Product Inhibition and Dose-Dependent Bioavailability of Propranolol in the Isolated Perfused Rat Liver Preparation

HANY GHABRIAL*, ROMINA NAND*, CHERYL K. STEAD*, RICHARD A. SMALLWOOD*, AND DENIS J. MORGAN‡x

Received August 29, 1991, from the *Department of Medicine, Repatriation Hospital, University of Melbourne, Melbourne, Victoria, Australia, and ‡Department of Pharmaceutics, Victorian College of Pharmacy, 381 Royal Parade, Parkville, Melbourne, Victoria, Australia. Accepted for publication March 7, 1994®.

Abstract □ We investigated in the isolated perfused rat liver (IPRL) whether product inhibition of metabolism contributes to the dose-dependent bioavailability of propranolol, a drug with a high, but saturable, hepatic first-pass effect. (±)-Propranolol was infused in the IPRL, using a recirculating design, for three 36-min periods (n = 9). Mean steady-state reservoir, i.e. hepatic inflow concentrations (C_{in}), were 4.97, 10.4, and 20.4 μM, respectively. Mean reservoir concentrations of the metabolites 4′-hydroxypropranolol, 5′-hydroxypropranolol, N-desisopropylpropranolol, and naphthoxylactic acid (NLA), a major side-chain-oxidation metabolite, increased disproportionately with propranolol dose, but their production rate did not reach steady state. In separate experiments (n = 4), perfusate containing 7.1, 12.8, and 21.6 μM (±)-propranolol, corresponding to administration rates of 114, 205, and 346 nmol/min, respectively, was passed through the liver for 30 min each using a single-pass design. The bioavailability (hepatic outflow concentration/C_{in}) of propranolol increased with C_{in} from 0.012 to 0.150 to 0.288 in the recirculating IPRL. In the single-pass IPRL the increase (0.0077 in 0.0669 to 0.136) was significantly less (P < 0.001). The greater bioavailability of propranolol in recirculating experiments was attributed to product inhibition since metabolites do not accumulate with the single-pass design. NLA did not appear to be the inhibiting metabolite because in further single-pass experiments with propranolol C_{in} of 21.6 μM the presence of NLA (21.6 μM) in perfusate had no effect on propranolol bioavailability (n = 7) compared with control experiments (n = 5). These data suggest that, with the recirculating IPRL, dose-dependent bioavailability of propranolol is due to competitive inhibition of propranolol metabolism by propranolol metabolites, which is distinct from the noncompetitive product inhibition that has been reported to accompany chronic propranolol administration.

Introduction

It has been stated that for most drugs that undergo substantial hepatic first-pass metabolism bioavailability will increase with increasing oral dose due to the high concentrations of drug entering the liver and saturating drug metabolism.[1,2] Similarly, bioavailability of such drugs can also increase when the drugs are given repeatedly, due to the higher concentrations of drug resulting from the accumulation that normally accompanies chronic drug administration.[1,2] Saturation of drug metabolism by high concentrations of the drug is known as substrate inhibition of drug metabolism. Dose-dependent bioavailability could also be caused by inhibition of metabolism of the parent drug by one or more of its metabolites as they accumulate (product inhibition).[3,4] The possibility of product inhibition does not appear to have been considered as a cause of the dose-dependent bioavailability of high first-pass effect drugs. It is difficult to distinguish between substrate and product inhibition because with product inhibition it is difficult to discern the relationship between the elimination rate of the parent drug and the concentration of the metabolic product, the latter varying with the parent drug concentration.[5]

Propranolol is a drug that undergoes extensive first-pass hepatic metabolism upon oral administration. In humans this drug exhibits increasing bioavailability with increasing single oral doses[6-8] and bioavailability increases when the drug is given repeatedly.[8-13] Dose-dependent bioavailability has also been observed *in vivo* in rats[14] and in the isolated perfused rat liver preparation.[15] Propranolol is metabolized by ring oxidation, side-chain oxidation, and glucuronidation in rat and human liver (Figure 1), with the rate of ring oxidation being the principal determinant of the drug's bioavailability in both species.[8,16-18]

The aim of this study was to determine whether product inhibition contributed to the dose-dependent bioavailability of propranolol in the isolated perfused rat liver preparation. This was done by comparing the dose dependence of propranolol bioavailability using a recirculating perfusate circuit, in which metabolites will accumulate, with that using a single-pass circuit, in which metabolites cannot accumulate in perfusate and re-enter the liver. We hypothesized that, if product inhibition of propranolol were present, the dose dependence of propranolol bioavailability using the single-pass design would be less pronounced compared with that using the recirculating circuit design, or even absent.

Experimental Section

Materials—(±)-Propranolol hydrochloride and labetalol hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO). α-Naphthoxylactic acid (NLA) was kindly donated by ICI Pharmaceuticals (Cheshire, England), and 4′-hydroxypropranolol (4OHP), 5′-hydroxypropranolol (5OHP), and N-desisopropylpropranolol (DIP) were synthesized as described previously.[19,20] Bovine serum albumin (Fraction V) was purchased from Commonwealth Serum Laboratories (Melbourne, Australia). β-D-Glucuronidase from helix pomatia type H-1 was purchased from Sigma Chemical Co. (St. Louis, MO) and contained a β-D-glucuronidase to sulfatase activity ratio of 20:1. All chemicals used were of analytical grade and all solvents used were HPLC grade and were purchased from Mallinckrodt Australia (Melbourne, Australia).

Experimental Preparation—The details of the procedure used have been described previously.[21] In brief, the livers of nonfasted male Sprague-Dawley rats (age, 6–8 weeks, weight; 193–278 g; mean weight, 236 ± 20 g) were surgically removed, transferred to a humidified glass chamber, and connected to the perfusion circuit. The 100-mL circuit was housed in a thermostatically controlled cabinet (37 °C). The perfusate consisted of a Krebs–Henseleit solution containing 10% washed human red cells, 0.1% glucose, and 1% albumin. In the recirculating design, stable bile flow was maintained by a constant infusion of sodium taurocholate (Calbiochem, San Diego, CA, 0.5 μmol/min) into the perfusate reservoir. In the single-pass design, taurocholate was added to the reservoir to a concentration of 30 μM. The perfusate was infused at 16 mL/min using a peristaltic pump (Model 2115 Multipurpex, LKB).

The preparation's viability was judged by monitoring oxygen consumption (>2.0 μmol/min per g of liver) bile flow (0.5–1.0 mL/h) and perfusion back-pressure (<5 cm H_2O).

Experimental Design—*Recirculating Design*—In a preliminary experiment to establish the stability of the preparation, a bolus of 2.6 μmol of propranolol was added to the reservoir and propranolol was then infused at the rate of 231 nmol/min for 90 min. Reservoir (C_{in}) and hepatic venous outflow (C_{out}) perfusate samples (1 mL) were collected

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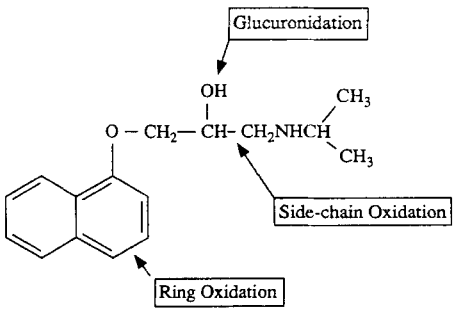

Figure 1—Primary pathways of propranolol metabolism.

every 7 min from 15 min. In nine liver preparations, with a 100-mL recirculating circuit, three sequential 36-min infusions of propranolol (77, 154, and 231 nmol/min) were administered into the reservoir in ascending order. Bolus doses of propranolol of 0.845, 1.54, and 2.63 μmol were added to the perfusate at the beginning of each period to ensure rapid attainment of steady state. Reservoir (1 mL) and hepatic venous outflow (2 mL) samples were taken at 15, 22, 29, and 36 min from the start of each infusion period, steady state having been attained by 15 min. An equal volume of drug-free perfusate was added to the reservoir after each sampling to compensate for loss due to sampling.

Single-Pass Design—In four preliminary experiments to establish the stability of the preparation, perfusate containing propranolol (21.6 μM) was passed through the liver for 90 min. In four liver preparations, three perfusates containing propranolol at concentrations of 7.1, 12.8, and 21.6 μM were passed through the liver sequentially for 30 min each in ascending order. These three concentrations resulted in propranolol administration rates of 114, 205, and 346 nmol/min, respectively. The hepatic venous effluent was allowed to drain to waste (i.e., single-pass mode). Perfusate samples were taken from the portal vein (C_{in}) (1 mL) and hepatic vein (C_{out}) (2 mL) at 15, 20, 25, and 30 min from the start of each period.

A further 12 liver preparations were perfused consecutively for two 40-min periods with two perfusates each containing 21.6 μM propranolol (administration rate of 346 nmol/min). In seven of the preparations the second perfusate also contained an equimolar concentration of NLA prepared by adding the NLA dissolved in 0.875 mL of NaOH solution (10 mM). In the other five preparations (controls), only 0.875 mL of 10 mM NaOH solution was added to the second perfusate. Portal and hepatic venous samples were taken at 20, 25, 30, and 40 min from the start of each period.

Varied Infusion Patterns—To determine whether the order of administration was important, in further recirculating ($n = 2$) and single pass ($n = 2$) preparations, the order of administration was altered such that the low infusion rate (recirculating = 77 nmol/min, single pass = 115 nmol/min) was given in the first period, the high infusion rate (recirculating = 231 nmol/min, single pass = 346 nmol/min) in the second period, and the low infusion rate again in the third period. In a further recirculating preparation, and a further single-pass preparation, no propranolol was administered during the third period, although sampling was continued. Sampling protocols were as described above.

Drug Analysis—Perfusate samples were collected into tubes containing 100 mg of ascorbic acid, protected from light and stored at −20 °C until assay. Propranolol, 4OHP, 5OHP, DIP, and NLA were assayed as previously described[22,23] by using HPLC with fluorimetric detection (excitation at 295 nm and emission at 360 nm for propranolol and NLA and 420 nm for 4OHP, 5OHP, and DIP). Perfusate samples were treated with β-glucuronidase and sulfatase prior to analysis for 4OHP and 5OHP.[22] The coefficient of variation for replicate perfusate assays ($n = 6$) of propranolol (0.2 μM) was 4.64%, and for 4OHP, 5OHP, DIP (all at 0.4 μM), and NLA (0.015 μM) the coefficient of variation was approximately 10%. At 7 μM concentration the coefficient of variation was approximately 5% for propranolol and each metabolite.

Calculations—Hepatic availability (F) of propranolol was calculated as C_{out}/C_{in}. The rate of metabolite formation was calculated as $Q(C_{out,m} - C_{in,m})$, where $C_{out,m}$ and $C_{in,m}$ are hepatic output and reservoir metabolite concentrations, respectively.

Data are presented as means ± the standard deviation. Statistical comparison was carried out using Student's t-test and regression analysis by least-squares linear regression. A probability of <0.05 was considered statistically significant.

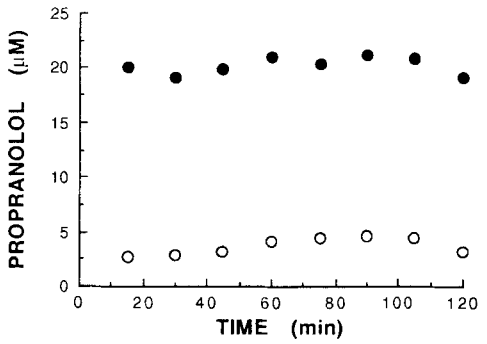

Figure 2—Perfusate propranolol concentrations in reservoir (hepatic inflow) (●) and hepatic venous outflow (○) during constant infusion of propranolol at 231 nmol/min into the reservoir of a recirculating isolated perfused rat liver preparation.

Results

Recirculating Design—In the preliminary experiment in which a 231 nmol/min propranolol infusion was continued for 90 min, stable C_{in} and C_{out} concentrations were attained within 20 min and did not rise during the 90-min period (Figure 2).

In the nine recirculating experiments in which three increasing infusion rates of propranolol were used, steady state was reached in all three periods by 20 min and this is shown for a single experiment in Figure 3A. The three infusion rates resulted in mean steady-state reservoir concentrations of 4.97, 10.4, and 20.4 μM, i.e., C_{in} increased 3–4-fold. However, mean C_{out} increased in a nonlinear manner, viz. 0.0597, 1.45, and 6.13 μM for the three infusion rates, respectively, i.e. a 100-fold increase, and this is illustrated for the single experiment in Figure 3A. Thus, bioavailability increased markedly with C_{in} (Figure 4). In five of these experiments perfusate metabolite concentrations were also measured. Figure 5 shows C_{in} and C_{out} for 4OHP, 5OHP, DIP, and NLA for a single experiment. Calculation of metabolite formation rates showed that formation rate had not reached steady state for any of the metabolites for any propranolol infusion rate. Oxygen consumption did not change significantly in the nine experiments, but there was a small but significant fall in perfusate pH, from 7.39 ± 0.10 at the beginning to 7.20 ± 0.10 at the end of the experiment ($P < 0.01$).

Single-Pass Design—In the four preliminary experiments in which the liver was perfused with propranolol (21.6 μM) for 90 min, C_{out} reached the steady state by 20 min and remained stable thereafter. Perfusion with propranolol C_{in} concentrations of 7.1, 12.8, and 21.6 μM (administration rates of 114, 205, and 346 nmol/min, respectively) for 30 min each resulted in the attainment of steady state by 20 min in each period, illustrated for a single experiment in Figure 3B. While C_{in} increased 3-fold, mean steady-state C_{out} values for each period again increased disproportionately, from 0.0338 to 0.283 to 1.73 μM, respectively (Figure 3B). However, the increase in C_{out} was not as marked as that observed with the recirculating design, and this was reflected in the respective increases in bioavailability (Figure 4). The slope of the plot of F versus C_{in} for the single-pass experiments was significantly less than that for the recirculating experiments (0.009 versus 0.017; $P < 0.001$). Oxygen consumption and outflow perfusate pH did not change significantly throughout the experiments.

In the seven further single-pass experiments, in which the effect of added NLA on propranolol bioavailability was examined, mean steady-state propranolol bioavailability during the first phase (propranolol C_{in} = 21.6 μM, administration rate = 346 nmol/min) was 0.114 ± 0.047. During the second phase, in which an equimolar concentration of NLA was present, mean steady-state propranolol bioavailability increased significantly to 0.181 ± 0.083 ($P < 0.019$). However, in the five control experiments

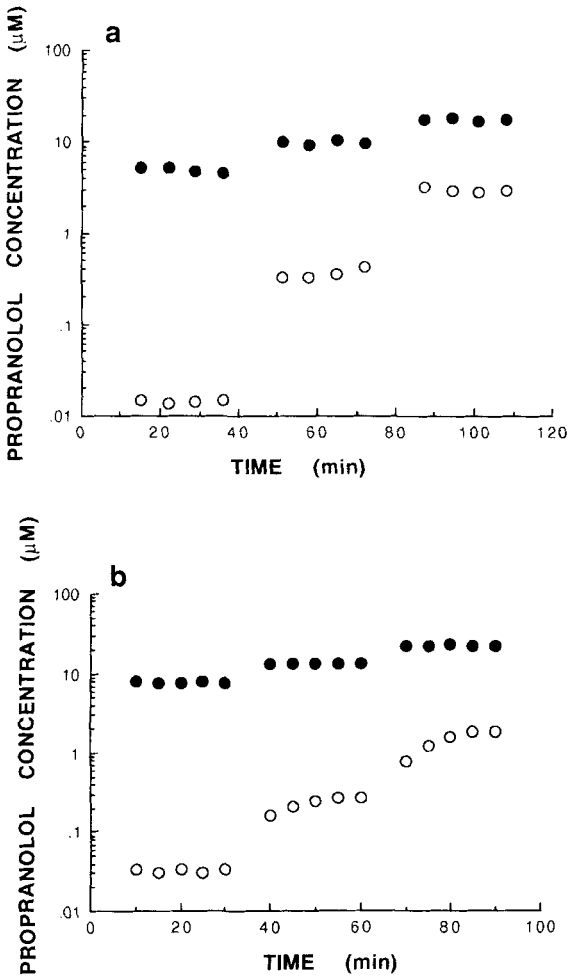

Figure 3—Perfusate propranolol concentrations in hepatic inflow (●) and hepatic venous outflow (O) (a) during three consecutive 36-min infusions of propranolol at 77, 154, and 231 nmol/min into the reservoir of the recirculating isolated perfused rat liver preparation and (b) during three consecutive 30-min perfusions of propranolol at perfusate inflow concentrations of 7.1, 12.8, and 21.6 μM (administration rates 115, 205, and 346 nmol/min, respectively) in the single pass isolated perfused rat liver preparation.

in which no NLA was present in the second phase, propranolol bioavailability also increased significantly from 0.0567 ± 0.037 in the first phase to 0.122 ± 0.042 in the second phase (P = 0.003). This suggests that the addition of NLA in the second phase of the first seven experiments was not the cause of the increase in propranolol bioavailability.

Varied Infusion Patterns—In both the recirculating and the single-pass designs, where the infusion rate was the same in the first and third phases, steady-state C_{out} in the third phase was approximately 10 times higher than in the first phase (Figure 6). In the experiments where no drug was given in the third phase, propranolol was found to have a half-life of 10.1 and 9.97 min in the recirculating and single-pass experiments, respectively. The elevated C_{out} in the third phase of the low–high–low dose experiments could not therefore be attributed to the persisting propranolol concentrations from the previous phase.

Discussion

In the present study, the bioavailability of propranolol in the isolated perfused rat liver preparation was markedly dose dependent in that a 3–4-fold increase in propranolol C_{in} resulted in about a 25-fold increase in bioavailability (i.e. C_{out}/C_{in}). This

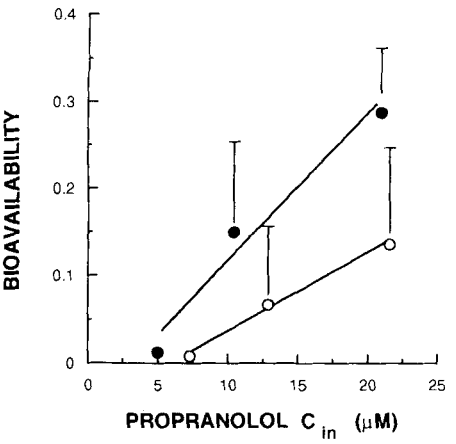

Figure 4—Relationship between hepatic bioavailability and propranolol C_{in} in recirculating experiments (●) ($y = 0.017x - 0.053$; $r^2 = 0.94$) and in single-pass experiments (O) ($y = 0.009x - 0.053$; $r^2 = 0.99$). Slope for single-pass experiments was significantly different than that for recirculating experiments, $P < 0.001$).

behavior is consistent with previous findings in humans,[6–8] *in vitro* in rats,[14] and in the isolated perfused rat liver preparation.[15,22]

When the dose dependence of propranolol bioavailability in the recirculating and single-pass experiments was compared, it was apparent that the dose dependence was more marked in the recirculating experiments. It was not possible to obtain reliable Michaelis–Menten parameters because there were only three doses used in each experiment. The increase in bioavailability with increasing C_{in}, which could be approximated to a straight line over the C_{in} range examined, was significantly greater in the recirculating than in the single-pass experiments (Figure 4).

We take these findings as a strong *prima facie* case for the presence of product inhibition in the recirculating experiments in addition to substrate inhibition. With the isolated perfused rat liver preparation, the recirculating design is analogous to drug administration *in vivo* in that drug metabolites can accumulate in perfusate and have the potential to re-enter the liver and compete with the parent drug for drug-metabolizing enzyme. By contrast, in the single-pass perfused liver, there are no metabolites in inflow perfusate since formed metabolites go to waste. Although endogenously produced metabolites could potentially inhibit metabolism of the parent drug before leaving the liver, less pronounced product inhibition of metabolism would be expected.

It could be argued that the difference between the recirculating and single-pass experiments could have been due to other factors, such as accumulation of endogenous substances, perfusate pH changes, or deterioration of the preparation in the recirculating experiments. However, control recirculating experiments showed that C_{out} was constant over a 90-min period during administration of propranolol at both the lowest (77 nmol/min)[15] and highest (231 nmol/min (Figure 2)) rates. Moreover, oxygen consumption was unchanged and perfusate pH decreased only slightly during the recirculating experiments. It was not possible to randomize the order of administration of the three propranolol doses because with both experimental designs C_{out} at the lowest dose was much greater when this dose followed the highest dose (Figure 6). It is unlikely that the order of dose administration could account for the difference between the findings in the recirculating and single-pass experiments, however, because the same order of administration was used in both sets of experiments. It is also possible that the difference between the recirculating and single-pass experiments could in some way be related to the use of racemic propranolol. However, we have performed several similar experiments with the individual enantiomers of propranolol and the difference was still apparent (unpublished data).

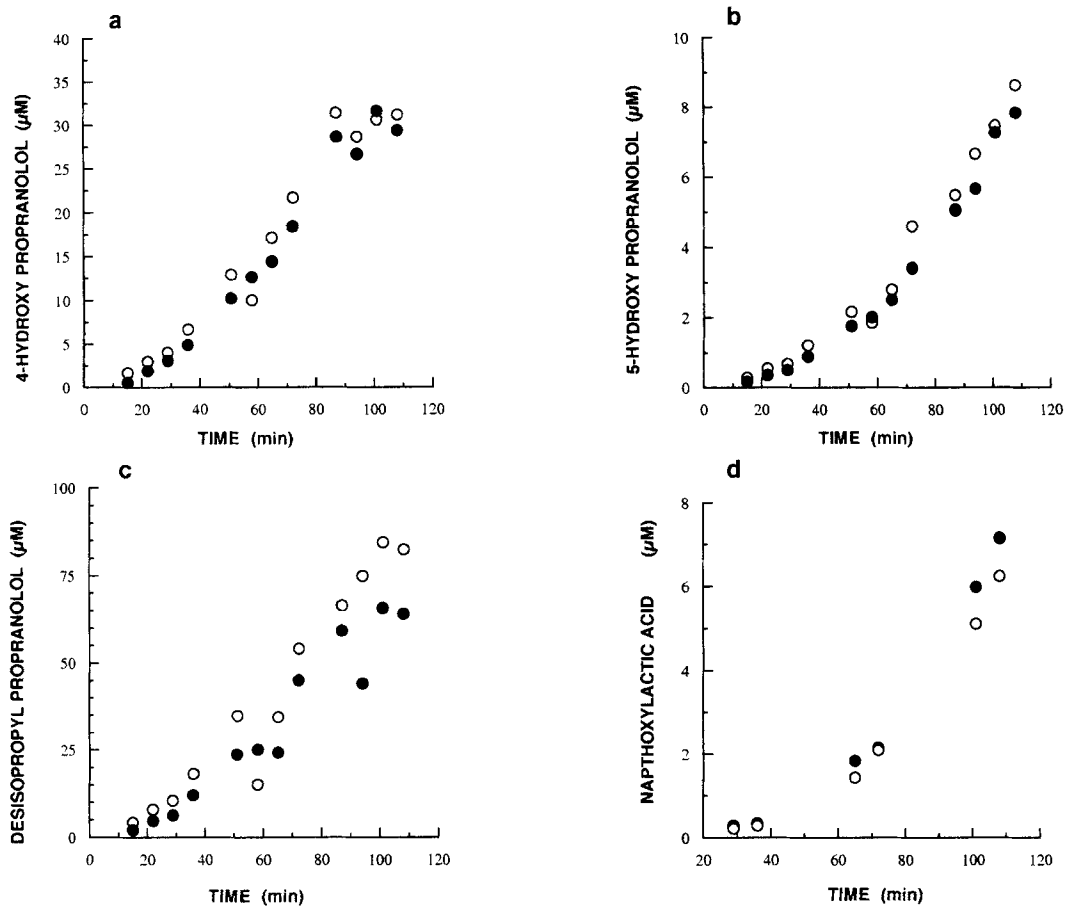

Figure 5—Perfusate metabolite concentrations in hepatic inflow (●) and hepatic venous outflow (○) during three consecutive 36-min infusions of propranolol at 77, 154, and 231 nmol/min into the reservoir of the recirculating isolated perfused rat liver preparation: (a) 4-hydroxypropranolol, (b) 5-hydroxypropranolol, (c) desisopropylpropranolol, (d) naphthoxylactic acid.

Propranolol is metabolized in rats and humans to a multitude of metabolites via three primary pathways, viz. ring oxidation, side-chain oxidation, and glucuronidation (Figure 1).[16,17] The main determinant of propranolol bioavailability in both rat and human is the rate of ring oxidation.[8,16,18] One of the main products of side-chain oxidation, NLA, can undergo ring oxidation,[17] but one of the main products of ring oxidation, 4OHP does not undergo side-chain oxidation. Moreover, the nonlinear bioavailability of propranolol in the perfused rat liver has been ascribed to saturation of ring oxidation.[22] Therefore, NLA and propranolol may compete with each other for ring oxidation. In recirculating experiments, perfusate C_{out} of each metabolite increased with increasing propranolol infusion rate (Figure 5). However, a large component of C_{out} for each metabolite is derived from previously formed metabolite which had recirculated via the reservoir (i.e. metabolite C_{in} also increased with increasing propranolol infusion rate, Figure 5). Despite the achievement of steady-state propranolol C_{in} and C_{out}, steady-state production was not reached for any of the four metabolites measured. This was probably due to slow hepatic uptake and/or release of these relatively polar metabolites. A further experiment was conducted in which the infusion of propranolol at the lowest rate was continued for 120 min and steady-state metabolite production still had not been reached by this time (unpublished data). It is therefore not possible to determine the mechanism of the postulated product inhibition from metabolite data in the recirculating experiments.

As an alternative approach, we examined the effect of added NLA on propranolol bioavailability in single-pass experiments at the highest propranolol inflow concentration (21.6 μM). An equimolar concentration of NLA was used, which was somewhat greater than the mean endogenous NLA concentration produced with the recirculating experiments (4.86 μM). This higher concentration was chosen in case exogenous NLA had reduced access to the hepatocyte. Although propranolol bioavailability increased from 0.114 to 0.181 upon addition of NLA, a similar change in the second phase was observed in those experiments in which only sodium hydroxide was added in the second phase. As control experiments had shown that bioavailability remained constant for 90 min without added NLA or sodium hydroxide, we conclude that the increase in bioavailability on addition of NLA was due to the sodium hydroxide added concomitantly. These experiments suggest that the postulated product inhibition in the recirculating experiments is not due to inhibition of ring oxidation by NLA. It was not possible to examine the effect of addition of other metabolites in single-pass experiments because insufficient quantities were available.

Evidence already exists that propranolol pretreatment results in selective inhibition of 4′-hydroxylation of propranolol in microsomal preparations from rats and humans.[24,25] This appears to be due to covalent binding to cytochrome P450IID1 of a reactive intermediate of propranolol oxidation.[26] It has been suggested[24] that this enzyme inactivation is the cause of the increase of propranolol bioavailability during chronic oral treatment[8-3] and the extended elimination half-life of propranolol (16–24 h) that follows discontinuation of chronic oral propranolol treatment.[11] It is not clear whether the difference in dose-dependent bioavailability between the recirculating and single-pass experiments in the present study could be explained by enzyme inactivation. The animals were not pretreated with propranolol, nor was there any evidence of a time-dependent change in propranolol elimination (Figure 2), as seen in the

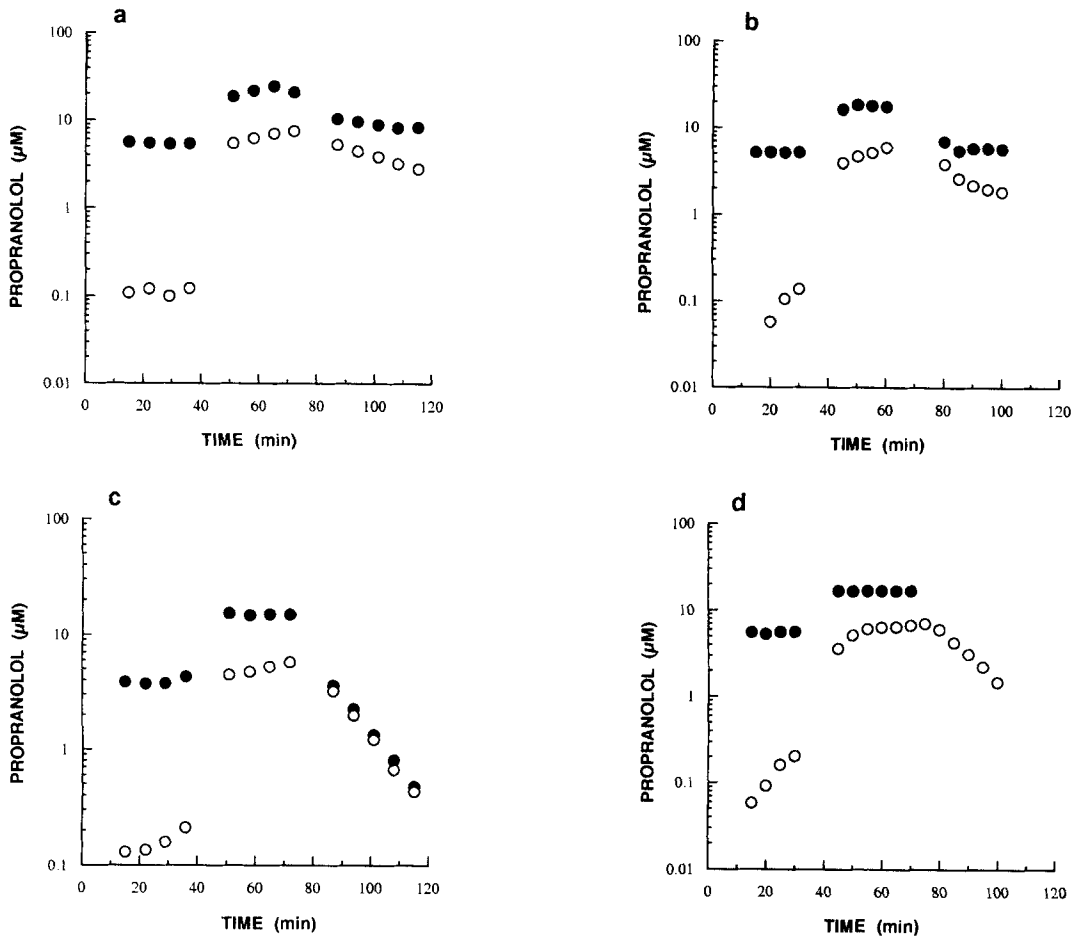

Figure 6—Perfusate propranolol concentrations in hepatic inflow (●) and outflow (○) (a) during three consecutive 36-min infusions of propranolol at 77, 154, and 231 nmol/min in a recirculating system, (b) during three consecutive 30-min perfusions at propranolol C_{in} of 7.1, 21.6, and 7.1 μM (administration rates 115, 205, and 346 nmol/min, respectively) in a single-pass system, and (c, d) same as in a and b, respectively, except no propranolol was administered in the third period.

previous studies.[24-26] These considerations argue in favor of the presence of reversible inhibition of propranolol metabolism in our perfused liver experiments. However, with both recirculating and single-pass designs, when the order of propranolol administration rate was low–high–low, steady-state C_{out} values in the third phase were approximately 10 times higher than in the first phase at the same dose rate (Figure 6). Moreover, the elimination half-life of propranolol in these experiments was similar (about 10 min). While this could be explained by the presence of noncompetitive inhibition in accordance with the mechanism described previously,[24-26] these observations are also consistent with persistence of the inhibiting metabolite(s) in perfusate and/or hepatic tissue.

Previous methods for detecting product inhibition include comparison of elimination rates in the absence and presence of added metabolite in isolated cell or microsomal preparations[27] or *in vivo* in animals[28] or humans[29] or, under certain conditions, looking for curvature of the plot of clearance versus reciprocal of steady-state plasma drug concentration.[5] Since the submission of this manuscript, Jaruratanasirikul et al.[30] identified product inhibition of debrisoquine by finding a decrease in systemic drug clearance with repeat drug dosage into the reservoir of the recirculating perfused rat liver preparation. Clearance returned to normal after a single-pass washout period. These methods address the possibility of product inhibition as a cause of nonlinear kinetics following single-dose intravenous administration, or as a cause of the decrease in clearance of certain drugs during chronic administration. They do not address the possibility of product inhibition as a cause of nonlinear bioavailability of orally administered, high-first-pass drugs. However, recently Chiba et al.,[31] using intraportal infusions of imipramine in rats, showed that the dose-dependent bioavailability of imipramine in rats could be adequately explained in terms of product inhibition of 2-hydroxylation of imipramine by its N-demethylated metabolite desipramine. Likewise, the unique experimental design used in the present study suggests that competitive product inhibition may also be significant in the first-pass hepatic extraction of propranolol, in addition to the noncompetitive inhibition that has been reported to accompany chronic propranolol administration.

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