after oral administration of FP-PPA might depend on systemic hydrolysis, especially in liver, rather than presystemic hydrolysis and stereoselective absorption.

The fast absorption and minor hydrolysis of FP-PPA in the gastrointestinal tract might be preferable for reduction of the mucosal irritation induced by FP. Further, the reduction of mucosal irritation was assisted by the stereoselective hydrolysis toward the R-isomer in the gastric mucosa. The most important bioconversion of FP-PPA to FP occurred in plasma, and additional hydrolysis of the R-enantiomer in liver resulted in the stereoselectivity seen after both i.v. and p.o. administrations.

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