Accumulation Kinetics of Propranolol in the Rat: Comparison of Michaelis–Menten-Mediated Clearance and Clearance Changes Consistent with the "Altered Enzyme Hypothesis"

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Received November 2, 1992; accepted September 27, 1993

(+)-Propranolol was infused at two rates into the pyloric vein (a portal vein tributary) of 15 male Sprague Dawley rats until apparent steady-state conditions were established (i.e., 8 hr at each rate). One group ($n = 7$) received the high dose (40 μg/min/kg) first, and in the other group ($n = 8$) the low dose (20 μg/kg/min) was used to initiate treatment. Free and total serum concentrations of propranolol were measured. When the low dose was given first, the apparent steady-state concentrations achieved during low- and high-rate infusion steps were 166 ± 37 and 774 ± 235 ng/mL, respectively. These data are consistent with a simple Michaelis–Menten kinetic model and the key parameters of such a model (V_{max} and K_m) were estimated. However, a crucial test of such a model (and one which should give insight regarding the relevance of an "altered enzyme hypothesis") is to reverse the order of infusion steps since, in a system controlled by Michaelis–Menten kinetics, the same steady-state concentrations should be achieved regardless of the order in which infusion steps are given. When the sequence of infusion rates was reversed, steady-state concentrations were 492 ± 142 and 298 ± 79 ng/mL for the high and low infusion rates, respectively. Clearly, a history of high-dose exposure reduces the intrinsic clearance of total drug (CL_{ss}) during a subsequent low-dose exposure (i.e., the apparent steady-state levels during the low-dose pyloric vein infusions were significantly different; $P < 0.001$). When these data were corrected for plasma protein binding, the same trends emerged. For example, the intrinsic clearance of free drug ($CL_{u_{ss}}$) during low dose treatment, when this treatment was given first, was 27.4 ± 7.3 L/min/kg. However, when the low dose was given as the last step, $CL_{u_{ss}}$ was only 14.4 ± 5.6 L/min/kg. This highly statistically significant decrease in $CL_{u_{ss}}$ ($P < 0.002$) is inconsistent with any simple Michaelis–Menten model, but it is consistent with an "altered enzyme hypothesis."

KEY WORDS: propranolol; Michaelis–Menten; altered enzyme hypothesis; intraportal infusion; rat.

INTRODUCTION

Propranolol is often used as a model substrate for the study of the hepatic extraction of high-intrinsic clearance drugs in man and laboratory animals (1). However, even after extensive investigation, considerable controversy exists regarding the characteristics of an adequate model to describe key elements of its disposition. For example, several authors (2,3) have suggested that a simple Michaelis–Menten model with either a single pooled V_{max} (maximum rate of metabolism) and K_m (plasma concentration at which the elimination rate is equal to 0.5 V_{max}) (2) or a similar model with multiple parallel Michaelis–Menten processes (3) provides an excellent description of steady-state plasma concentrations observed in humans treated for several days at each of a series of ascending doses. Also consistent with Michaelis–Menten kinetic behavior is the observation that sustained-release formulations of propranolol almost invariably exhibit a systemic bioavailability which is 50–70% of that observed with conventional-release tablets (2,4). Since it might be anticipated that lower delivery rates of drug to the liver could increase the efficiency of hepatic extraction for a drug which exhibits Michaelis–Menten kinetics, this observation is also consistent with, but not proof of, such behavior. However, another theory subsequently referred to as the "altered enzyme hypothesis" is capable of explaining these data and is inconsistent with the notion that the Michaelis–Menten equation adequately describes the elimination of this drug. Some of the evidence which supports this hypothesis includes the observation that when radiolabeled propranolol is administered to rats, a portion of the injected label appears to be covalently bound to hepatic microsomal proteins and these hepatic microsomes exhibit a selective reduction in the rate at which they catalyze the formation of aromatic oxidation products from propranolol (5). Of significance is the fact that these changes in oxidation are not well described by a Michaelis–Menten equation and that recent *in vitro* studies using human hepatic microsomal preparations have yielded similar findings (6). Furthermore, a "threshold dose" (i.e., a dose below which no measurable drug appears to enter the body) for propranolol has been reported in humans (7), dogs (8), and rats (9,10). At least in the rat, these data have been explained on the basis of tight but reversible intrahepatic binding of the parent drug (9). Clearly, consideration of such thresholds must be added to any comprehensive model of the pharmacokinetics of propranolol (9). Fortunately, in *in vivo* studies focusing on the elimination kinetics of propranolol, the consequences of such threshold effects can be minimized by performing experiments under (near-) steady-state conditions. Indeed studies at steady state can allow differentiation between a system whose pharmacokinetics are dominated by enzyme saturation (Michaelis–Menten system) and one in which the drug treatment appears to have altered the metabolic enzyme system such that a history of pretreatment decreases the total drug metabolizing activity of the liver.

Because of the implications of Michaelis–Menten pharmacokinetic behavior for propranolol dosing and the frequent use of propranolol as a model substrate, the disposition of this drug was studied following prolonged infusion to rats. The results of these experiments support the "altered enzyme hypothesis" and argue against the notion that Michaelis–Menten kinetics adequately explain the disposition of propranolol in this rodent model.

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0724-8741/94/0300-0420$07.00/0

MATERIALS AND METHODS

Reagents

(+)-Propranolol-HCl and 4-methyl propranolol-HCl were kindly supplied by Ayerst Laboratories Inc. (New York, NY). Heptane-sulfonic acid was purchased from Sigma Chemical Corp. (St. Louis, MO), and ether for anesthesia from J. T. Baker Inc. (Jackson, TN). All other reagents and HPLC grade solvents were supplied by Fisher Scientific Co. (Pittsburgh, PA).

Animal Experiments

Male Sprague Dawley rats weighing between 190 and 235 g were used. Six days before the study, catheters were implanted into the pyloric vein and the right jugular vein under light ether anesthesia. Both catheters were constructed from a section of polyethylene tubing (PE 50) and either a segment of more narrow (PE10) polyethylene tubing (for the pyloric vein cannula) or a segment (ID, 0.025 in.) of Silastic tubing (for the jugular vein cannula). The rats were fasted for 2 hr before and during the study but had free access to water. (+)-Propranolol-HCl was dissolved in saline and infused via the pyloric vein cannula for a total of 16 hr. Infusion rates of 20 and 40 μg/min/kg were used. Each step was 8 hr in duration. Group A rats (n = 8) received (+)-propranolol HCl at a dose of 20 μg/min/kg as the first step and 40 μg/min/kg as the second step. Group B animals (n = 7) received these infusion steps in reversed order. An infusion pump (Model 940, Harvard Apparatus) was employed to provide constant drug delivery at rates of 0.0034 and 0.0068 mL/min. Blood samples (180–250 μL) were collected via a right jugular vein cannula before and at 30, 120, 240, 320, 360, 400, 440, 480 (immediately before changing the infusion rate), 510, 600, 720, 800, 840, 880, 920, and 960 min after the start of the infusion. All samples were obtained after withdrawal of blood (approx 150 μL) to clear the catheter. After sample acquisition, the presample blood was reinjected, followed by 300 μL of saline. These injections were given to minimize artifacts caused by contamination, dehydration, or excessive blood loss. Blood was centrifuged for 5 min immediately after it was taken from the rat and serum was stored at −20°C. The hematocrit of the animals in each group fell during the experiment from an average of 36% to an average of 24%. Twelve hours after the end of the experiment and immediately before the rats were sacrificed, a large blood sample (approx 7 mL) was obtained from the abdominal aorta of each animal (under light ether anesthesia) for studies of the plasma protein binding of propranolol. The placement of the portal vein catheter was checked by injection of a methylene blue solution (0.2 mg/mL, v/v), with a change in liver color and the absence of leakage into the peritoneum being used as indicators of proper catheter placement (11).

Propranolol Assay

Serum concentrations of propranolol were determined using a reverse-phase HPLC assay with fluorescence detection as described previously (11). The fluorescence response was simultaneously recorded on a Model 3392 A integrator (Hewlett Packard, Avondale, PA) and on a strip-chart recorder (Omni Scribe Recorder, Houston Instruments, Austin, TX). If the area-to-height ratio of most of the chromatographic peaks was less than 0.64 (an indication of deteriorating column performance), then peak area values were used to construct standard curves. If the ratio was greater than 0.64, then the peak height ratio was used since it was found to yield better-quality standard curves (correlation coefficients were greater). Propranolol and 4-methyl propranolol eluted with retention times of 6 and 8.75 min, respectively. Linear propranolol standard curves were obtained over a concentration range of 20–600 ng/mL. Serum samples with defined propranolol concentrations unknown to the analyst were routinely assayed together with the study samples to provide an unbiased assessment of the performance of the assay. The average error in the estimated concentration of these unknown samples was ±8%.

Plasma Protein Binding

The binding of propranolol to rat serum proteins was evaluated using the micropartition Centrifree ultrafiltration technique (Amicon Corp., Cambridge, MA) (11). Propranolol was added to 1 mL of serum to yield a final concentration of about 2000 ng/mL and the pH of the sample was adjusted to 7.40 with 0.5 N phosphoric acid (10–50 μL per sample). Immediately after pH adjustment, the tubes were sealed and centrifuged at 1500g for 10 min at 25°C. The volume of the filtrate recovered was 200–250 μL. Propranolol was extracted and the concentration was measured as described above. Linear standard curves were obtained in the range of 5 to 25 ng/mL for free concentrations of propranolol (samples for standard curves were prepared in phosphate buffer).

Based on previous detailed studies of the relationship between the propranolol-free fraction and the plasma concentration of total drug (12), it is probable that animals 6 days postsurgery would exhibit a free fraction of 300–500 ng/mL, which is 50–70% of that we measured at the spiked concentration (2000 ng/mL).

Calculations

The intrinsic clearance of free propranolol ($CL_{u_{ss}}$) was calculated under apparent steady-state conditions from the relationship

$$CL_{u_{ss}} = \frac{k_o}{fC_{ss}} \quad (1)$$

where k_o is the rate at which drug was infused into the pyloric vein (a convenient tributary which flows directly into the portal vein), C_{ss} is the steady-state concentration of total drug (estimated from the mean of the propranolol concentrations at 400, 440, and 480 min into each infusion step), and f is the free fraction in serum (estimated at a total drug concentration of 2000 ng/mL). The ratio of k_o/C_{ss}, which could be called the intrinsic clearance of total drug (CL_{ss}), was estimated because it is a useful parameter from the perspective of mass balance considerations. The apparent values for V_{max} and K_m were estimated using the relationship (13)

$$k_o = V_{max} - K_m \cdot k_o/C_{ss} \quad (2)$$

Student's t test for unpaired samples was used for statistical

comparison, taking $P < 0.05$ as the minimal level of significance. Unless stated otherwise, all data are presented as the group mean ± 1 standard deviation.

RESULTS AND DISCUSSION

The propranolol serum concentrations observed during the two infusion protocols are summarized in Fig. 1. Steady-state appeared to be reached during each step of each protocol and Tables I and II contain the data for each individual animal with respect to steady-state serum concentration, free fraction, intrinsic clearance of free drug, and intrinsic clearance of total drug.

The data show that the apparent steady-state concentrations achieved are highly dependent on the sequence of infusion rates. For example, if the low dose (20 μg/min/kg) was infused as the first step, a steady-state concentration of 166 ± 37 ng/mL was achieved. If the same infusion rate was given as the second step, the steady-state concentration was almost doubled (298 ± 79 ng/mL; significantly different at the $P < 0.001$ level). The calculated intrinsic clearances of total drug for the low infusion rate were 0.126 ± 0.031 and 0.073 ± 0.025 L/min/kg if given as the first step and second step, respectively (statistically different at the $P < 0.003$ level). Correction of these values for the free serum concentration of propranolol also resulted in a statistically significant difference between the two groups of animals ($P < 0.002$). The same effect was observed for the higher infusion rate. The steady-state concentrations were 492 ± 142 and 774 ± 235 ng/mL if given as the first step and second step, respectively (statistically different at the $P < 0.02$ level). The corresponding intrinsic clearances of total drug and free drug were different at the $P < 0.02$ and $P < 0.08$ level, respectively.

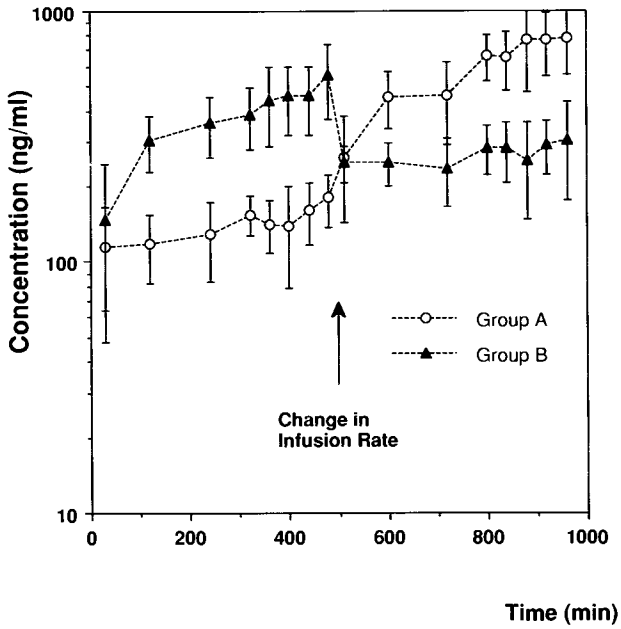

Fig. 1. Time course of propranolol serum concentrations. The data are presented as the observed mean (±SD) serum concentrations of (+)-propranolol in rats which received (a) 20 μg/min/kg for 480 min as the first infusion step and 40 μg/min/kg from 480 to 960 min for the second step (group A; ○) and (b) 40 μg/min/kg for 480 min as the first infusion step and 20 μg/min/kg from 480 to 960 min as the second step (group B; ▲). For purposes of comparison, it has been reported (2) that human volunteers receiving 320 mg of (+)-propranolol per day (or ≈5 mg/kg/day) achieved average steady-state serum concentrations of approximately 120 ng/mL.

Table I. Apparent Steady-State Serum Concentration of Total Propranolol[a,b] (ng/mL): Data from Individual Animals

Animal no.	Steady-state serum concentration (ng/mL): During low-rate (20 μg/min/kg) infusion steps	During high-rate (40 μg/min/kg) infusion steps
Group A	First step	Second step
A1	179	844
A2	183	886
A3	171	606
A4	165	602
A5	135	944
A6	158	592
A7	233	1200
A8	105	518
Mean (SD)	166 (37.2)	774 (235)
Group B	Second step	First step
B1	309	613
B2	359	660
B3	412	625
B4	161	322
B5	264	347
B6	307	484
B7	274	389
Mean (SD)	298 (79.1)*	492 (142)**

[a] The estimates of steady-state serum concentrations were calculated as mean values of the concentrations at 400, 440, and 480 min after the initiation of each infusion step. This kinetic system appears to be so complex and dynamic that any practical nonlinear regression model unlikely could predict the time course of changes in serum concentration that would result from changes in the rate and/or route of drug input. Therefore this simple approach was used.

[b] That the average serum concentration–time curves were quite flat at the end of each infusion step is suggested by the data in Fig. 1. However, it should be noted that there was a modest tendency for the serum concentration to increase between 400 and 480 min into each infusion step. When simple linear regression analysis of these data points is performed on the 30 sets of available data, the average slope over this time interval is +0.129 ± 0.297%/min; i.e., 11 of 30 sets of data had negative slopes and 19 had positive slopes. Based on this calculation, it is reasonable to assume that 8 hr of infusion allows a reasonable approach to steady-state.

* $P < 0.001$ for comparison of group A with group B (20 μg/min/kg).

** $P < 0.02$ for comparison of group A with group B (40 μg/min/kg).

The free fractions of propranolol in rat serum determined *in vitro* were very low (0.003–0.007) and this caused concentrations in the ultrafiltrate to be near the sensitivity limit of the assay. Furthermore, based on the data of Yasuhara and co-workers (12), it can be assumed that the free fraction for propranolol at concentrations of 200–800 ng/mL (i.e., the total drug concentrations which are encountered *in vivo*) would be approximately 50–70% of those observed at

Table II. Intrinsic Clearance and Free Fraction of Propranolol: Data from Individual Animals

Animal no.	Low rate (L/min/kg)		High rate (L/min/kg)		Percentage free
	CL_{ss}	$CL_{u_{ss}}$	CL_{ss}	$CL_{u_{ss}}$	
Group A	First step		Second step		
A1	0.112	37.3	0.047	15.8	0.30
A2	0.109	19.5	0.045	8.1	0.56
A3	0.117	27.2	0.066	15.3	0.43
A4	0.122	28.3	0.067	15.5	0.43
A5	0.148	30.8	0.042	8.8	0.48
A6	0.127	17.3	0.068	9.3	0.73
A7	0.086	22.6	0.033	8.8	0.38
A8	0.190	35.9	0.077	14.6	0.53
Mean	0.126*	27.4**	0.055***	12.0****	0.48
SD	0.031	7.3	0.016	3.5	0.13
Group B	Second step		First step		
B1	0.065	19.0	0.065	19.2	0.34
B2	0.056	9.3	0.061	10.1	0.60
B3	0.049	8.0	0.064	10.5	0.61
B4	0.125	23.9	0.124	23.9	0.52
B5	0.076	12.6	0.115	19.2	0.60
B6	0.065	12.5	0.083	15.9	0.52
B7	0.073	15.2	0.103	21.4	0.48
Mean	0.073*	14.4**	0.088***	17.2****	0.52
SD	0.025	5.6	0.026	5.3	0.10

* $P < 0.003$, comparison of CL_{ss} estimates when 20 μg/min/kg infusions were given to groups A and B.
** $P < 0.002$, comparison of $CL_{u_{ss}}$ estimates when 20 μg/min/kg infusions were given to groups A and B.
*** $P < 0.02$, comparison of CL_{ss} estimates when 40 μg/min/kg infusions were given to groups A and B.
**** $P < 0.08$, comparison of $CL_{u_{ss}}$ estimates when 40 μg/min/kg infusions were given to groups A and B.

a total drug concentration of 2000 ng/mL (as used in the present study). Unfortunately, it was not practical to define precisely the relationships between total serum drug concentration and free fraction over a broad range of concentrations in individual rats because of limits on the amount of blood which could be taken. Thus our approximation of $CL_{u_{ss}}$ probably represents a moderate systematic underestimate of the true value of this parameter but these approximations should be satisfactory for between-group comparisons. Indeed, it can be concluded that when the same infusion rates are employed, the differences in $CL_{u_{ss}}$ between our two treatment groups are even larger than suggested by the data presented in Table II, since free fractions would be greater when total drug concentrations are greater (12,14). It is quite probable that the low free fractions of propranolol which were observed were the result of very high α-1 acid glycoprotein serum concentrations, which are known to be a consequence of the acute-phase response which follows surgery (12,14). The free fraction of propranolol is assumed to have been nearly stable during our 16-hr experiment 6 days after surgery because Hanano's group (14) reported that the propranolol free fraction is stable 36 hr after surgery, and unpublished preliminary results of our own group also support this assumption. Since blood loss was comparable in both groups of rats, no systematic bias should be introduced into the data using this sampling schedule. Furthermore, such a blood loss over 16 hr should neither influence the hepatic blood flow rate in rats (15) nor change the protein binding of propranolol (14).

In the past, several efforts to explain the nonlinear pharmacokinetics of propranolol in man have focused on the possibility that this drug exhibits simple Michaelis–Menten pharmacokinetics (2,3), therefore attempts to calculate pooled V_{max} and K_m values were made. Plausible results (i.e., positive numbers for V_{max} and K_m) were obtained from this analysis if data from group A were used. Based on these data, a V_{max} of 57 ± 6 μg/min/kg and a K_m value of 308 ± 82 ng/mL were obtained (see Fig. 2 and Table III). That this drug clearly does not exhibit simple Michaelis–Menten kinetics in the rat is shown by the data from group B (see Fig. 2), where the clearance either remained constant or *decreased* further after *decreasing* the infusion rate (i.e., negative K_m values were obtained in four of seven rats). Consistent with this finding is the "altered enzyme hypothesis," which assumes that changes in the metabolic enzyme system are caused by drug treatment such that the total drug metabolizing activity of the liver is decreased. The underlying biochemical event(s) responsible for this "enzyme alteration" is(are) not known. Realistically it can be speculated that an alteration in the heme moiety or the apoprotein (or both) could cause irreversible (or pseudo-irreversible) inhibition of one or more cytochrome P-450 isozymes, presumably while having little or no effect on other isozymes (hence the residual drug metabolizing capacity even after 16 hr of infusion; 28.8 mg/kg total dose). Indeed, there was sufficient evidence a decade ago to justify a review of heme alkylation in cytochrome P-450 by a wide variety of compounds including a number of lipophilic bases (16). Of particular interest

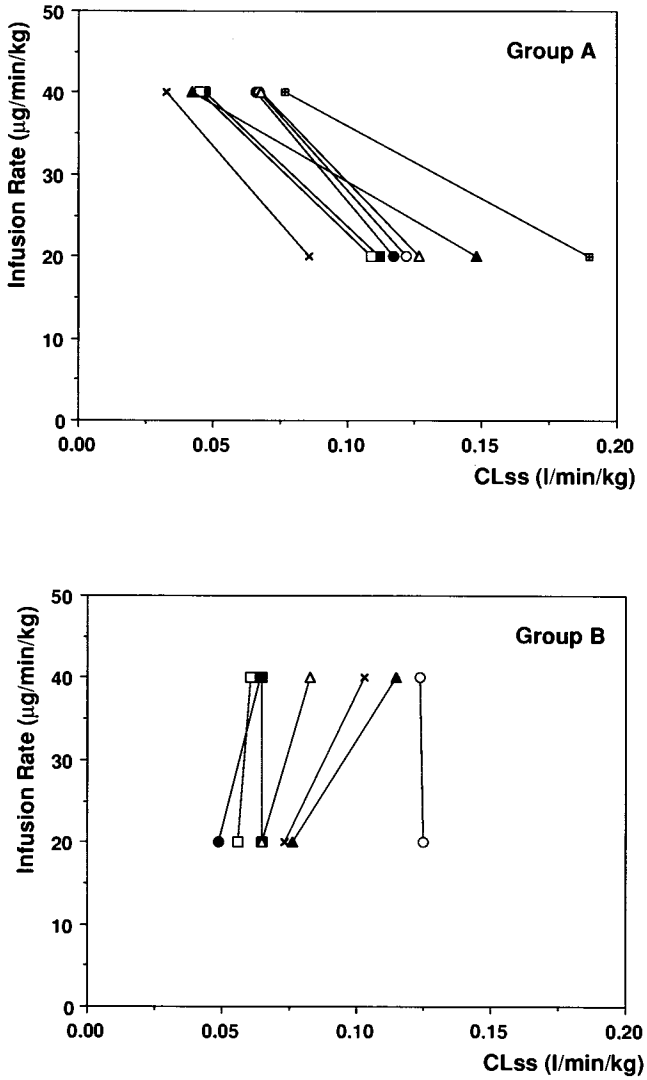

Fig. 2. Plot of infusion rate vs steady-state intrinsic clearance of propranolol. Relationship between the infusion rate and the ratio of infusion rate divided by steady-state serum concentration according to Eq. (2). Top: The individual data of group A rats (low-dose treatment first). Bottom: The individual data of group B rats (high-dose treatment first). Each different symbol in the upper and lower panel represents one individual animal. If simple Michaelis–Menten pharmacokinetics adequately described this system, the "y" intercept should be V_{max}/fraction of drug escaping first-pass metabolism and the slope of this line should be $-K_m$. Note: For the animals which received the high-dose treatment first, this parameter estimation technique suggests that the K_m is a negative number in four of seven rats (which is physically impossible).

are two recent reports (17,18). Osawa and Coon (17) found that the lipophilic tertiary amine phencyclidine or 1-(1-phenylcyclohexyl)-piperidine inactivated rabbit cytochrome P-450 form 2 while having virtually no effect on forms 3a, 3b, 4, and 6 (analogous to P450's CYP2E1, CYP2C3, CYP1A1, and CYP1A2 in man). If propranolol metabolism in the rat can be associated with a small number of P450 isozymes, it should be possible to identify which isoform or isoforms are affected. The data from the phencyclidine study (17) provide direct evidence that the heme moiety is altered by treatment

Table III. Michaelis–Menten Parameters Estimated for Each Animal in Group A

Animal no.	V_{max} (µg/min/kg)	K_m (ng/mL)
A1	55	310
A2	54	313
A3	66	391
A4	64	363
A5	48	190
A6	63	340
A7	53	380
A8	54	177
Mean	57	308
SD	6	82

with this lipophilic base (with an iminium ion intermediate being involved) and that there is also indirect evidence suggesting reaction with the apoprotein. Masubuchi *et al.* (18) concluded from their experiments in rats that repetitive oral administration of propranolol leads to tight binding of a reactive metabolite to one or more of the P450 isozyme(s) belonging to the CYP2D gene subfamily and that CYP2D enzymes might be involved in the metabolic pathway forming that metabolite. Thus if this selective inactivation of isozymes occurs, it may be possible to clarify the relevant molecular events that reduce the liver's ability to clear propranolol (5,6).

In conclusion, the results of this study demonstrate that the use of pooled Michaelis–Menten parameters is not adequate to predict steady-state concentrations of propranolol in the rat. The underlying cause for this behavior is still unknown. However, one mechanism consistent with the available data is that one or more propranolol metabolites covalently bind to a key component of the cytochrome P-450 system and that this results in the irreversible inhibition (5,6) of its enzymatic activity.

ACKNOWLEDGMENTS

This work was supported in part by Grant GM-20852 from the Institute of General Medical Sciences, National Institutes of Health and by a Grant from F. Hoffmann-La Roche Ltd., Basel, Switzerland.

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