7. Hanhijärvi H, Ylinen M, Kojo A and Kosma V-M, Elimination and toxicity of perfluorooctanoic acid during subchronic administration in the Wistar rat. *Pharmacol Toxicol* **61**: 66–68, 1987.
8. Reddy JK and Lalwani ND, Carcinogenesis by hepatic peroxisome proliferators: evaluation of the risk of hypolipidemic drugs and industrial plasticizers to humans. *CRC Crit Rev Toxicol* **12**: 1–58, 1983.
9. Hawkins JM, Jones WE, Bonner FW and Gibson GG, The effect of peroxisome proliferators on microsomal, peroxisomal, and mitochondrial enzyme activities in the liver and kidney. *Drug Metab Rev* **18**: 441–515, 1987.
10. Rao MS and Reddy JK, Peroxisome proliferation and hepatocarcinogenesis. *Carcinogenesis* **8**: 631–636, 1987.
11. Kawashima Y, Katoh H, Nakajima S, Kozuka H and Uchiyama M, Effects of 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid on peroxisomal enzymes in rat liver. *Biochem Pharmacol* **33**: 241–245, 1984.
12. Kawashima Y, Horii S, Matsunaga T, Hirose A, Adachi T and Kozuka H, Co-induction by peroxisome proliferators of microsomal 1-acylglycerophosphocholine acyltransferase with peroxisomal β-oxidation in rat liver. *Biochim Biophys Acta* **1005**: 123–129, 1989.
13. Ubel FA, Sorenson SD and Roach DE, Health status of plant workers exposed to fluorochemicals—a preliminary report. *Am Ind Hyg Assoc J* **41**: 584–589, 1980.
14. Griffith FD and Long JE, Animal toxicity studies with ammonium perfluorooctanoate. *Am Ind Hyg Assoc J* **41**: 576–583, 1980.

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Plasma protein binding and blood cell distribution of propranolol enantiomers in rats

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Although propranolol (PL*) is commercially available as a racemic mixture, (−)-PL is about 100 times more potent than (+)-PL as a β-blocker [1]. It is, therefore, clinically important to clarify any enantiomeric differences in the disposition of PL. As factors which contribute to the stereochemistry of the delivery and actions of β-blocking drugs, Walle *et al.* [2] have stressed the processes involving hepatic metabolism, plasma protein binding, and the storage and release of β-antagonists by adrenergic nerve endings. A number of studies have demonstrated stereoselectivity in the hepatic metabolism of PL [3–5], but limited information is available on the distribution of the two PL enantiomers. As the volume of distribution (V_d) of PL depends on the binding of PL to plasma protein [6], the difference in plasma protein binding between the two PL enantiomers may produce a distinct difference in their distribution patterns. Several reports have already demonstrated the stereoselective plasma protein binding of PL in humans as well as dogs [7–9]. However, all of the studies investigated stereoselectivity at only one constant concentration (around 100–150 ng/mL). Therefore, it is not clear whether the difference between the PL enantiomers in plasma binding is due to the difference in binding affinity, to the capacity, or to both parameters, and also whether or not enantiomer–enantiomer interaction exists at the binding site(s) in the plasma. The purpose of the present study was to clarify the characteristics of the stereoselective plasma protein binding observed in rats by applying a competitive inhibition model and to investigate the effect of stereoselective plasma binding of PL enantiomers on their distribution into blood cells.

Methods

Materials. (±)-PL hydrochloride and (−)-penbutolol sulfate were obtained from I.C.I.-Pharma Ltd. (Osaka, Japan) and Hoechst Japan Ltd. (Tokyo, Japan) respectively. Free (±)-PL base was resolved into its enantiomers by fractional crystallization of its diastereomer, which was obtained by reaction with di-*p*-toluoyl-L-(+)tartaric acid [10]. The hydrochloride salts of both enantiomers were then prepared. The purities of the enantiomers, as confirmed by optical density, melting point and HPLC resolution, were over 92 and 95% for (+)- and (−)-PL respectively.

Plasma protein binding. ^{3}H-Labeled (±)-PL (sp. act. 26.6 Ci/mmol; Amersham International Ltd.) was resolved into (+)-[^{3}H]PL and (−)-[^{3}H]PL (radiochemical purity 98%) by adopting a procedure similar to that used for measuring PL enantiomers in biological fluids and tissues as reported previously [11], employing a Chiralcel OD analytical column (Daicel Chemical Industries, Tokyo, Japan) with variable-wavelength UV (280 nm, Shimadzu SPD-2A) and radioisotope (Beckman 171) detectors. Binding of PL to rat plasma was measured by ultrafiltration (Ultrafree C3-LGC, Nihon Millipore Ltd., Tokyo, Japan). The influence of the column separation of (±)-[^{3}H]PL and the evaporation of the eluent on the binding properties of PL was assessed by comparing the binding of racemic [^{3}H]PL with that of a pseudoracemate, which was prepared by combining equal amounts of each radiolabeled enantiomer resolved [12]. The difference between f_p obtained with racemic [^{3}H]PL and that with a pseudoracemate was not statistically significant at a concentration of 15.4 μM (N = 4). Pooled heparinized plasma was obtained from male Wistar rats (body weight 220–320 g). A tracer amount of (+)-^{3}H-labeled PL enantiomer and the unlabeled (+)-PL were added to 0.5 mL of plasma to yield a final plasma (+)-PL concentration ranging from 0.386 to 38.6 μM. Following incubation at 37° for 5 min, the plasma was centri-

* Abbreviations: PL, propranolol; C_p, plasma total concentration of PL enantiomer; C_f, plasma free concentration of PL enantiomer; C_b, plasma bound concentration of PL enantiomer; f_p, plasma free fraction of PL enantiomer; N_p, binding capacity of plasma protein; K_d, dissociation constant of PL enantiomer–plasma protein complex; α, proportionality constant for non-specific binding of PL enantiomer; I_t, plasma total concentration of inhibitor (the opposite PL enantiomer); I_f, plasma free concentration of inhibitor; I_b, plasma bound concentration of inhibitor; K_i, dissociation constant of inhibitor–plasma protein complex; β, proportionality constant for non-specific binding of inhibitor; C_B, blood concentration of PL enantiomer; C_{bc}, blood cell concentration of PL enantiomer; Hc, hematocrit; R_b blood-to-plasma concentration ratio of PL enantiomer; and K_p^{bc}, blood cell-to-plasma free concentration ratio of PL enantiomer.

fuged at 7000 rpm for 10 min at 37°. Radioactivity was counted in a liquid scintillation counter (LSC-700, Aloka), and correction for quenching was done by external standardization. The experiment was repeated in the presence of (−)-PL at concentrations of 1.16 and 11.6 μM. The plasma protein binding of (−)-PL was carried out at the same concentration range [from 0.386 to 38.6 μM (−)-PL] and the same inhibitor concentrations [1.16 and 11.6 μM (+)-PL] as those employed in the experiments for (+)-PL. In a preliminary experiment, no significant adsorption of PL onto the membrane was observed (0.3% at 11.6 μM and 0.1% at 1.16 μM on the average). 4-Hydroxy PL (10.9 μM to 1.09 M), one of the main metabolites of PL in rats and possessing β-blocking activity comparable to that of PL [13], had no effect on the plasma protein binding of PL over a concentration range of 0.386 to 11.6 μM PL. The binding parameters were calculated by the non-linear least squares method [14], according to the following equations [15], which were derived from the Scatchard plots shown in Fig. 1:

$$C_b = (N_p \times C_f/K_d + C_f) + \alpha C_f \quad (1)$$

where C_f and C_b are the free and the bound concentrations of PL enantiomers in plasma, respectively; N_p is the binding capacity of plasma protein; K_d is the dissociation constant of the PL enantiomer–plasma protein complex; and α is the proportionality constant for non-specific binding of PL enantiomer. If it is assumed that only the specific binding site is inhibited competitively in the presence of the opposite enantiomer of PL,

$$C_b = [N_p \times C_f/K_d(1 + I_f/K_i) + C_f] + \alpha C_f \quad (2)$$

where I_f is the free concentration of the inhibitor (the opposite PL enantiomer) and K_i is the dissociation constant of the inhibitor–plasma protein complex. If the binding of inhibitor at the specific binding site is also assumed to be inhibited in a similar manner,

$$I_b = [N_p \times I_f/K_i(1 + C_f/K_d) + I_f] + \beta I_f \quad (3)$$

where I_b is the bound concentration of the inhibitor and β is the proportionality constant for the non-specific binding of the inhibitor. Then, the total inhibitor concentration (I_t) can be expressed as:

$$I_t = I_b + I_f. \quad (4)$$

Rearrangement of equations (3) and (4) gives,

$$(\beta + 1)I_f^2 + \{N_p - I_t + K_i(\beta + 1 + C_f/K_d + \beta C_f/K_d)\} I_f - I_t \times K_i(1 + C_f/K_d) = 0 \quad (5)$$

Therefore,

$$I_f = [-q + \{q^2 + 4I_t \times K_i(\beta + 1) \times (1 + C_f/K_d)\}^{\frac{1}{2}}]/2(\beta + 1) \quad (6)$$

where

$$q = N_p - I_t + K_i(\beta + 1 + C_f/K_d + \beta C_f/K_d). \quad (7)$$

The values of N_p, K_d, K_i, α and β were obtained by simultaneous curve-fitting of C_f, C_b and I_t to equations (1) and (2) combined with equations (6) and (7).

Blood cell distribution. Various amounts of (+)-PL (1.93 to 19.3 μM) were added to 2.0 mL of blood from the pooled heparinized sample. The blood was centrifuged after incubation at 37° for 30 min. The experiment was repeated in the presence of (−)-PL at concentrations of 1.93 and 19.3 μM. The experiments to determine the blood cell distribution of (−)-PL were carried out by employing a procedure identical to that for (+)-PL. The plasma concentrations of PL enantiomers were determined by chiral stationary-phase liquid chromatography with fluorescence detection using (−)-penbutolol sulfate as an internal standard. Accuracy and recovery of the analysis were excellent, as the between-day coefficient of variation (*c.v.*) was 1.6% for (+)-PL and 2.6% for (−)-PL and the average recovery from rat plasma was 102.1% for (+)-PL and 98.6% for (−)-PL. The analytical method for PL enantiomers has been described in detail elsewhere [11]. Then, an aliquot (0.5 mL) of plasma was used for the determination of plasma protein binding of PL enantiomer as described above. The blood cell-to-plasma free concentration ratio (K_{pf}^{bc}) for PL enantiomers was calculated using the following equations [15, 16]:

$$R_b = C_B/C_p \quad (8)$$

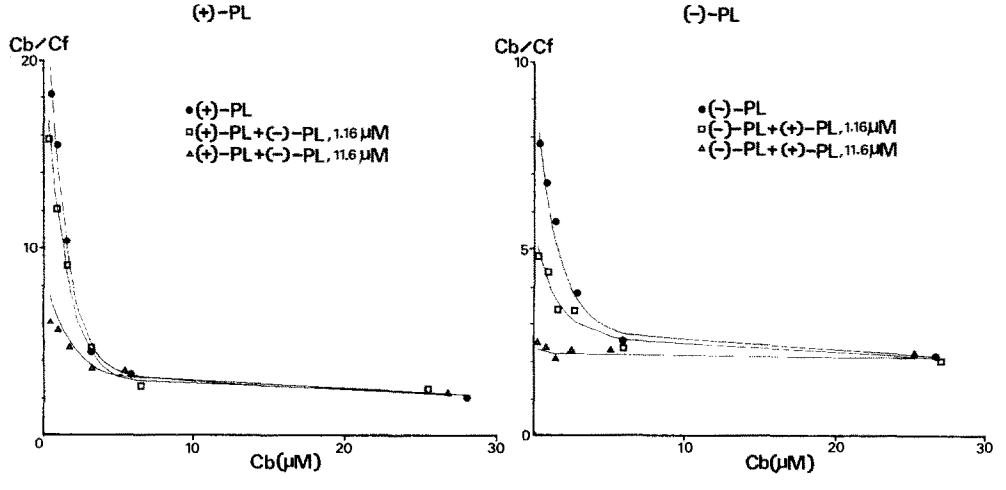

Fig. 1. Left panel: representative Scatchard plots of the binding of (+)-propranolol alone and in the presence of (−)-propranolol at concentrations of 1.16 and 11.6 μM. Right panel: plots of the binding of (−)-propranolol alone and in the presence of (+)-propranolol at concentrations of 1.16 and 11.6 μM. The curves were drawn with a computer simulation. N_p values (± SD), generated by the computer, were 1.992 ± 0.672 and 1.654 ± 0.234 for (+)- and (−)-PL, and K_d values (± SD) were 0.093 ± 0.116 and 0.231 ± 0.053 for (+)- and (−)-PL respectively.

$$C_{bc} = \{C_B - (1-\mathrm{Hc})C_p/\mathrm{Hc} \quad (9)$$

$$K_{pf}^{bc} = C_{bc}/C_f = \{C_B/C_p - (1-\mathrm{Hc})\}/f_p \times \mathrm{Hc} \quad (10)$$

where C_B, C_{bc} and C_p are PL enantiomer concentrations in blood, blood cells and plasma, respectively; Hc is the hematocrit; and f_p is the plasma free fraction of PL enantiomer. An average distribution of each PL enantiomer into blood cell was obtained in duplicate.

Statistical analysis. To compare the binding parameters of enantiomers to plasma, statistical analysis was performed using Student's *t*-test. For all the analyses, the minimum level of statistical significance was taken as $P < 0.05$.

Results and discussion

Plasma protein binding of PL enantiomers. The free percentage of both enantiomers showed a concentration dependency, that of (−)-PL being significantly ($P < 0.05$) higher than that of (+)-PL over the concentration range observed, which was opposite to previous observations in humans and dogs [7–9]. This non-linear plasma binding of PL enantiomers was also observed *in vivo* in rats [17]. Since PL is the highly cleared drug, unbound concentrations and thereby pharmacological response after i.v. administration would be sensitive to change in plasma binding [18]. The curvilinear Scatchard plots shown in Fig. 1 indicate the presence of two distinctive binding sites for both (+)- and (−)-PL. Therefore, specific and non-specific binding sites were assumed for the calculation of binding parameters. Considering the decreased slopes and intercepts of the Scatchard plots of (+)- and (−)-enantiomers by the presence of each opposing enantiomer (Fig. 1), only the specific binding site in both (+)- and (−)-PL appears to be inhibited competitively, rather than non-competitively. The extent of interaction between enantiomers depended on the concentration of the opposite enantiomer. The binding parameters obtained by a competitive inhibition model are listed in Table 1. The average binding capacity (N_p) was 1.951 μM for (+)-PL and 1.633 μM for (−)-PL, respectively, and the dissociation constant (K_d) was 0.087 μM for (+)-PL and 0.253 μM for (−)-PL, respectively. These values were in the same order as those obtained from *in vitro* binding of PL racemate (N_p, 2.6 μM; and K_d, 0.21 μM) [16] and *in vivo* binding of PL enantiomers in rats [17]. There was no significant difference in the capacities for either specific (N_p) or non-specific (α and β) binding between (+)- and (−)-PL. On the other hand, K_d was significantly larger in (−)-PL than in (+)-PL ($P < 0.05$), indicating that the specific binding site had a higher affinity for (+)-PL than for (−)-PL. Therefore, the affinity rather than the capacity in the specific binding site seems to be responsible for the higher binding percentage of (+)-PL to plasma proteins compared with (−)-PL. Such difference in affinity for plasma protein has also been reported for disopyramide enantiomers [12]. Although the calculated values of K_i showed a large variance for both (+)- and (−)-PL, (+)-PL appears to be a far more potent displacer than (−)-PL in rat plasma [$K_i = 1.672$ in the presence of (−)-PL and $K_i = 0.045$ in the presence of (+)-PL, which is contrary to the case for binding of PL enantiomers to human α_1-acid glycoprotein [19], indicating a species difference in the stereoselective plasma protein binding of PL enantiomers. This species difference in the stereoselective plasma binding of PL between humans and rats is important to recognize, because the rat is commonly used in drug evaluations as a substitute for humans.

Table 1. Binding parameters of propranolol enantiomers to plasma protein

Parameter	(+)-PL	(−)-PL
N_p (μM)	1.951 ± 0.085	1.633 ± 0.132
K_d (μM)	0.087 ± 0.006	0.253 ± 0.060*
K_i (μM)	1.672 ± 0.149	0.045 ± 0.045†
α	2.259 ± 0.200	2.195 ± 0.380
β	2.235 ± 0.424	5.290 ± 2.448

Abbreviations: N_p, binding capacity of plasma protein; K_d and K_i, dissociation constants of propranolol enantiomer– and inhibitor–plasma protein complex; α and β, proportionality constants for the non-specific binding of PL enantiomer and inhibitor respectively. Data are means ± SD of three experiments.

* $P < 0.05$, compared (+)-PL.

† $P < 0.01$, compared to (+)-PL.

Table 2. Distribution of propranolol enantiomers into blood cells

	C_B (μg/mL)	C_p (μg/mL)	R_b	f_p	K_{pf}^{bc}
(+)-PL	0.5	0.584	0.856	0.046	14.7
	2.5	2.003	1.248	0.088	17.7
	5.0	2.859	1.749	0.199	13.4
(+)-PL + (−)-PL (0.5 μg/mL)	0.5	0.580	0.862	0.054	13.0
	2.5	1.702	1.469	0.128	16.0
	5.0	3.242	1.542	0.210	10.5
(+)-PL + (−)-PL (5.0 μg/mL)	0.5	0.534	0.936	0.085	10.1
	2.5	1.745	1.433	0.178	11.0
	5.0	3.223	1.552	0.211	10.5
(−)-PL	0.5	0.392	1.277	0.101	15.9
	2.5	1.616	1.547	0.127	17.5
	5.0	2.799	1.786	0.171	16.1
(−)-PL + (+)-PL (0.5 μg/mL)	0.5	0.359	1.394	0.116	16.2
	2.5	1.203	2.079	0.178	19.1
	5.0	2.757	1.814	0.223	12.6
(−)-PL + (+)-PL (5.0 μg/mL)	0.5	0.312	1.603	0.201	11.6
	2.5	1.111	2.250	0.318	11.9
	5.0	2.050	2.439	0.299	14.0

Abbreviations: C_B and C_p, the concentrations of propranolol enantiomers in blood and plasma; f_p, plasma free fraction of propranolol enantiomer; $R_b = C_B/C_p$; $K_{pf}^{bc} = R_b - (1 - \mathrm{Hc})/(f_p \times \mathrm{Hc})$; and Hc, hematocrit.

Distribution of PL enantiomers into blood cells (Table 2). The blood-to-plasma concentration ratio (R_b) showed the same concentration dependency as observed with f_p for both (+)- and (−)-PL. Because the values of C_f/C_{bc} (the reciprocal of K_{pf}^{bc}) were almost constant over the concentration range observed, the binding of PL enantiomers to blood cells would be a linear process. Consequently, the nonlinearity observed in C_B/C_p can be explained largely by that in the plasma protein binding of PL enantiomers. Therefore, in the presence of the opposite enantiomer, the increased f_p observed at a low blood concentration of (+)- and (−)-PL, as a consequence of enantiomer–enantiomer interaction in plasma protein binding, may produce the increased R_b. The red blood cell-to-plasma free concentration ratio (K_{pf}^{bc}) remained almost constant over the concentration range observed and showed no significant difference between PL enantiomers. The mean values (± SD) were 13.0 ± 2.7 for (+)-PL and 15.0 ± 2.6 for (−)-PL. Our results suggest that the uptake and/or binding of PL by red blood cells is linear over this concentration range and not stereoselective in nature. These findings from rats are consistent with those observed previously in humans and dogs [9, 20]. However, the values of K_{pf}^{bc} reported in dogs and humans (around 2.5 and 3.1) were much smaller than those in rats. Since it has been reported that f_p has almost the same values in humans and rats at therapeutic concentrations [6], a species difference in the uptake and/or binding process(es) to blood cells is suggested.

In conclusion, the stereoselective plasma protein binding observed in rats is due mainly to the difference in the affinity for plasma protein rather than the capacity between PL enantiomers. Moreover, the two enantiomers would appear to compete with each other for a specific binding site on plasma, and (+)-PL may be the more potent displacer in rats. Furthermore, the results of blood cell distribution indicated that the concentration dependency in plasma protein binding would be largely responsible for the nonlinearity of the blood-to-plasma concentration ratio (R_b) observed for both of the PL enantiomers, and no stereoselectivity existed in the distribution of PL enantiomers into blood cells (K_{pf}^{bc}) over the concentration range employed. Therefore, the apparent difference in the value of R_b between PL enantiomers (R_b:(+)-PL < (−)-PL) seems to be caused mainly by the stereoselective plasma protein binding of the PL enantiomers (f_p: (+)-PL < (−)-PL). These results are in good agreement with those observed in the tissue distribution study of the PL enantiomers in rats, where plasma protein binding of the PL enantiomers was a major determinant of their stereoselective tissue distribution [17].

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Department of Biopharmaceutics
Meiji College of Pharmacy
Tanashi-Shi
Tokyo 188, Japan

HARUMI TAKAHASHI
HIROYASU OGATA*

REFERENCES

1. Barrett AM and Cullum VA, The biological properties of the optical isomers of propranolol and their effects on cardiac arrhythmias. *Br J Pharmacol* **34**: 43–55, 1968.
2. Walle T, Webb JG, Bagwell EE, Walle UK, Daniell HB and Gaffney TE, Stereoselective delivery and actions of beta receptor antagonists. *Biochem Pharmacol* **37**: 115–124, 1988.
3. von Bahr C, Hermansson J and Lind M, Oxidation of (*R*)- and (*S*)-propranolol in human and dog liver microsomes. Species differences in stereoselectivity. *J Pharmacol Exp Ther* **222**: 458–462, 1982.
4. Silber B, Holford NHG and Riegelman S, Stereoselective disposition and glucuronidation of propranolol in humans. *J Pharm Sci* **71**: 699–704, 1982.
5. Nelson WL and Bartels MJ, N-Dealkylation of propranolol in rat, dog, and man. Chemical and stereochemical aspects. *Drug Metab Dispos* **12**: 345–352, 1984.
6. Evans GH, Nies AS and Shand DG, The disposition of propranolol. III. Decreased half-life and volume of distribution as a result of plasma binding in man, monkey, dog and rat. *J Pharmacol Exp Ther* **186**: 114–122, 1973.
7. Walle UK, Walle T, Bai SA and Olanoff LS, Stereoselective binding of propranolol to human plasma, α_1-acid glycoprotein, and albumin. *Clin Pharmacol Ther* **34**: 718–723, 1983.
8. Albani F, Riva R, Contin M and Baruzzi A, Stereoselective binding of propranolol enantiomers to human α_1-acid glycoprotein and human plasma. *Br J Clin Pharmacol* **18**: 244–246, 1984.
9. Bai SA, Walle UK, Wilson MJ and Walle T, Stereoselective binding of the (−)-enantiomer of propranolol to plasma and extravascular binding sites in the dog. *Drug Metab Dispos* **11**: 394–395, 1983.
10. Howe R and Shanks RG, Optical isomers of propranolol. *Nature* **210**: 1336–1338, 1966.
11. Takahashi H, Kanno S, Ogata H, Kashiwada K, Ohira M and Someya K, Determination of propranolol enantiomers in human plasma and urine and in rat tissues using chiral stationary-phase liquid chromatography. *J Pharm Sci* **77**: 993–995, 1988.
12. Lima JJ, Interaction of disopyramide enantiomers for sites on plasma protein. *Life Sci* **41**: 2807–2813, 1987.
13. Fitzgerald JD and O'Donnell SR, Pharmacology of 4-hydroxypropranolol, a metabolite of propranolol. *Br J Pharmacol* **43**: 222–235, 1971.
14. Yamaoka K, Tanigawara Y, Nakagawa T and Uno T, A pharmacokinetic analysis program (MULTI) for microcomputer. *J Pharmacobiodyn* **4**: 879–885, 1981.
15. Harashima H, Sawada Y, Sugiyama Y, Iga T and Hanano M, Analysis of nonlinear tissue distribution of quinidine in rats by physiologically based pharmacokinetics. *J Pharmacokinet Biopharm* **13**: 425–440, 1985.
16. Yasuhara M, Fujiwara J, Kitade S, Katayama H, Okumura K and Hori R, Effect of altered plasma protein binding on pharmacokinetics and pharmacodynamics of propranolol in rats after surgery: Role of alpha-1-acid glycoprotein. *J Pharmacol Exp Ther* **235**: 513–520, 1985.
17. Takahashi H, Ogata H, Kanno S and Takeuchi H, Plasma protein binding of propranolol enantiomers as a major determinant of their stereoselective tissue distribution in rats. *J Pharmacol Exp Ther*, in press.
18. Rowland M and Tozer TN, *Clinical Pharmacokinetics: Concepts and Applications*. Lea & Febiger, Philadelphia, PA, 1980.
19. Brunner F and Müller WE, The stereoselectivity of the 'single drug binding site' of human α_1-acid glycoprotein (orosomucoid). *J Pharm Pharmacol* **39**: 986–990, 1987.
20. Olanoff LS, Walle T, Walle UK, Cowart TD and Gaffney TE, Stereoselective clearance and distribution of intravenous propranolol. *Clin Pharmacol Ther* **35**: 755–761, 1984.

* All correspondence should be addressed to: Professor Hiroyasu Ogata, Department of Biopharmaceutics, Meiji College of Pharmacy, Yato 1-22-1, Tanashi, Tokyo 188, Japan.