Selective Effect of Adjuvant Arthritis on the Disposition of Propranolol Enantiomers in Rats Detected Using a Stereospecific HPLC Assay

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Nonstereospecific studies have indicated that the pharmacokinetics of propranolol (PR) are altered in inflammatory conditions such as arthritis. However, as the kinetics and dynamics of PR are stereoselective, we examined the effect of adjuvant arthritis (AA) on the disposition of the individual enantiomers. A novel normal-phase stereospecific HPLC assay for PR was developed involving chiral derivatization with *S*-(naphthyl)ethyl isocyanate and fluorescence detection. Oral and iv doses of racemic PR were administered to control and AA rats (n = 6). AA had no significant effect on either clearance or S:R ratio after iv doses. On the other hand, after oral doses, clearance was significantly decreased in AA. Although significant for both enantiomers, this effect was more pronounced on the less active R-enantiomer. The AUC R:S ratio was, therefore, significantly altered (AA, 14 ± 3.0; control, 4.3 ± 1.2). Increased total (S + R) plasma concentrations of PR in AA, possibly due to a reduced intrinsic clearance, therefore, reflect mainly increased concentrations of the less active R-enantiomer.

KEY WORDS: propranolol; inflammation; stereoselective; adjuvant arthritis; enantiomer; HPLC; pharmacokinetics.

INTRODUCTION

β-Adrenoceptor antagonists are frequently used in the treatment of cardiovascular disorders such as hypertension. Many hypertensive patients also have other common diseases such as arthritis. One of the most commonly prescribed drugs in this class, propranolol (PR), is highly bound to the acute-phase reactant, α-1-acid glycoprotein (AAG), concentrations of which are elevated in inflammatory conditions (1). PR also undergoes extensive metabolism. Thus, acknowledged pathophysiological changes in rheumatoid diseases such as increased AAG and decreased intrinsic clearance may significantly influence the disposition kinetics of drugs such as PR. Studies utilizing nonstereospecific methods have shown significantly higher than normal plasma concentrations of PR in patients with inflammatory diseases after oral administration (2–4). Similarly, 10-fold increases in PR concentrations after oral doses have been reported for rats with adjuvant-induced arthritis and turpentine-induced inflammation (5–7). These increases have been explained by both decreased metabolism and increased protein binding. Based on such findings, dosage adjustment in patients suffering from inflammatory conditions such as arthritis appears to be warranted. However, although PR is commercially available as a racemate, the antihypertensive activity of *S*-(−)-PR is about 100 times greater than that of the R-(+)-isomer (8). In addition to the pharmacological activity, the pharmacokinetics of PR are stereoselective in humans and rats (9–11). Therefore, as therapeutic activity is dependent mainly upon concentrations of the S-enantiomer, it is important to examine whether the observed changes in disposition kinetics of PR reflect those of the therapeutically more relevant enantiomer. The objective of this study was to examine the effect of adjuvant-induced arthritis (AA) on the stereoselective disposition of PR and report an HPLC method suitable for quantification of PR enantiomers.

MATERIALS AND METHODS

Animal Experiments. Female Lewis rats weighing approximately 150 g were intraperitoneally inoculated with 35 mg of heat-killed, freeze-dried *Mycobacterium butyricum* (Difco Lab, Detroit, MI) in 1 mL normal saline (n = 6). Control rats (n = 6) were not pretreated. Approximately 21 days after injection of adjuvant, mild to moderate swelling of the hind paws was evident in the AA rats. In AA all erythrocyte sedimentation rates, as measured using the Wintrobe method, were greater than 3 mm in the first hour. No physical changes were observed in the control rats. On the day of PR administration, the mean weight of the AA rats (220 ± 28 g) was slightly but not significantly less than that of controls (256 ± 36 g).

The day before administration of PR, Silastic catheters were implanted into the right jugular vein under light anesthesia with methoxyflurane and the animals were allowed to recover overnight. Racemic PR in saline solution was administered orally (30 mg/kg) and intravenously (2 mg/kg) to unanesthetized AA and control rats in a crossover fashion, with a 48-hr washout period between doses. Blood samples (175 μL) were collected through the jugular vein at 0, 15, 30, 45, 60, 120, 180, 240, and 360 min after oral and at 0, 5, 15, 30, 45, 60, 90, 120, 180, and 240 min after intravenous doses. The catheter was flushed with an equal volume of heparin in saline (100 U/mL) after each sample. Rats were fasted for about 16 hr prior to drug administration, with free access to water. Each blood sample was immediately centrifuged and the plasma was separated and stored at −20°C until assayed for PR enantiomers. Due to ethical reasons, only five of the six AA rats could be used for the pharmacokinetic study.

Assay. Enantiomers of PR were quantitated utilizing the following HPLC assay. To 0.1 mL of plasma, 50 μL of internal standard (bupranolol, 2.5 μg/mL; Logeais, Issy-les-Molineaux, France) and 250 μL of 0.2 *M* NaOH were added. The mixture was then extracted with 5 mL of diethyl ether, vortex-mixed (Vortex Genie 2 mixer; Fisher Scientific, Edmonton, Canada) for 30 sec, and centrifuged at 1800*g* for 5 min. The organic layer was transferred to clean tubes and evaporated to dryness using a Savant Speed Vac concentrator-evaporator (Emerston Instruments, Scarborough, Canada). The residue was then derivatized with 185 μL of 0.02% *S*-(+)-1-(−1-naphthyl)ethyl isocyanate (Aldrich, Milwaukee, WI) in chloroform:hexane (50:50) and vortex-mixed for 60 sec. Aliquots of 100 to 150 μL were injected onto the HPLC. The formed diastereomeric derivatives were chro-

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matographed using a mobile phase consisting of hexane:chloroform:methanol (75:25:0.4) pumped at 2 mL/min through a 25-cm stainless-steel silica column (Whatman Partisil 5; Clifton, NJ). The HPLC system consisted of a Model M-45 pump and a Model 712 WISP autosampler (Waters, Missisauga, Canada). The detector was a fluorescence Applied Biosystems Model 980 detector and was set at 225 and 280 nm for excitation and emission, respectively. Hexane, chloroform, diethyl ether, and methanol (BDH, Toronto, Canada) were all analytical grade.

Identification of peaks corresponding to PR isomers was accomplished by derivatization and chromatography of racemate and pure R- and S-enantiomers of PR (Sigma, St. Louis, MO). Linear calibration curves were prepared by least-squares regression analysis of peak-height ratios of PR enantiomers to internal standard against standard concentrations of PR.

Microsomal Isolation and Incubation. Microsomal oxidation reactions were carried out using fresh liver microsomes prepared as previously described (12) from livers of control and arthritic rats. Briefly, freshly obtained rat liver was immediately placed in ice-cold 100 m*M* phosphate–sucrose buffer (pH 7.4) and homogenized. Homogenates were centrifuged at 10,000*g* for 20 min. The supernatant was then collected and centrifuged at 105,000*g* for 60 min, resulting in a small protein pellet, which was suspended in buffer and again centrifuged at 105,000*g* for 60 min. The final pellet was resuspended in phosphate–sucrose buffer. Protein concentration was determined by the Lowry method (Sigma kit). The incubation reaction mixture was similar to that used by Fujita *et al.* (13) for microsomal oxidation of PR. Reaction mixtures (total volume, 3 mL) containing 5 m*M* $MgCl_2$, 10 m*M* glucose 6-phosphate, 0.75 m*M* $NADP^+$, 2 U/ml glucose 6-phosphate dehydrogenase, and 15 μg liver microsomes in 0.05 *M* Tris–HCl buffer (pH 7.4) were spiked with 2 mg/L racemic PR and shaken in a water bath at 37°C (reagents purchased from Sigma). At 0, 0.5, 1 and 2 hr after incubation, the reaction was stopped by transferring aliquots (0.5 mL) to tubes containing 0.5 mL 1 *N* NaOH. Samples were stored at −20°C until assayed for PR enantiomers. Microsomal metabolism of AA was compared to that of controls by measuring the percentage loss of PR versus time in the microsomal mixture.

Data Analysis. The area under the plasma concentration-versus-time curve (AUC) for each enantiomer was determined using the linear trapezoidal method. The extrapolated AUC from the time of the last plasma sample to time infinity was calculated as C_{last}/β. Elimination rate constants (β) were calculated using the regression slope of the log-linear terminal elimination phase. Systemic clearance (Cl_s) was calculated by dividing the administered intravenous dose with the corresponding AUC. The volume of distribution (V_d) was calculated by Cl_s/β. Assuming complete absorption, the oral clearance (Cl_o) was calculated by dividing the administered oral dose by the corresponding AUC. The Cl_s, Cl_o, and V_d were corrected for weight in all animals. Bioavailability (*F*) was calculated for each rat by dividing AUC after oral administration by that after intravenous administration, taking into account the doses used.

Differences between pharmacokinetic parameters of AA and those of control rats and differences between pharmacokinetic parameters of the individual enantiomers of PR were tested using two-tailed unpaired and paired Student's *t* tests, respectively ($\alpha = 0.05$). Data are expressed as mean ± standard deviation.

RESULTS

Assay

Chromatographic traces of blank rat plasma, with added PR and internal standard, and plasma following PR dosing are shown in Fig. 1. Peaks corresponding to R- and S-PR

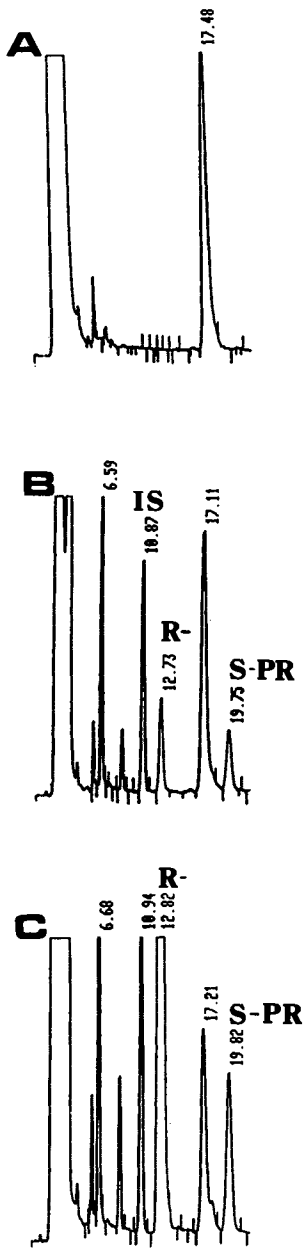

Fig. 1. HPLC chromatograms of (A) drug-free plasma, (B) plasma spiked with 500 ng/mL of racemic PR, and (C) a plasma sample taken 4 hr following oral administration of a 30 mg/kg racemic dose of PR in AA. Peaks at times 6.6 and 10.9 min, internal standard; at 12.7 min, R-PR; and at 19.8 min, S-PR.

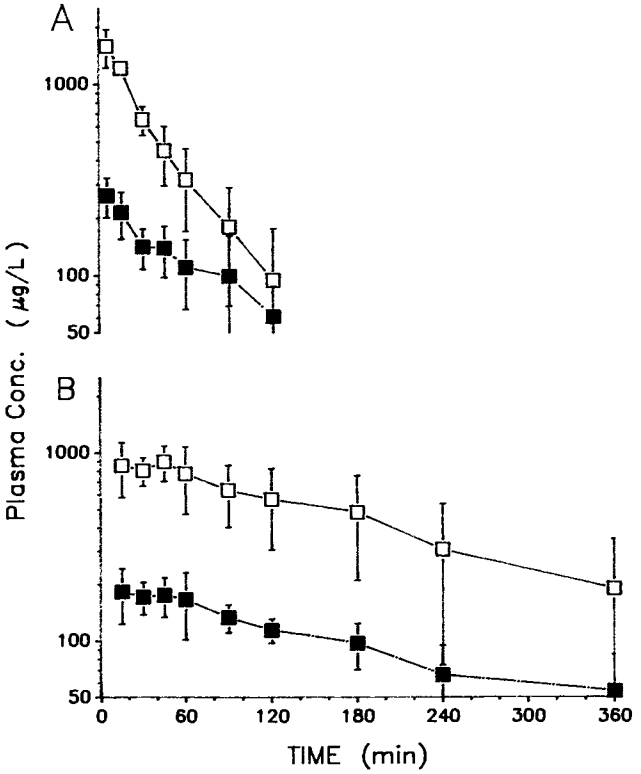

Fig. 2. Mean (n = 6) plasma concentration-versus-time curves in controls after (A) iv administration and (B) oral administration. (□) R-PR; (■) S-PR.

were free from any interference and eluted with a resolution factor >2 at approximately 12 and 19 min. Internal standard peaks eluted at approximately 6 and 10 min.

Calibration curves were linear over the concentration range of 50–10,000 ng/mL of PR enantiomers (correlation coefficient $r > 0.99$). Coefficients of variation less than 10% were determined for concentrations of 50, 500, 1000, and 2500 ng/mL (n = 6). However, using a signal-to-noise ratio of 4:1, the limit of detection was 10 ng/mL.

Pharmacokinetics

In order to account for first-pass metabolism, we chose larger doses of PR for oral administration as compared with iv doses. Accordingly, in the control rats, plasma levels after an oral dose of 30 mg/kg were comparable to those after a 2 mg/kg iv dose (Fig. 2). A pronounced stereoselectivity in the plasma concentrations was observed in all rats, with concentrations of R-PR being consistently greater than those of the active S-enantiomer.

After iv administration, the R:S concentration ratio was observed to decline with time, from 6.3 ± 1.4 at 5 min to 0.65 ± 0.86 at 180 min. Overall, the AUC of the R-enantiomer was significantly greater (AUC R:S, 3.0 ± 1.1) and its Cl_s was less than half that of S-PR. The V_d of S-PR was over six times greater than that of R-PR. Similarly, $t_{1/2}$ values of S-PR were longer than those of R-PR (Table I).

After oral administration of PR to control rats, an AUC R:S ratio of 4.3 ± 1.2 was observed. Similarly the peak plasma level (C_{max}) of R-PR was significantly greater than that of S-PR in all animals (C_{max} R:S, 4.9 ± 1.3), while the time to reach C_{max} (T_{max}) was not significantly different between the enantiomers (Table II). Similar $t_{1/2}$ values for R- and S-enantiomers were observed. These $t_{1/2}$ values, however, were longer than those observed after iv administration.

In all rats, the Cl_o of S-PR was significantly greater than that of its antipode. Hence, bioavailability was stereoselective and was calculated to be 21 ± 10% for R-PR and 12 ± 9% for S-PR in controls.

Effect of Adjuvant Arthritis

Intravenous Dose. As depicted in Table I, the disease did not affect the disposition of PR or the S:R ratio of PR in plasma (AUC S:R—AA, 0.39 ± 0.23; control, 0.37 ± 0.11) (Fig. 3).

Oral Dose. After oral administration, the Cl_o of both enantiomers was significantly decreased, resulting in a significantly greater AUC and bioavailability of both R- and S-PR (Fig. 4, Table II). Although significant for both enantiomers, this effect was more pronounced on the less active R-enantiomer: 13- and 4-fold increases in the AUC of R- and S-PR were seen, respectively. This resulted in a significant change in the AUC R:S ratio of PR, from 4.3 ± 1.2 in controls to 14 ± 3.0 in arthritic rats.

The T_{max} was not significantly different from that of controls, although AA resulted in a 4- and 10-fold greater C_{max} for S- and R-PR, respectively (C_{max} R:S—AA, 11 ± 0.81; control, 4.9 ± 1.3). The effect of AA on $t_{1/2}$ was insignificant.

Table I. Pharmacokinetic Parameters After iv Administration

	AUC (μg * hr/L)		Cl_s (L/hr/kg)		$t_{1/2}$ (hr)		V_d (L/kg)	
	R	S	R	S	R	S	R	S
Control								
Mean	1185	432*	0.93	2.7*	0.62	1.32	0.74	4.62*
SD	374	175	0.29	0.86	0.36	0.59	0.23	1.17
Arthritic[a]								
Mean	1518	525*	0.80	2.3*	0.40	1.22	0.48	3.56*
SD	689	259	0.34	1.0	0.14	0.89	0.22	1.71

[a] Not significantly different from control.
* Significantly different from R-PR.

Table II. Pharmacokinetic Parameters After Oral Administration

	AUC (μg * hr/L)		C_{max} (μg/L)		T_{max} (min)	Cl_o (L/hr/kg)		$t_{1/2}$ (hr)		F	
	R	S	R	S		R	S	R	S	R	S
Control											
Mean	3,200	722*	1,068	229*	38	6.31	22.4*	1.94	2.27	0.21	0.12
SD	1,540	181	196	59	14	4.24	6.20	1.12	0.73	0.10	0.04
Arthritic											
Mean	43,330	3,180*	11,040	1,030*	30	0.36	4.82*	2.74	2.76	1.56	0.46*
SD	7,860	386	2,560	235	19	0.08	0.52	0.94	1.14	0.59	0.21
	**	**	**	**		**	**			**	**

* Significantly different from R-PR.
** Significantly different from controls.

In Vitro Metabolism

Microsomal oxidative activity was apparent in livers from both AA and control rats. The time course of *in vitro* metabolism of R- and S-PR is shown in Fig. 5. Loss of PR was significantly slower in AA microsomes compared to controls. This effect appeared to be stereoselective; in AA, there was a significantly greater percentage of the S-enantiomer remaining at each sampling time than of R-PR. In control microsomes, however, no difference between enantiomers was observed.

DISCUSSION

Separation of PR enantiomers with this assay was found to be more convenient than previously reported stereospecific methods (14–16), as sample preparation was rapid (approximately 20–30 min) and only a 25-min run time was required. Sensitivity and accuracy were adequate for pharmacokinetic studies. In addition, excellent resolution of diastereomer peaks allowed accurate enantiomer quantification in plasma samples possessing high R:S ratios.

Disposition of propranolol is stereoselective in rats. This stereoselectivity is dependent upon the route of administration: in controls, plasma levels of the active S-enantiomer account for only 27% of the total (S + R) plasma concentrations after iv and 18% after oral doses. This likely reflects both a greater systemic and a greater presystemic clearance of S-PR. Our data correspond to a 16% bioavailability for total (S + R) PR, which is consistent with previous reports (17). In addition, a stronger binding affinity of

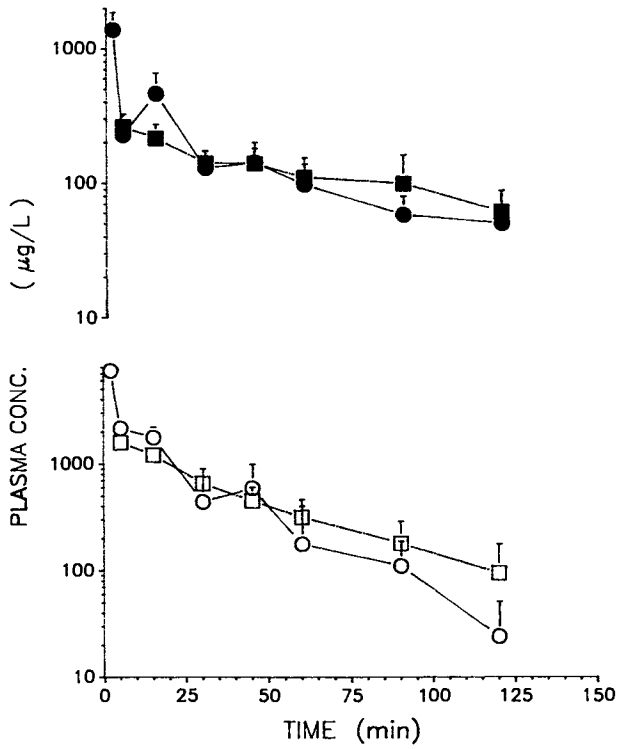

Fig. 3. Mean plasma concentration-versus-time curves of (A) S-PR and (B) R-PR in arthritic (n = 5) and control (n = 6) rats after iv administration. AA: (○) R-PR; (●) S-PR. Controls: (□) R-PR; (■) S-PR.

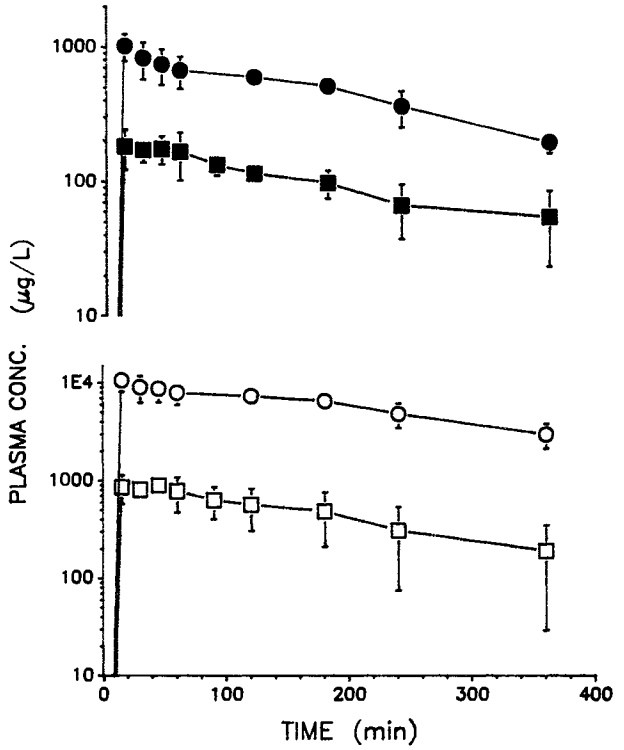

Fig. 4. Mean plasma concentration-versus-time curves of (A) S-PR and (B) R-PR in arthritic (n = 5) and control (n = 6) rats after oral administration. AA: (○) R-PR; (●) S-PR. Controls: (□) R-PR; (■) S-PR.

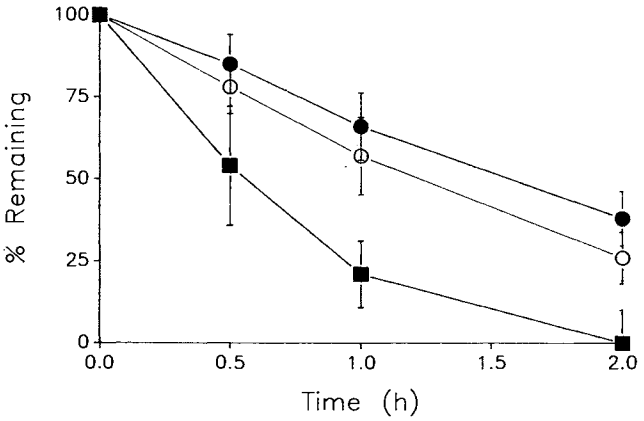

Fig. 5. Mean percentage PR remaining versus time in microsomes from AA and controls (n = 4). AA: (○) R-PR; (●) S-PR. Controls: (■) both R- and S-PR.

R-PR to plasma proteins, as reported recently by Takahashi *et al.* (18), is most likely responsible for differences in enantiomer distribution.

In AA, plasma concentrations of both PR enantiomers were found to be significantly raised after oral but not after iv doses. This route-related effect indicates that presystemic rather than systemic clearance is responsible for the observed disposition changes. This is not surprising, as decreases in intrinsic clearance of highly extracted drugs generally result in a decreased presystemic rather than systemic clearance (19). Presystemic changes may be attributed to absorption and/or metabolic processes. An increased absorption is unlikely to be the cause of the elevated plasma concentration of PR, as it has been shown that in both control and AA rats the drug readily crosses the jejunum (20).

Metabolism of PR, a highly cleared drug, is known to be dependent mainly upon the liver blood flow (Q) (19). It has been shown that Q remains unchanged in AA (6). However, a substantial reduction in the metabolic capacity of liver may render the drug more sensitive to changes in other factors such as protein binding and intrinsic clearance (Cl_{int}). Previous reports suggest increased plasma concentrations of AAG and reduced Cl_{int} in AA (5,6). Our *in vitro* study demonstrated a decrease in the disappearance of PR in arthritic rats. Furthermore, stereoselective differences in the metabolism of the two enantiomers were evident only in the microsomal fractions we obtained from arthritic rats. Provided that the results of the microsomal study reflect those of the intact animal, it suggests a nonparallel decrease in the oxidative metabolism of PR enantiomers. A reduction in the metabolism of PR has also been previously demonstrated in the 9000g liver supernatant and isolated hepatocytes of rats with inflammation (21,22). Although injection of various adjuvants may temporarily result in altered hepatic metabolism, Beck and Whitehouse (23) have shown that the effect on drug metabolism lasts for less than 14 days (23). As we studied our animals 21 days after the *M. butyricum* injection, the diminished PR metabolism is likely due to an arthritic rather than an acute inflammatory response.

Basic drugs such as PR are mainly protein bound to the acute-phase reactant AAG, the levels of which are greatly increased in inflammatory conditions (1). Indeed, increased F values of PR and oxprenolol in human inflammation are attributed to an increase in AAG levels (4,24). Our data do not exclude the possibility that changes in the free fraction may affect PR disposition. After iv administration, a 20–30% reduction in volume of distribution was observed in AA, and although not significant, it may suggest altered protein binding. However, an elevated plasma AAG cannot solely account for our observations: The percentage unbound of total propranolol has been reported to change from 10.7% in control rats to 2.4% in rats with turpentine-induced inflammation (7). Considering that at comparable concentrations there is a 50% lower protein binding of the R- compared to the S-enantiomer (25), and assuming that $F = Q/Q + (\text{Dose}/AUC_{po})$ (26), $Cl_o = f_u \cdot Cl_{int}$, and Q = 3.5 L/hr/kg, such an increase in protein binding without a change in Cl_{int} should result in only a 100 and 200% increase in F of R- and S-PR, respectively. This is not in agreement with our results, as the F of R- and S-PR was increased by 640 and 280%, respectively. Thus, in addition to an increased protein binding in AA, a suppressed Cl_{int} is likely to play a significant role in the increased bioavailability of PR.

The significantly prolonged $t_{1/2}$ of R-PR after oral (2.8 ± 0.94 hr) compared to iv (0.40 ± 0.14 hr) doses in arthritic animals is suggestive of non-linear kinetics. As a dose-dependent presystemic elimination has been described for PR (17), it is plausible that a decrease in the microsomal enzyme capacity in AA could result in saturation of both presystemic and systemic metabolism of R-PR after oral administration. However, this reduction in enzyme capacity in AA may not affect Cl_s when given intravenously, as much lower concentrations are presented to the eliminating organ, levels which may not exceed the liver saturation threshold. Consistent with this hypothesis is our bioavailability data, which indicate greater than expected F values after oral doses in AA. Such an effect was not observed for S-PR.

The effect of AA on the pharmacokinetics of PR was stereoselective. Hence, plasma enantiomer ratios increased in AA. Interestingly, the stereoselectivity *in vitro* was opposite to that observed *in vivo*. PR is metabolized through a multitude of pathways including N-dealkylation, hydroxylation, and glucuronidation. Several enzyme systems for each of these pathways have been proposed, many of which have been found to be stereoselective for different enantiomers (27–29). Thus it is plausable that the stereochemical ratio observed in AA *in vivo* may be influenced by impairment of additional pathways of metabolism such as glucuronidation.

The purpose of this investigation was to examine the effect of diseases such as arthritis on the stereoselective disposition of a racemic drug. PR was chosen as a model drug, as it has been studied extensively through nonstereoselective methods. Furthermore, its pharmacokinetics and dynamics are reportedly affected by various types of inflammation in humans and rats. Decreased presystemic metabolism of both enantiomers of PR due to a reduction of intrinsic clearance in AA is likely responsible for the increased plasma concentrations. Dissimilarity in the extent of effect this disease had on the enantiomers resulted in altered stereoselectivity. More importantly, in AA, measurements of total PR concentrations were more indicative of plasma levels of the inactive R-enantiomer, as S-PR accounted only for less than 7% of the total (R + S) AUC after oral adminis-

tration. However, this may be reversed in human arthritic subjects. Whereas plasma levels of the R-enantiomer are greater in rats, it is the active S-enantiomer concentration which is dominant in humans (9). In addition, in humans, binding to AAG is greater for the S-isomer (30). This underlines the importance of measuring individual enantiomers when examining the effect of disease or drug interactions on the disposition of racemic drugs.

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NOMENCLATURE

$t_{1/2}$	Half-life of elimination
β	Elimination rate constant
Cl_s	Systemic clearance
V_d	Volume of distribution
Cl_o	Oral clearance
F	Bioavailability
C_{max}	Peak plasma concentrations
Cl_{int}	Intrinsic clearance
Q	Liver blood flow

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