Effects of the Ester Moiety on Stereoselective Hydrolysis of Several Propranolol Prodrugs in Rat Tissues

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The stereochemical characteristics of the hydrolysis of several ester-type prodrugs of propranolol, *O*-acetyl, *O*-propionyl, *O*-butyryl, *O*-pivaloyl and succinyl esters, were studied in phosphate buffer (pH 7.4), rat plasma and rat tissue homogenates. In phosphate buffer, no differences were observed in the hydrolysis rate between the esters of (*R*)- and (*S*)-propranolol. The effects of the ester moieties on the hydrolysis rate in phosphate buffer were in the following order: acetate > propionate > butyrate > succinate > pivalate. In rat plasma and tissue homogenates, the hydrolysis of the esters was accelerated, and stereoselective hydrolysis was observed. In plasma, the hydrolysis of the (*R*)-isomer was faster than that of the (*S*)-isomer except for the succinate ester. On the other hand, in the liver and intestine homogenates, the (*S*)-isomer was hydrolyzed faster than the (*R*)-isomer except for the succinate and pivalate esters in the liver homogenate. Also, the ratio of the hydrolysis rates (*S/R*) changed with the type of prodrug. As the length of the alkyl chain of the ester increased, the *S/R* ratio approached unity in liver and intestine homogenates but stayed almost constant in plasma. For the pivalate ester, stereoselective hydrolysis was observed in plasma and intestine homogenate but not in liver homogenate. The stereoselective hydrolysis of the succinate ester was observed only in intestine homogenate, but the *S/R* ratio was almost 1 in plasma, liver and intestine homogenates. No interconversion between (*R*)- and (*S*)-isomer was observed during the hydrolysis of any of the ester prodrugs. These results indicate that hydrolysis of ester-type prodrugs of propranolol occurs stereoselectively in rat tissues, and that its selectivity is dependent on the kind of tissue and prodrug.

Key words propranolol; prodrug; stereoselective hydrolysis; rat tissue

Propranolol (PL) is a nonselective β-adrenergic receptor blocker and has two optical isomers, dextro (*R*)- and levo (*S*)-PL, with the (*S*)-isomer being about 100 times more potent than the (*R*)-isomer as a β-blocker.[1] PL has been shown to undergo extensive first-pass metabolism after oral administration.[2,3] Garceau *et al.*[4] and Anderson *et al.*[5] have shown that the hemisuccinate or acetate ester of PL after oral administration to beagle dogs or rats yielded higher plasma PL concentrations than an equivalent dose of PL hydrochloride. Although a number of studies have demonstrated stereochemical differences in the disposition of PL,[6–12] there has been no report of such stereochemical differences in the disposition of PL prodrugs.

Recently, Mork and Bundgaard[13] demonstrated stereoselective hydrolysis of ester prodrugs of ibuprofen and flurbiprofen in human plasma. They also studied the effects of changes in the ester moieties of these prodrugs on this stereoselective hydrolysis. In our previous studies,[14,15] stereoselective hydrolysis of *O*-acetyl PL was demonstrated in human plasma and in some rat tissues. We also suggested that the enantioselectivity of hydrolysis differed in various tissues. In the present study, the effects of ester moieties of PL prodrugs on stereoselective hydrolysis were studied in phosphate buffer, rat plasma and rat tissue homogenates.

MATERIALS AND METHODS

Animals Male Wistar rats (180—220 g) were used throughout the experiments. All rats were fasted overnight (for 16 h), with free access to water.

Materials Racemic-propranolol (PL) hydrochloride was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). (*R*)- and (*S*)-PL hydrochlorides were obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). All other reagents used were of analytical grade. Racemic *O*-acyl PL hydrochloride was prepared by the previously published procedure.[16] By the same procedure, *O*-acyl (*R*)- and *O*-acyl (*S*)-PL hydrochloride were prepared from (*R*)- and (*S*)-PL hydrochloride, respectively. Following recrystallization, the identity and purity of the synthesized materials were established by IR, TLC and HPLC.

Preparation of Mucosal Cells from the Small Intestine Rats were killed by decapitation. The upper two-thirds portion of the small intestine was removed rapidly, immersed in ice-cold 0.9% NaCl and cut into approximately 5-cm lengths, trimmed free of fat and omentum and the lumen was washed with saline. All subsequent manipulations were carried out at about 4 °C. Each tube was cut open longitudinally, and the mucosal layer was removed by scraping with a glass slide.

Preparation of Enzyme Solution The mucosal cells and liver were homogenized in 5 volumes of 1.15% KCl solution with a Potter-Elvehjem glass homogenizer equipped with a Teflon pestle, and centrifuged at 9000 × *g* for 20 min at 4 °C. To adjust the protein content (1.3 mg/ml in the liver samples and 0.5 mg/ml in the intestine samples), the supernatant fraction was diluted with 1.15% KCl solution. This solution was further diluted nine times with phosphate buffer solution (pH 7.4, 20 mM). Linear relationships were observed between the concentration of protein in the enzyme solution (under 0.26 mg/ml in the liver and 0.10 mg/ml in the intestine samples) and the pseudo first-order rate constant. Blood was collected in

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heparinized tubes, and the plasma was separated by centrifugation at $10000 \times g$ for 15 min at 4 °C.

Hydrolysis of Propranolol Prodrugs One hundred μl aliquots of each propranolol prodrug (300 μM for the racemic compound or 150 μM for the pure isomer) were added to 900 μl of enzyme solution and 100 μl of enzyme solution) and incubated at 37 °C. Aliquots of 100 μl were collected at predetermined intervals, and 200 μl of acetonitrile was added to quench the hydrolysis reaction. The agitated mixture was centrifuged at $10000 \times g$ for 10 min, and the supernatant (100 μl) was mixed with 200 μl of the mobile phase for HPLC and stored in an ice-water bath until the assay. Hydrolysis of the propranolol prodrugs in phosphate buffer (pH 7.4, 20 mM, $\mu = 0.5$) was also studied according to the procedures described above, but without the centrifugation process.

Chromatography The system consisted of a Jasco pump (Trirotar-V, Tokyo, Japan), a 20 μl rheodyne loop (Model 7125, Cotati, CA, U.S.A.), and a Jasco fluorescence detector set (FP-210, Tokyo) at excitation and emission wavelengths of 285 and 340 nm, respectively. An ovomucoid-conjugated column (Ultron ES-OVM, Shinwa Kako Co., Ltd., Kyoto, Japan) was used. The PL prodrugs were assayed by a mobile phase of acetonitrile (10% (v/v) for acetyl PL, 15% (v/v) for propyl PL, 18% (v/v) for butyl PL, 20% (v/v) for pivaloyl PL or 15% (v/v) for succinyl PL) in 20 mM sodium dihydrogen phosphate. The flow rate was 1.0 ml/min and the separation was performed at ambient temperature. The concentration of protein in the enzyme solution was determined by the method of Lowry *et al.*[17]

Statistical Analysis Data are represented as the mean with standard deviation (mean ± S.D.). Statistical evaluation of the data was carried out using Student's *t*-test.

RESULTS

Hydolysis of Prodrugs in Buffer The hydrolysis of the pure isomers of propranolol (PL) esters was studied in phosphate buffer (pH 7.4) at 37 °C. The disappearance of the PL esters displayed strict first-order kinetics. The pseudo first-order rate constant was determined from the slope of linear plots of the logarithm of residual prodrug against time. The hydrolysis rate constants are summarized in Table 1. There were no significant differences between the hydrolysis rates of (*R*)- and (*S*)-PL prodrugs. The rates decreased with increasing alkyl chain length of the esters, and as the steric hindrance and hydrophobicity increased, the rate constant decreased further. Total rate constants (sum of rate constant of (*R*)- and (*S*)-) were similar to the values reported by Buur *et al.*[18] No significant differences were observed between the hydrolysis rates of the pure isomers and the racemates of the acetate, propionate or butyrate esters. No interconversion between (*R*)- and (*S*)-isomer was observed during the hydrolysis of the prodrugs in phosphate buffer.

Effects of the Ester Moiety on Stereoselective Hydrolysis in Rat Tissues The disappearance of PL esters also displayed strict first-order kinetics in rat tissues. The pseudo first-order rate constants were determined from the slopes of linear plots of the logarithm of residual prodrug against time.

The hydrolysis rate constants in plasma are summarized in Table 2. The rate of hydrolysis of the (*R*)-isomer was faster than that of the (*S*)-isomer except for the succinate ester in the phase of the study using pure enantiomer preparations. The same stereoselectivity was observed previously in human serum using the acetate ester of PL.[14] The rates of hydrolysis differed among the prodrugs, and the maximum rate among those used was that of the propionate ester. The hydrolysis rates decreased markedly as steric hindrance and hydrophobicity increased. The same stereoselective hydrolysis was observed when racemates were used instead of pure isomers (Table 2). However, the observed rate constants for the (*R*)-isomer were lower in racemate than in pure isomer preparations, although the rate constants for the (*S*)-isomers did not change even though the final concentration of substrate used was 15 μM for the enantiomerically pure samples and 30 μM for racemates.

In the intestine homogenate and the liver homogenate (Table 2), the hydrolysis rate constants of the (*S*)-isomers in enantiomerically pure preparations were faster than those of the (*R*)-isomers, with the exception of the pivalate ester in liver homogenate. This stereoselectivity was reversed when plasma was used as the medium. The hydrolysis rate constants increased as the alkyl chain length of the esters increased. The same stereoselectivities of these hydrolysis reactions were observed with the respective racemic compounds, although the observed rate constants were lower than those obtained with individual enantiomers in the intestine homogenate. No differences in the liver homogenate were observed between the rate constants of enantiomerically pure and racemic preparations.

Table 1. Rate Constants of Propranolol Prodrug Hydrolysis in Phosphate Buffer

	Rate constant ($\times 10^3$ min^{-1})				
	Acetate	Propionate	Butyrate	Pivalate	Succinate
Enantiomer					
R	4.65 ± 0.13	4.23 ± 0.17	2.67 ± 0.09	0.50 ± 0.02	1.01 ± 0.03
S	4.60 ± 0.09	4.22 ± 0.22	2.68 ± 0.09	0.50 ± 0.03	1.02 ± 0.04
Racemic mixture					
R	4.39 ± 0.26	4.13 ± 0.22	2.58 ± 0.09	N.D.[a]	N.D.[a]
S	4.47 ± 0.22	4.04 ± 0.22	2.64 ± 0.07	N.D.[a]	N.D.[a]

a) Not determined. Each value represents mean ± S.D. ($n = 3$).

Table 2. Rate Constants of Propranolol Prodrug Hydrolysis in Plasma, Intestine and Liver Homogenates

	Rate constant (min^{-1} mg protein^{-1})				
	Acetate	Propionate	Butyrate	Pivalate	Succinate
Plasma					
Enantiomer ($\times 10^3$)					
R	15.91±0.51	21.29±0.61	8.69±0.53	2.06±0.10	0.16±0.01
S	2.91±0.12*	2.81±0.10*	1.73±0.08*	1.16±0.07*	0.18±0.01
Racemic mixture ($\times 10^3$)					
R	6.72±0.34	8.87±0.38	4.76±0.11	N.D.	N.D.
S	2.00±0.11*	3.02±0.09*	1.52±0.06*	N.D.	N.D.
Intestine					
Enantiomer					
R	0.51±0.01	4.88±0.11	13.68±0.62	0.23±0.01	$5.03\times10^{-3}\pm0.07\times10^{-3}$
S	1.02±0.02*	8.16±0.18*	15.88±0.51*	0.29±0.02*	$6.15\times10^{-3}\pm0.07\times10^{-3}$*
Racemic mixture					
R	0.42±0.02	2.68±0.04	8.30±0.11	N.D.	N.D.
S	0.94±0.05*	4.51±0.09*	9.58±0.08*	N.D.	N.D.
Liver					
Enantiomer					
R	0.11±0.004	0.89±0.02	1.66±0.08	0.18±0.01	$2.19\times10^{-3}\pm0.09\times10^{-3}$
S	0.34±0.006*	2.52±0.11*	3.31±0.13*	0.18±0.01	$2.34\times10^{-3}\pm0.11\times10^{-3}$
Racemic mixture					
R	0.07±0.002	0.87±0.02	1.36±0.07	N.D.	N.D.
S	0.20±0.004*	2.02±0.09*	2.99±0.12*	N.D.	N.D.

N.D. not determined. Each value represents mean±S.D. ($n=3$). * $p<0.05$ *versus* (*R*)-isomer.

DISCUSSION

In our previous studies,[14,15] the stereoselective hydrolysis of the acetate ester of PL was demonstrated in human serum and in rat tissues. We also reported that the stereoselectivity of this differed in various tissues (*i.e.*, rat plasma, liver or intestine). It is well known that the relative activity of esterases changes in accordance with different ester moieties on the substrate. Salmona *et al.*[19] suggested that oxazepam succinate was stereoselectivity hydrolyzed, and that the stereoselectivity of this reaction differed between liver microsomes and brain homogenates. To study the effects of ester moieties on the stereoselective hydrolysis of propranolol prodrugs, five prodrugs were synthesized and used. The concentration of the substrate used was determined from the total blood concentration after oral administration of acetate and succinate esters in rats.[5]

In phosphate buffer, no differences in the hydrolysis rates between the esters of (*R*)- and (*S*)-propranolol were observed in any prodrug (Table 1). In rat plasma and tissue homogenates, however, hydrolysis was accelerated and stereoselectivity was observed (Table 2), also, the hydrolysis rates differed with different ester moieties. In the intestine and liver homogenates, the hydrolysis rate constants tended to increase in proportion to increases in acyl C-chain length (C_1 to C_3) of the prodrugs. The same tendency was reported for a purified esterase from human intestine and liver using α-naphthyl esters as substrate.[20] In plasma, the maximum hydrolysis rate among the prodrugs used was observed for the propionate ester. The hydrolysis rates decreased as steric hindrance and hydrophobicity increased in the present study. This may have been due to a decrease in the affinity of the prodrug for the esterases. Among the enzyme solutions used in the present study, the maximum hydrolysis rate was observed in the rat intestine homogenate. This may indicate the possibility that the prodrugs are rapidly hydrolyzed after oral administration.

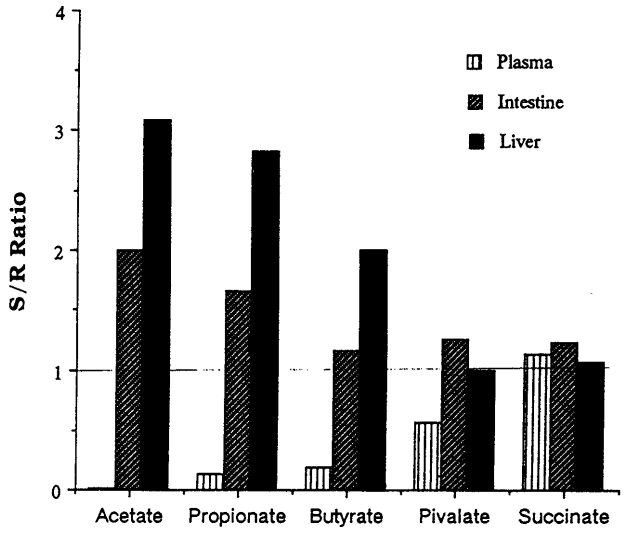

Fig. 1. Enantiomeric Ratios of Rate Constant of Hydrolysis for Various Esters of Propranolol in Rat Tissue Homogenates

The *S*/*R* ratio was calculated from the rate constants of pure isomers shown in Table 2.

Opposite stereoselective hydrolysis was observed in plasma and intestine/liver homogenate (Fig. 1). In the intestine and liver homogenates, the *S*/*R* ratio decreased as the length of the alkyl chain of the ester increased, and this *S*/*R* ratio in the intestine homogenate was higher than that in the liver homogenate. However, the ratio in plasma increased as the length of the alkyl chain of the ester increased. For the pivalate and succinate esters, the *S*/*R* ratio was almost 1, except for that of the pivalate ester in

palsma, suggesting that these prodrugs may have low affinity for the esterases in these crude preparations.

The esterases, as a group, can hydrolyze a wide range of endogenous as well as exogenous ester substrates.[21,22] Many esterases have been purified from tissues,[23-29] and various esterases may have different catalytic activity and stereoselectivity toward esters of different structures. But there has been no report of stereoselectivity hydrolysis using those esterases. Anderson *et al.*[5] suggested that the oral bioavailability of PL was significantly increased when PL succinate and acetate were used as the prodrugs. They also suggested that the prodrugs were absorbed from the gastrointestinal tract in the intact form, since they were detected in blood. In the present study, two types of hydrolysis were observed; the stereoselective hydrolysis (acetate, propionate and butyrate esters), and the non-stereoselective hydrolysis (pivalate and succinate esters). Further enzymatic investigation may be needed to clarify the stereoselective hydrolysis properties of esterases.

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