Propranolol oxidation by human liver microsomes – the use of cumene hydroperoxide to probe isoenzyme specificity and regio- and stereoselectivity

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1 Three oxidations of the enantiomers of propranolol were studied in human liver microsomes under two reaction conditions. Previous *in vitro* studies had established that two of the livers were from poor metaboliser (PM) phenotypes for the debrisoquine 4-hydroxylase (cytochrome P-450IID6) and the remaining seven were from extensive metaboliser (EM) phenotypes.

2 In the presence of NADPH and oxygen 4- and 5-hydroxylation of propranolol occurred in microsomes from all nine livers, as did propranolol *N*-desisopropylation. R(+)-propranolol was oxidized preferentially along the three pathways, although enantioselectivity observed for *N*-desisopropylation may have arisen not only from stereoselectivity in formation rates, but also from stereoselectivity in subsequent microsomal metabolism, possibly by monoamine oxidase. The involvement of monoamine oxidase in the further microsomal metabolism of *N*-desisopropylpropranolol was indicated by inhibition of the metabolism of this compound when incubated with phenelzine.

3 Cumene hydroperoxide has been proposed to support only the activity of cytochrome P450IID6. This is consistent with the observations that a) propranolol 4- and 5-hydroxylation occurred in microsomes from the EM livers only and b) side-chain oxidation was not observed under these conditions in either PM or EM livers.

4 Using cumene hydroperoxide to support the reactions, the 4-hydroxylation of propranolol showed little enantioselectivity, whereas S(–)-propranolol was 5-hydroxylated about twice as fast as the R(+)-enantiomer. There were highly significant correlations between the rates of 4- and 5-hydroxylation of R(+)-propranolol ($r = 0.96$, $P < 0.001$, $n = 7$ livers) and of S(–)-propranolol ($r = 0.98$, $P < 0.001$). Both oxidations were described by single-site Michaelis-Menten kinetics.

5 The findings suggest that P450IID6 is involved in both the 4- and 5-hydroxylations of propranolol, but that these metabolites can also be formed by other P450 isoenzymes. It is confirmed that P450IID6 does not contribute to the *N*-desisopropylation of propranolol. Furthermore, the finding that mephenytoin did not inhibit the appearance of this metabolite is not consistent with the results of *in vivo* studies suggesting the involvement of the same enzyme in the side-chain oxidation of propranolol and the 4-hydroxylation of mephenytoin.

Keywords propranolol metabolism cytochrome P-450IID6 cumene hydroperoxide

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Introduction

In clinical practice, propranolol is used as a racemate, although most of the β -adrenoceptor antagonist activity resides in the S(-)-enantiomer (Barrett & Cullum, 1968; Stoschitzky *et al.*, 1989). The pharmacokinetics of propranolol enantiomers also differ, primarily as a result of preferential hepatic metabolism of the R(+)-antipode (Olanoff *et al.*, 1986; Silber *et al.*, 1982; Ward *et al.*, 1989). Propranolol is metabolised to a large number of products, some of which are pharmacologically active. These arise from three initial reactions (Walle *et al.*, 1985, 1986), namely, direct glucuronidation, ring hydroxylation at the 4-position and side-chain oxidation to form *N*-desisopropylpropranolol (DIP) (Figure 1). DIP is metabolised further by monoamine oxidase to an intermediate aldehyde, which is then either oxidized to naphthoxylactic acid (NLA) or reduced to propranolol glycol (Chen & Nelson, 1982; Goldzer *et al.*, 1981). The side-chain oxidation accounts for about 40% of the dose (Walle *et al.*, 1985), half of which appears in the urine as NLA.

In vitro (von Bahr *et al.*, 1982) and *in vivo* (Ward *et al.*, 1989) the side-chain oxidation of propranolol is not catalysed by the polymorphic debrisoquine cytochrome P450, termed P450IID6 (Nebert *et al.*, 1989). However, Ward *et al.* (1989) have suggested that the partial clearance of propranolol to NLA may be subject to oxidative polymorphism of the mephenytoin-type, catalysed by a cytochrome of the P450IIC

subfamily. Propranolol is a weak competitive inhibitor of mephenytoin 4-hydroxylase activity in human liver microsomes (Goodhart *et al.*, 1987; Inaba *et al.*, 1985), but is reported to have no effect on mephenytoin pharmacokinetics *in vivo* (Goodhart *et al.*, 1987).

Ring hydroxylation to form 4-hydroxypropranolol (4HP) is impaired in poor metabolisers (PM) of debrisoquine (Lennard *et al.*, 1984; Raghuram *et al.*, 1984). In this phenotype, P450IID6 is absent as a result of splicing errors during transcription of the gene (Gonzalez *et al.*, 1988). PMs convert about 5% of an oral dose of propranolol to 4HP, compared with 25% in extensive metabolisers (EM) (Raghuram *et al.*, 1984; Shaheen *et al.*, 1989). It is assumed that the small amount of 4HP formed by PMs arises from the activity of other P450 isoenzyme(s). Thus, polyclonal antibodies to the rat orthologue of P450IID6 cause partial immunoinhibition of propranolol 4-hydroxylase activity in human liver microsomes (Distlerath & Guengerich, 1984).

It is possible that the h.p.l.c. assays used in these studies failed to resolve 4HP from its regioisomer, 5-hydroxypropranolol (5HP). This latter metabolite accounts for only 1–2% of a dose (Walle *et al.*, 1982), but it is highly fluorescent. Therefore, if 5HP is not formed by P450IID6, co-elution of this metabolite with 4HP could alter the interpretation of the relationship between debrisoquine oxidation phenotype and 4HP formation.

The aim of the present study was to characterise the P450 enzymes in human liver microsomes which catalyse the production of 5HP, 4HP and DIP. A particular aim was to assess the contribution of P450IID6 to the hydroxylations of propranolol. In order to examine the activity of P450IID6, we have used cumene hydroperoxide (CumOOH) as an oxidant instead of NADPH and molecular oxygen (NADPH/O₂). This approach, which exploits the ability of P450IID6 to function as a peroxxygenase at low concentrations of CumOOH, was used by Zanger *et al.* (1988a) to characterise the high affinity component of bufuralol 1-hydroxylase and has been described by Fonne-Pfister & Meyer (1988) as being isoenzyme specific for P450IID6.

Figure 1 Major routes of propranolol metabolism.

Methods

Drugs and chemicals

The hydrochloride salts of R(+)- and S(-)-propranolol, 4HP and DIP were kindly supplied

by D. E. Riley, I.C.I. Pharmaceuticals (Macclesfield, Cheshire, UK). 5HP HCl and 6-hydroxypropranolol HCl were gifts from Professor W. L. Nelson, Department of Medicinal Chemistry, University of Washington, Seattle, WA, USA. Stock solutions of propranolol enantiomers were prepared in 0.1N HCl and were stored at 4° C. Stock solutions of metabolites were prepared in 0.1N HCl containing 10 mg ml⁻¹ ascorbic acid, and these were stored at -20° C. Mexiletine HCl (the h.p.l.c. internal standard) was a gift from Boehringer Ingelheim (Bracknell, Berks., UK). Cumene hydroperoxide (80% v/v in cumenol) and phenelzine sulphate were purchased from Sigma Chemical Co. (Poole, Dorset, UK). Mephénytoin was obtained from Sandoz (Canada) Ltd, Dorval, PQ. G6P-dehydrogenase (grade II) and the disodium salts of G6P and NADP were purchased from Boehringer Mannheim (Lewes, East Sussex, UK). All other chemicals were of analytical reagent grade.

Human liver samples

Samples of human liver were obtained from nine renal transplant donors with the approval of the hospital Ethics Committee and the local Coroner. Clinical details of the donors have been published previously (Otton *et al.*, 1988; Shaw *et al.*, 1987). There is strong evidence based on analyses of debrisoquine 4-hydroxylase and/or metoprolol oxidase activity performed in our own and in independent laboratories that the donors of livers HL1 and HL3 were phenotypically PMs and that the remainder were phenotypically EMs (Otton *et al.*, 1988 and unpublished observations).

Liver microsomes

Liver microsomes were prepared as described previously (Otton *et al.*, 1988). Microsomal protein content was measured by the method of Lowry *et al.* (1951) using bovine serum albumin (fraction V) as the standard.

Incubation conditions

The enantiomers of propranolol were diluted with 1.15% (w/v) KCl from the stock solutions. Each enantiomer was studied separately. Final concentrations of each enantiomer for the inter-liver comparisons were 40 µM, and for kinetic studies (using microsomes from livers HL4, HL9 and HL3) they ranged from 0.05 µM to 50 µM (CumOOH system) or from 1 µM to 500 µM (NADPH/O₂ system). In studies of the

NADPH/O₂-supported reactions, the incubation mixture comprised 0.1 ml microsomal suspension (equivalent to 0.2 mg of microsomal protein), 0.2 ml 1.15% KCl (w/v) containing either R(+)- or S(-)-propranolol, and 0.2 ml of the NADPH generating system dissolved in 0.2M potassium phosphate buffer (pH 7.4). The NADPH generating system comprised 2 µM G6P, 0.2 µM NADP, 0.2 u G6P-dehydrogenase and 2 M MgCl₂. The reaction was started by the addition of microsomes and was carried out at 37° C for 10 min in a shaking water bath. In studies of CumOOH-supported reactions, only 0.1 mg microsomal protein was added to the incubation mixture, and the NADPH generating system was replaced by CumOOH in 0.2M potassium phosphate buffer diluted as described by Zanger *et al.* (1988a) to a final concentration of 75 µM. This mixture was incubated at 20° C for 2 min.

The reactions were stopped by adding 50 µl 6% (v/v) perchloric acid (PCA). Approximately 25 mg ascorbic acid was added to prevent the decomposition of 4HP. Mexiletine (40 µl of a 0.03 mg ml⁻¹ solution) was added as the h.p.l.c. internal standard. It was found that the samples could be stored at 4° C for up to 1 week, without degradation of propranolol metabolites.

The effect of mephénytoin on the conversion of propranolol to DIP was investigated by including 200 µM of racemic mephénytoin (dissolved in propylene glycol at a final concentration 0.5% v/v) in incubations of propranolol enantiomers (2 µM or 75 µM) and microsomes from livers LP9 or HL4. Mephénytoin dissolved completely under these conditions, as verified by h.p.l.c. analysis (Parkin & Lennard, unpublished method).

The disappearance of DIP added as substrate was followed in incubations containing 28 µM DIP and microsomes from four livers (HL4, M1, H1 or LP9). The incubation mixtures contained 0.3 mg protein ml⁻¹ but no NADPH generating system. After incubating for 15 min at 37° C, concentrations of DIP were compared with those in incubations to which PCA had been added to stop enzyme activity.

Propranolol metabolite assay

Most assays for 4HP in biological fluids involve solvent extraction from an alkalized sample (Koshakji & Wood, 1987; Kwong & Shen, 1987; Pritchard *et al.*, 1979). However, 4HP is unstable at high pH. To avoid metabolite decomposition, samples were centrifuged (900 g for 10 min) and aliquots of the supernatant were injected directly onto the chromatograph. The

h.p.l.c. comprised a Model 6000A pump (Waters Associates, Northwich, UK), a Model 7125 Rheodyne injector (H.p.l.c. Technology, Macclesfield, UK), a Z-Module column system containing a cartridge packed with Nova-Pak C₁₈ (5 µm particle size) reverse-phase packing material (Waters Associates) and a Model 970FS Schoeffel/Kratos fluorescence detector (H.p.l.c. Technology). The mobile phase was water/acetonitrile (80:20, v/v) containing 1% (w/v) triethylamine and was adjusted to pH 2.7 with orthophosphoric acid. Chromatography was performed isocratically at a flow rate of 2.5 ml min⁻¹. The detector was operated at an excitation wavelength of 205 nm with a 340 nm emission cut-off filter.

Standards

Working solutions of 4HP were prepared every 2 weeks by diluting the stock solution with 0.1N HCl containing ascorbic acid (10 mg ml⁻¹) and were stored at 4° C. Calibration curves were constructed by spiking PCA-treated microsomes with 4HP, 5HP and DIP and analysis was carried out as described above. Final concentrations ranged from 0.032 to 0.13 nmol ml⁻¹ (5HP and DIP) and from 0.26 to 1.00 nmol ml⁻¹ (4HP). Plots of the peak height ratios of metabolite to internal standard were linear and passed through the origin. The limits of detection of 4HP, 5HP and DIP were 15, 3 and 3 pmol ml⁻¹, respectively. The coefficients of variation for the assay were less than 5% for 5HP and DIP at 0.064 nmol ml⁻¹ and for 4HP at 0.52 nmol ml⁻¹.

Data analysis

Data from kinetic experiments were analysed by NONLIN, an iterative least squares fitting program (Metzler *et al.*, 1974), using initial estimates of K_m and V_{max} obtained from Eadie-Hofstee plots. One- and two-site models were fitted to the data and best fit was assessed by the significance of the F ratio. Rate constants for the disappearance of DIP were calculated using the integrated form of the first order rate equation, $2.3 \times \log (S_0/S_t) = kt$, where S_0 and S_t are the DIP concentrations at zero-time and 15 min.

Results

Patterns of propranolol metabolism

Under the chromatographic conditions described in the **Methods**, three metabolites of propranolol were resolved. Their retention times matched those of authentic standards of 5HP, 4HP and DIP (Figure 2). 6-Hydroxypropranolol, which eluted immediately after 5HP in this system, was not produced in detectable amounts by the human liver microsomes.

Chromatograms obtained with the CumOOH and NADPH/O₂-mediated oxidations of propranolol by EM and PM liver microsomes are shown in Figure 2. CumOOH did not support the *N*-desisopropylation of propranolol by microsomes from the nine livers examined. When the same microsomal preparations were incubated with an NADPH generating system,

Figure 2 Representative chromatograms of reaction products formed in incubations of EM (HL4) or PM (HL3) liver microsomes with R(+)-propranolol (40 µM) in the presence of CumOOH or NADPH/O₂. 5 = 5HP, 4 = 4HP, I = internal standard, D = DIP, P = propranolol. (The peak marked by a dot is related to the presence of CumOOH). The arrow indicates the time of injection.

DIP was produced by microsomes from all livers. The rate of appearance of DIP from 40 μM concentrations of R(+)- or S(-)-propranolol ranged from 0.21 ± 0.02 (mean $\pm$ s.d., $n = 3$ determinations) to 1.72 ± 0.01 nmol mg^{-1} protein 10 min^{-1} , and from 0.18 ± 0.02 to 0.93 ± 0.03 nmol mg^{-1} protein 10 min^{-1} , respectively.

In the presence of CumOOH, microsomes from livers HL3 and HL1 (the livers phenotyped *in vitro* as PM) did not catalyse the 4- or the 5-hydroxylation of propranolol. When an NADPH generating system was added to incubations of HL3 and HL1 microsomes, however, both hydroxylated metabolites were produced (Figure 2).

In microsomal incubates from the remaining seven livers, CumOOH-mediated 5-hydroxylation of R(+)- and S(-)-propranolol varied from 0.010 ± 0.004 to 0.116 ± 0.009 nmol mg^{-1} protein 2 min^{-1} , and from 0.016 ± 0.002 to 0.245 ± 0.004 nmol mg^{-1} protein 2 min^{-1} , respectively. Similarly, CumOOH-mediated 4HP formation from R(+)- and S(-)-propranolol ranged from 0.021 ± 0.009 to 1.87 ± 0.08 nmol mg^{-1} protein 2 min^{-1} and from 0.031 ± 0.016 to 1.73 ± 0.02 nmol mg^{-1} protein 2 min^{-1} , respectively. As shown in Figure 3, there were highly significant correlations between the rates of 4- and 5-hydroxylation of R(+)-propranolol ($r = 0.96$, $P < 0.001$, $n = 7$ livers) and of S(-)-propranolol ($r = 0.98$, $P < 0.001$) in the CumOOH system. The regioselectivity of these hydroxylations is indicated by the slopes of the regression lines. Thus, the ratio of 5HP/4HP formed from S(-)-propranolol was 0.15, compared with a value of 0.06 for R(+)-propranolol.

There was no relationship between NADPH/ O_2 -mediated DIP appearance and CumOOH-

Figure 3 A comparison of the rates of CumOOH-mediated hydroxylation (4HP vs 5HP) of R(+)-propranolol (●) and S(-)-propranolol (○) in microsomes from seven human livers (mean $\pm$ s.d. of three determinations). The equations of the lines derived by linear regression were $y = 0.06x + 0.008$ and $y = 0.15x + 0.015$ for the R(+)- and S(-)-enantiomers, respectively.

mediated 4-hydroxylation by microsomes from the seven livers ($r = 0.16$ for R(+)-propranolol and $r = 0.08$ for S(-)-propranolol).

4HP and 5HP production

Enantioselectivity The 4- and 5-hydroxylation of propranolol both displayed enantioselectivity under the two types of oxidation conditions (Table 1). On average, CumOOH-mediated 4HP formation was slightly greater from S(-)-propranolol, whereas this enantiomer was 5-hydroxylated about twice as fast as R(+)-propranolol.

Table 1 Enantioselectivity of oxidative pathways of propranolol in human liver microsomes mediated by CumOOH or NADPH/ O_2

Liver specimen	CumOOH-mediated		R(+)/S(-) ratios		DIP
	4HP	5HP	4HP	5HP	
HL3	— ^a	— ^a	2.21	> 30	1.96
HL1	— ^a	— ^a	2.71	> 30	2.32
H1	0.68	0.63	1.64	> 30	1.87
HL2	1.05	0.48	1.54	27	1.77
M1	0.87	0.39	1.41	3.45	1.85
F45	0.97	0.40	1.15	1.49	2.08
W21	0.98	0.40	1.23	1.32	1.54
LP9	1.08	0.47	1.15	1.61	1.82
HL4	0.76	0.40	0.99	0.99	1.17
Mean $\pm$ s.d.	0.91 ± 0.14	0.44 ± 0.09	1.56 ± 0.56		1.82 ± 0.31

40 μM R(+)-propranolol or S(-)-propranolol was used as the substrate.

^a Microsomes from HL3 (a PM liver) and HL1 formed no detectable 4HP or 5HP.

The opposite enantioselectivity was observed when microsomes were incubated with the NADPH generating system (Table 1). Under these conditions, R(+)-propranolol was hydroxylated at both the 4- and 5-positions faster than S(-)-propranolol, although the degree of enantioselectivity varied considerably between livers. This variation was related inversely to the absolute amount of metabolite formed, with the highest enantioselectivity seen in the two PM livers (HL3 and HL1) and the EM liver with the least activity (H1). Using microsomes from these three livers (HL3, HL1 and H1), formation of 5HP from S(-)-propranolol was so close to the limits of detection of the assay that the extent of enantioselectivity could not be determined reliably.

Enzyme kinetics Preliminary experiments established that the formation of 4HP and 5HP was linear during the incubation time that was used and was linear with respect to microsomal protein concentration when R(+)- or S(-)-propranolol was incubated with microsomes and an NADPH generating system at 37° C.

Using the CumOOH-mediated system, the formation of 5HP and 4HP from either propranolol enantiomer (1 μM) was linear with time for only 2 min at 20° C. Under these conditions metabolite formation was linear over a protein concentration range of 0.04 mg ml^{-1} to 0.2 mg

ml^{-1} . The rates of hydroxylation were maximal at a CumOOH concentration of about 75 μM .

Kinetic analysis of propranolol 4- and 5-hydroxylation by microsomes from three livers (HL4, LP9 and HL3) was performed. Both the CumOOH- and NADPH/O₂-mediated hydroxylations of propranolol enantiomers were best described by single-site Michaelis/Menten kinetics over the range of substrate concentrations examined. Representative Eadie-Hofstee plots of the CumOOH-supported hydroxylations are shown in Figure 4. The apparent kinetic constants are given in Table 2. The K_m values calculated from the data for liver microsomes from the two EM livers (HL4 and LP) were similar under both oxidation conditions. However, values of apparent K_m were an order of magnitude greater in microsomes from the PM liver (HL3).

DIP appearance

Enantioselectivity The recovery of DIP from R(+)-propranolol was greater than that from S(-)-propranolol in microsomes from all nine livers (Table 1).

Relationship to mephenytoin oxidation The mephenytoin hydroxylase activity of the liver microsomes used in these studies has not been determined. Therefore, a direct comparison of

Figure 4 Eadie-Hofstee plots of the kinetics of the 4-hydroxylation (a) and 5-hydroxylation (b) of propranolol enantiomers by microsomes from liver LP9. The lines are the computer-generated lines of best fit to the velocity, V (nmol mg^{-1} protein 2 min^{-1}) and substrate concentration, S (μM) data. S(-)-propranolol ($\circ$, $\square$) and R(+)-propranolol ($\bullet$, $\blacksquare$).

Table 2 Apparent kinetic parameters of the microsomal 4- and 5-hydroxylations of propranolol enantiomers, mediated by CumOOH or by NADPH/O₂

		<i>CumOOH-mediated</i>		<i>NADPH/O₂-mediated</i>	
		<i>R(+)-Prop</i>	<i>S(-)-Prop</i>	<i>R(+)-Prop</i>	<i>S(-)-Prop</i>
<i>4-Hydroxylation</i>					
Liver HL4	K_m^a	2.7	7.2	3.1	5.4
	V_{max}^b	1.01	1.37	1.60	1.76
Liver LP9	K_m^a	1.9	4.6	3.4	5.9
	V_{max}^b	2.88	2.87	1.95	2.13
Liver HL3	K_m^a	—	—	70	107
	V_{max}^b	—	—	0.93	0.54
<i>5-Hydroxylation</i>					
Liver HL4	K_m^a	2.5	5.2	3.7	5.8
	V_{max}^b	0.08	0.13	0.21	0.25
Liver LP9	K_m^a	1.79	4.85	7.5	7.6
	V_{max}^b	0.16	0.34	0.32	0.21
Liver HL3	K_m^a	—	—	42	41
	V_{max}^b	—	—	0.27	0.05

^a μM

^b nmol mg⁻¹ protein 2 min⁻¹ in CumOOH system, and nmol mg⁻¹ protein 10 min⁻¹ in NADPH/O₂ system

this activity with DIP production cannot be made. However, the addition of racemic mephentyoin (200 μM) did not affect the rates of DIP appearance by microsomes from livers HL4 and LP9.

Further metabolism When DIP (28 μM) was incubated with microsomes, its concentration decreased rapidly with time, a process that was independent of the presence of NADPH. The

first order rate constants for DIP disappearance varied between microsomal preparations from different livers, but not between preparations of the same liver (Table 3). Addition of phenelzine (1 μM) completely inhibited DIP metabolism by human liver microsomes.

Discussion

Our findings provide evidence that two of the monohydroxylated metabolites of propranolol are formed in human liver microsomes by P450IID6 and by at least one other cytochrome P450 enzyme. Although this has been proposed previously for 4HP (Distlerath & Guengerich, 1984), possible analytical interference by 5HP had not been considered.

The activity of P450IID6 towards propranolol 4- and 5-hydroxylation was examined using CumOOH supported oxidation. This assay was used originally to study microsomal bufuralol 1'-hydroxylase Zanger *et al.* (1988a) who observed that, with respect to K_m value and enantioselectivity, CumOOH-supported bufuralol 1'-hydroxylation by microsomes closely resembled that of the purified bufl enzyme and of the high affinity component of this reaction when supported by NADPH/O₂.

In contrast to the oxidation of bufuralol, propranolol hydroxylations were not monophasic when supported by CumOOH and biphasic

Table 3 First-order rate constants for DIP disappearance in different preparations of microsomes from four human livers

<i>Liver specimen</i>	$k \text{ (min}^{-1}\text{)}$
LP9	0.043
	0.042
H1	0.094
	0.087
M1	0.086
	0.072
HL4	0.036
	0.037

Each value is the mean of three determinations using microsomes prepared at least two months apart from each human liver.

when supported by NADPH/O₂, but were monophasic under both reaction conditions. However, the apparent K_m values for 4HP and 5HP formation by microsomes of a PM liver in the presence of NADPH/O₂ were an order of magnitude higher than those obtained using EM liver microsomes. Presumably, this reflects the lower affinity of alternate form(s) of P450 for propranolol hydroxylation compared with P450IID6. Such a difference in the affinity of propranolol for different isoenzymes may be too small to allow the detection of biphasic kinetics when EM microsomes are incubated with NADPH/O₂.

It is not possible to assess the extent of the contribution of P450IID6 to the 4- and 5-hydroxylation of propranolol from our data because oxidation in the two assay systems involves different mechanisms and different incubation temperatures. In an earlier paper, we used inhibition by quinidine to isolate the contribution of P450IID6 to metoprolol *O*-demethylation from that of other enzyme(s) present in human liver microsomes (Otton *et al.*, 1988). This approach was not possible with propranolol because of interference by quinidine with the h.p.l.c. analysis. The use of antibodies raised to P450IID6 (Distlerath & Guengerich, 1984; Zanger *et al.*, 1988b) may help to define further the role of this isoenzyme in the metabolism of propranolol. However, until the absolute specificity of these antibodies for P450IID6 can be demonstrated, they may offer no advantage over other probes for the activity of this enzyme. The apparent specificity of CumOOH for P450IID6 also needs further evaluation and the possibility that CumOOH-supported reactions could generate a pattern of metabolites different from that seen with the native NADPH/O₂ system cannot be excluded.

The absence of DIP in microsomal incubations of propranolol with CumOOH is consistent with the view that CumOOH is relatively specific for the activity of P450IID6. Furthermore, the lack of inhibition by mephenytoin of DIP production argues against the involvement of mephenytoin hydroxylase in propranolol side-chain oxidation, suggested by *in vivo* studies (Ward *et al.*, 1989). At the concentrations of propranolol and mephenytoin used in the present work, and if mephenytoin is assumed to have a K_i equal to its K_m value of 100 μ M (Jurima *et al.*, 1985), then between 20% to 35% inhibition of propranolol *N*-dealkylation should have been observed.

Our observations of enantioselectivity in the two major NADPH/O₂-mediated oxidations of propranolol (Table 1) are similar to those of von

Bahr *et al.* (1982). Thus, in their study, the mean R(+)/S(-) ratio of 4HP formation in microsomes from six human livers was 1.3 ± 0.2 , although it is possible that the h.p.l.c. assay used did not resolve 4HP and 5HP. Von Bahr *et al.* (1982) also observed that R-propranolol was preferentially *N*-dealkylated, the mean R(+)/S(-) ratio being 1.9 ± 0.4 , a value almost identical to that found in the present study (R(+)/S(-) = 1.8 ± 0.3).

In another investigation of the substrate stereoselectivity of propranolol metabolism by human liver microsomes (Nelson & Shetty, 1986), the production of 4HP, 5HP and DIP was measured in incubations of pseudoracemic propranolol. The mean R(+)/S(-) ratio for 4HP was 1.07 ± 0.12 ($n = 6$ unphenotyped livers) and that for 5HP was 1.96 ± 0.62 . The R(+)/S(-) ratio observed for DIP was 0.91 ± 0.05 , a value similar to that obtained when the individual enantiomers were incubated with microsomes (Nelson & Shetty, 1986). This value differs considerably from that observed in the present study (1.82 ± 0.31). The reason for the discrepancy between our data and that of Nelson & Shetty (1986) is unclear, although we found greater variability. One possibility may be differences in the extent of monoamine oxidase (MAO) activity in the respective microsomal preparations, and stereoselective metabolism of DIP by this enzyme. DIP is metabolised by MAO to an intermediate aldehyde (Chen & Nelson, 1982; Goldzer *et al.*, 1981), and there is evidence from studies in rats that DIP formed from S(-)-propranolol is oxidized preferentially to the aldehyde (Nelson & Bartels, 1984). MAO activity has been observed in microsomal preparations of rat liver (Gomez *et al.*, 1988), and the present finding that phenelzine, an irreversible inhibitor of MAO, prevents the disappearance of DIP suggests that MAO activity is also associated with human liver microsomes. MAO-catalysed metabolism of DIP may explain the recent observation that the appearance of the end product of the side-chain oxidation of propranolol in humans is impaired by the co-administration of debrisoquine (Anthony *et al.*, 1989), a known inhibitor of MAO activity (Medina *et al.*, 1969).

In both EMs and PMs, the clearance of R(+)-propranolol to its 4-hydroxy product was about 2.5-times faster than that of the S(-)-enantiomer along this pathway (Ward *et al.*, 1989). Our *in vitro* findings that propranolol 4- and 5-hydroxylation catalysed by the enzymes acting in the NADPH/O₂ system is also enantioselective for R(+)-propranolol agree with the *in vivo* data. The inter-liver variation observed in R(+)/

S(-) ratios for the NADPH/O₂-supported microsomal reactions (Table 1) may be due to the varying contributions of P450IID6 and other P450 isoenzymes, each with different enantioselectivities. As indicated by the results of CumOOH-supported hydroxylations, P450IID6 shows little enantiomeric preference for propranolol 4-hydroxylation, but highly favours S(-)-propranolol for 5-hydroxylation. Different steric preferences of P450IID6 for different sites of oxidation of bufuralol (Meyer *et al.*, 1986) and metoprolol (Otton *et al.*, 1988) have also been observed.

In conclusion, several aspects of the *in vitro* oxidation of propranolol have been studied. These relate to enantioselectivity, regioselectivity, isoenzyme specificity (CumOOH vs NADPH/O₂), enzyme kinetics and relationship to oxida-

tion phenotype. The findings indicate that at least two cytochrome P450 isoenzymes catalyse each of the hydroxylation pathways examined. Detailed *in vitro* studies of the multiplicity and interdependence of enzymes involved in propranolol oxidation provide a basis for a more complete understanding of the disposition of this drug *in vivo*.

We are grateful to Dr Paul E. B. Reilly of the University of Queensland, Australia, who brought to our attention the importance of subsequent microsomal metabolism of *N*-desisopropylpropranolol. We also thank Professor Wendel Nelson for the donation of samples of 5- and 6-hydroxypropranolol. These studies were supported by the Hallamshire Therapeutics Research Trust. Part of this work was presented at the IVth World Conference on Clinical Pharmacology and Therapeutics held in Mannheim-Heidelberg, F.R.G., July 1989.

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(Received 5 March 1990,
accepted 18 June 1990)